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Liver-Targeted Drug Delivery Using HepDirect™ Prodrugs

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Running Title:

Prodrugs for Liver-Targeted Nucleotide Delivery

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Abbreviations:

MB06866, (2*R*, 4*S*)-9-{2-[4-(3-chlorophenyl)-2-oxo-1,3,2-dioxaphosphorinan-2-yl]methoxyethyl}adenine, compound **8**; **8A**, racemic mixture of MB06866; MB07133, (2*R*, 4*S*)-4-amino-1-[5-*O*-[2-oxo-4-(4-pyridyl)-1,3,2-dioxaphosphorinan-2-yl]-β-D-arabinofuranosyl]-2(1*H*)-pyrimidinone, compound **9**; **9A**, 1:1 mixture of *R*- and *S*-isomers of MB07133; PMEA, 9-(2-phosphonylmethoxyethyl)adenine, adefovir; PMEAp, PMEA monophosphate; PMEApp, PMEA diphosphate; araC, cytarabine; CYP, cytochrome P_{450}; GSH, glutathione; NMP, nucleoside monophosphate; POM, pivaloyloxymethyl; araU, arabinosyl uracil; LDH, lactate dehydrogenase; AST, aspartate aminotransferase; ALT, alanine transaminase; AUC, area under the curve; LOQ, limit of quantitation; PMEA-RM, PMEA-related metabolites; ACP, acetaminophen; NTP, nucleoside triphosphate; dCK, deoxycytidine kinase; MRP, multidrug resistance protein.

Section:

Absorption, Distribution, Metabolism and Excretion

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Abstract: Targeting drugs to specific organs, tissues or cells is an attractive strategy for enhancing drug efficacy and reducing side effects. Drug carriers such as antibodies, natural and man-made polymers and labeled liposomes are capable of targeting drugs to blood vessels of individual tissues, but often fail to deliver drugs to extravascular sites. An alternative strategy is to use low molecular weight prodrugs that distribute throughout the body but cleave intracellularly to the active drug by an organ-specific enzyme. Here we show that a series of phosphate and phosphonate prodrugs, called HepDirect™ prodrugs, results in liver-targeted drug delivery following a cytochrome P_{450}–catalyzed oxidative cleavage reaction inside hepatocytes. Liver targeting was demonstrated in rodents for MB06866, a HepDirect prodrug of the nucleotide analogue adefovir (PMEA), and MB07133, a HepDirect prodrug of cytarabine (araC) 5'-monophosphate. Liver targeting led to higher levels of the biologically active form of PMEA and araC in the liver and to lower levels in the toxicologically most sensitive organs. Liver targeting also confined production of the prodrug byproduct, an aryl vinyl ketone, to hepatocytes. Glutathione within the hepatocytes rapidly reacted with the byproduct to form a glutathione conjugate. No byproduct-related toxicity was observed in hepatocytes or animals treated with HepDirect prodrugs. A 5-day safety study in mice demonstrated the toxicological benefits of liver targeting. These findings suggest that HepDirect prodrugs represent a potential strategy for targeting drugs to the liver and achieving more effective therapies against chronic liver diseases such as hepatitis B, hepatitis C and hepatocellular carcinoma.

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Site-specific drug delivery is a concept actively pursued over the past thirty years to increase local drug concentrations and thereby produce more effective medicines with fewer side effects (Tomlinson, 1987). Despite the obvious attractiveness of drug targeting, few marketed drugs depend on a targeting mechanism. The most advanced strategies use site-specific drug carriers such as antibodies (Payne, 2003), peptides (Arap et al., 1998) natural and man-made polymers (Meijer et al., 1990), and carbohydrate- or peptide-labeled nanoparticles (Akerman et al., 2002) and liposomes (Wu et al., 2002) capable of recognizing cell- and tissue-specific proteins expressed on the surface of the targeted cells. In many cases, drugs conjugated to the carrier molecules gain high tissue selectivity through the ability of the carrier molecule to recognize blood vessels of individual tissues via tissue-specific vascular markers expressed on the endothelium. While impressive vascular specificity is achieved (Ruoslahti, 2002), drug exposure to extravascular sites is often severely compromised by limitations in drug conjugate exchange across the endothelial barrier and the slow rate of drug conjugate cleavage relative to the rate of drug removal from the vascular delivery site (Tomlinson, 1987; Stella and Himmelstein, 1980). One conjugate-based strategy capable of delivering drugs to an extravascular site uses glycoprotein and glycolipid-containing drug carriers that recognize the asialoglycoprotein receptor expressed on hepatocytes (Meijer et al., 1990; Wu et al., 2002; Meijer and Molema, 1995; Fiume et al., 1988). The conjugate gains access to hepatocytes by passing through the fenestrated endothelium of the sinusoid. Hepatocytes uptake the drug conjugate via receptor-mediated endocytosis which is followed by intracellular drug-conjugate cleavage. Despite some success in pre-clinical models, advancement of these and other drug conjugates remains slow due to concerns

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over manufacturing costs, conjugate-induced immunogenic reactions and limitations in drug loading and the route of administration.

To avoid these and other limitations associated with drug carriers, several small molecule strategies have been explored. Drug delivery usually entails use of prodrugs that are designed to cleave in a site-specific manner. Some prodrugs gain cell specificity by exploiting differences in local environment (e.g. oxidation/reduction potential, pH) (Bodor et al., 1981; Mulcahy et al., 1994) while others gain specificity through use of cell-specific surface receptors (e.g. bile acid transporter) that facilitate prodrug transport into cells (Kramer et al., 1992). The strategy with the most appeal, however, entails the design of prodrugs that cleave using tissue-specific intracellular enzymes. Such prodrugs have proven to be difficult to identify presumably because they require enzymes that: 1) are expressed in a limited number of tissues; 2) catalyze a reaction that is chemically useful for prodrug cleavage; and 3) efficiently cleave a broad range of structurally-diverse substrates. These difficulties subsequently stimulated efforts to deliver prodrug-cleaving enzymes to tissues using antibodies (Bagshawe, 1987) or gene therapy (Huber et al., 1994).

Recently we discovered a novel class of phosphate and phosphonate prodrugs, which we named HepDirect™ prodrugs. These prodrugs are cyclic 1,3-propanyl esters containing a ring substituent that renders them sensitive to an oxidative cleavage reaction catalyzed by a cytochrome P_{450} (CYP) (Erion et al., 2004). Prodrugs with a 4-aryl substituent (**1**, Fig. 1) are oxidized specifically by the CYP isoenzyme family CYP3A, which is expressed predominantly in the parenchymal cells of the liver and to a lesser extent the enterocytes of the small intestine (de Waziers et al., 1990). Oxidation results

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in ring-opening and the generation of a transient, negatively-charged intermediate (**3**), which is retained inside the cell. A subsequent β-elimination reaction produces the phosphate or phosphonate (**4**) and the prodrug byproduct, i.e. the aryl vinyl ketone **5**. The latter undergoes rapid conjugation with glutathione, which exists at millimolar levels in the liver and all tissues expressing CYPs as part of a natural defense system against oxidative free radicals (Meister, 1983).

In this study, we demonstrate in rats and mice the potential of HepDirect prodrugs for liver-targeted drug delivery using HepDirect prodrugs of two structurally different nucleoside monophosphate (NMP) analogues, namely adefovir (PMEA) and cytarabine (araC) 5'-monophosphate. Moreover, we show that the liver targeting leads to an improved safety profile and no byproduct-related toxicities.

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Materials and Methods

Radiolabeled MB06866 (**8**) [adenine-2, 8-^{3}H] and bispivaloyloxymethyl (POM) PMEA [adenine-8-^{3}H] were prepared at Moravek Biochemicals (Brea, CA). Compounds **8, 8A**, MB07133 (**9**) and **9A** were prepared and characterized in vitro using methods described previously (Erion et al., 2004). The mono- and diphosphorylated forms of PMEA, PMEAp and PMEA-pp, and araCTP were synthesized at Trilink Biotechnology, Inc (San Diego, CA).

Animal Treatments. Animals were given unrestricted access to food and water at all times unless otherwise indicated. Rat studies used overnight-fasted male Simonsen Albino rats (Sprague-Dawley) and mouse studies used *ad libitum*-fed male NIH Swiss mice. Experiment 1: Rats (n = 3/group) were gavaged with a solution of labeled MB06866 [adenine-2,8-^{3}H, 2.23 mCi/mmol] (46.5 mg/kg, 30 mg/kg PMEA molar equivalent) or labeled bisPOM PMEA [adenine 8-^{3}H, 4.52 mCi/mmol] (55 mg/kg, 30 mg/kg PMEA molar equivalent) in PEG 400/ethanol/water (2:1:1, v/v). Experiment 2: Mice (n = 4-5/group) were injected i.p. with araC (100 mg/kg) or MB07133 (188 mg/kg, 100 mg/kg araC molar equivalent) as a solution in 0.9% saline. Experiment 3: Mice (n = 7-8/group) were injected i.p. once daily for five days with araC (100, 30, 10 and 3 mg/kg/day) or **9A** (1848, 554, 185 and 55.4 mg/kg/day which equates to 1000, 300, 100 and 30 mg/kg araC molar equivalents/kg/day) dissolved in 0.9% saline (pH 4). Body weights were recorded on days 0 - 4 prior to treatment and on the last treatment day (day 5). Experiment 4: Mice (n = 5/group) were treated i.p. with **8A** as a suspension in 0.25% carboxymethyl cellulose (CMC) at doses of 300 and 1000 mg/kg or with acetaminophen

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as a solution in saline at doses of 50, 250, and 500 mg/kg. All studies were conducted in accordance with IACUC guidelines.

Tissue Collection. At the times indicated, animals were anesthetized with halothane and blood obtained from the vena cava (rats) or heart (mice). Liver samples were obtained by freeze clamping with liquid nitrogen-cooled tongs. Liver, kidneys and the small intestine were perfused with saline, dropped into liquid nitrogen, and stored at –80 ºC. Mouse bone marrow was obtained by flushing the dissected right femur with 1 mL of phosphate-buffered saline. Tissue collected for histology was fixed with formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin. The stained tissue sections were blinded and evaluated by a pathologist using a light microscope at Comparative Biosciences.

Radioactive Metabolite Measurements. Weighed tissue samples were homogenized in an equal volume of deionized water and then solubilized by addition of a 10- to 20-fold excess of a 1:1 solution of Soluene-350 (Packard, Madison, CT): isopropyl alcohol and heated for 4 hours at 50 ºC. Aliquots were added to 15 ml Hionic Fluor (Packard, Madison, CT), decolorized by addition of H_2O_2 (60 ºC, 30 min), and counted in a Beckman LS6000IC scintillation counter (Fullerton, CA). For the determination of liver and kidney metabolites, samples were homogenized in 3 volumes of 10% perchloric acid (v/w) and centrifuged (2500 x g, 5 min, 4 ºC). Supernatants were neutralized with 3M KOH/ 3M $KHCO_3$, centrifuged (16,000 x g, 20 minutes, 4 ºC), and analyzed by anion exchange HPLC using a Whatman Partisphere SAX (5 µm, 4.6 x 125 mm) column coupled to a Radiomatic Series A-100 detector. A 3:1 ratio of ULTIMA-FLO AP scintillant to mobile phase was employed in the detector unit. The mobile phase

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consisted of 10 mM ammonium phosphate pH 6.0 (Buffer A) and 1 M ammonium phosphate pH 3.5 (Buffer B). Samples were loaded onto the column in 100% buffer A, eluted with buffer A for 10 minutes, and then with a gradient to 80% B over 15 min. The flow rate was 1 mL/min. The retention times of the PMEA-related metabolites, i.e. PMEA, PMEA monophosphate (PMEAp) and PMEA diphosphate (PMEApp), were 7, 21, and 28 minutes, respectively. The concentrations were calculated in terms of PMEA equivalents based on specific activity.

Metabolite Profiles. Plasma samples (100 µL) were deproteinated by addition of acetonitrile (1 mL). After centrifugation (16,000 x g, 10 min, room temperature), the supernatant was removed and dried in a Savant SpeedVac Plus SC110A. Samples were reconstituted with 110 µL of mobile phase buffer (20 mM KH_2PO_4 buffer, pH 4.5), sonicated for 5 min, and centrifuged for 30 s. Liver samples were prepared as described above. For araCTP analysis, clarified liver homogenates were treated with periodate to remove endogenous CTP (Garrett and Santi, 1979). Bone marrow cell pellets were extracted with 12 volumes of 3% perchloric acid (v/v). The extracts were vortexed and centrifuged (16,000 x g, 5 min, room temperature). The supernatant (100 µL) was neutralized with 3 M KOH/3 M $KHCO_3$, centrifuged and treated as above.

Processed plasma samples were analyzed for araC and arabinosyl uracil (araU) by HPLC using an Alltech C_{18} column (5µm, 4.6mm x 250mm). After sample injection, the acetonitrile concentration was increased to 10 % over 10 min, then to 50% over 15 min. AraC, araU and MB07133 eluted at 5.0, 7.0 and 14.5 min, respectively (280 nm) and were quantified using standard curves obtained with spiked plasma samples processed as above. Neutralized tissue extracts were analyzed for PMEApp and araCTP on a

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Whatman Partisil SAX column (4.6 x 250 mm) eluted with a linear gradient from 70% Buffer A (10 mM ammonium phosphate pH 3.5 and 6% v/v ethanol), 30% Buffer B (1 M ammonium phosphate pH 3.5 and 6% v/v ethanol) to 80% Buffer B at a flow rate of 1.25 mL/min over 25 min. UV absorbance was monitored at 254 nm for PMEApp and at 270 nm for araCTP. The elution times were: PMEApp (19 min), araCTP (12 min). Standard curves were prepared by adding known amounts of araCTP or PMEApp into perchloric acid extracts from control liver or bone marrow, neutralizing, and analyzing by HPLC as above.

Hepatocyte Cytotoxicity. Hepatocytes were isolated as described (Berry and Friend, 1969) from rats administered dexamethasone once a day for four days (50 mg/kg, i.p.; corn oil suspension). Suspensions of cells (60 mg/mL) were treated with an aqueous solution of **8A** (final concentration of 250 µM and 1 mM) or acetaminophen (final concentration of 1, 3 and 10 mM). After 0, 2, 4, and 6 hours of incubation (37°C), aliquots (0.4 mL) of the cell suspensions were collected and centrifuged (16,000 x g, 30 seconds, room temperature). Supernatants (0.2 mL) were stored at -20 °C for later analysis of lactate dehydrogenase (LDH) and aspartate aminotransferase (AST). Cell pellets were extracted with 5% sulfosalicylic acid (0.2 mL) and stored on dry ice for glutathione analysis (Baker et al., 1990). Cell viability was measured at each time point by counting a 1:10 dilution of cells in 0.2% Trypan blue in a hemocytometer. Viability is defined as the percentage of trypan blue excluding cells (viable) vs. total cells. The leakage of LDH and AST from cells was measured using standard assay kits (Sigma Chemical Co., St. Louis, MO).

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Liver, Blood and Bone Marrow Measurements. Liver glutathione content was determined in tissue samples that were freeze-clamped *in situ* and homogenized in 5 volumes of sulfosalicylic acid. After centrifugation of the homogenate (16,000 x g, 10 min, room temperature), the supernatants were collected and glutathione measured as described (Baker et al., 1990). Quantitation of liver markers (albumin, alkaline phosphatase, alanine transaminase (ALT), AST, bilirubin, glutamyltranspeptidase, LDH, globulin) were measured from plasma samples (Labcorp, San Diego, CA). For the determination of cell numbers, EDTA-treated blood or bone marrow flush was mixed with 450 µL nuclear staining solution containing 0.19 mg/mL crystal violet in 1 M acetic acid, and incubated at room temperature for at least 5 min. After brief vortexing, the cells were counted in an improved Neubauer chamber on a Nikon Optiphot-2 microscope.

Data Analysis. For the tissue distribution studies, the areas under the concentration-time profiles (AUCs) were determined non-compartmentally by trapezoidal summation from zero to the last measured time point. For values below the limit of quantitation (LOQ), the LOQ was substituted into the equation. For the cytotoxicity study in hepatocytes and mice safety study, differences between treatment groups were evaluated using a one-factor ANOVA followed by a Dunnett's test and are considered significant when $p<0.05$. For the toxicity study in rats, data were analyzed using an unpaired Student's T test.

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Results

Tissue Metabolite Profiles for PMEA Prodrugs. To assess differences in the tissue metabolite profile for the HepDirect prodrug of PMEA, **8** (MB06866, Fig. *2A*), relative to the esterase-sensitive prodrug of PMEA, bisPOM PMEA, normal fasted rats were gavaged with PMEA-equivalent doses of the corresponding ^{3}H-labeled prodrugs. HPLC analysis of liver and kidney samples collected at 3, 8 and 24 h showed no intact prodrug whereas PMEA and the mono and diphosphorylated forms of PMEA (PMEAp and PMEApp) were consistently detected in approximately a 0.8:1:0.6 ratio. In the small intestine, neither prodrug nor PMEApp was detected, while PMEA and PMEAp were present at an approximate 1:1 ratio. The mean $AUC_{0\text{-}24h}$s for PMEA-related metabolites (PMEA-RM) (PMEA, PMEAp, PMEApp) in liver, kidney and intestine samples obtained from rats treated with MB06866 were 884, 196 and 118 (nmol/g*h), respectively (Fig. *2B-D*). The $AUC_{0\text{-}24h}$s for rats treated with bisPOM PMEA were 284, 742, and 3206 (nmol/g*h), respectively. Accordingly, MB06866 was associated with a 4.5- and 7.5-fold greater exposure of PMEA-related metabolites to the liver relative to the kidneys and intestine, respectively. In contrast, the liver exposure to PMEA-related metabolites in bisPOM-treated rats was 0.38- and 0.09-fold lower than for kidneys and intestine, respectively. These results correspond to an increase in the liver/kidney and liver/intestine exposure ratios of 12- and 84-fold, respectively, for MB06866 relative to bisPOM PMEA. The temporal profile of PMEA-related metabolites in the liver showed MB06866 relative to bisPOM PMEA produced a 5.1-fold higher peak level and a 3.1-

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fold greater $AUC_{0\text{-}24h}$. The opposite profile was observed in the kidney and intestine, where bisPOM PMEA produced a 2.9- and 23.3-fold greater $AUC_{0\text{-}24h}$, respectively.

Tissue and Plasma Metabolite Profiles for araC and araCMP Prodrug. A second, structurally-different prodrug, **9** (MB07133, Fig. *3A*), was studied in mice to further assess the liver/hepatocyte targeting ability of HepDirect prodrugs. Liver, bone marrow and plasma samples collected at 0.5, 1, 2 and 4 hours following i.p. administration of either MB07133 or araC at a 100 mg/kg araC equivalent dose showed clear evidence of liver targeting (Fig. *3B*). Mice treated with MB07133 exhibited 12.6-fold higher peak liver araCTP levels (96 vs. 7.6 nmol/g) and an 11.7-fold greater $AUC_{0\text{-}4h}$ (225 vs. 19 nmol/g*h). The increase in AUC represents a minimum value, since the AUC was calculated using the LOQ (3 nmol/g) for all time points with levels of araCTP below the LOQ. In bone marrow, araCTP levels were below the LOQ (3 nmol/g) at all time points in mice treated with MB07133 ($AUC_{0\text{-}4h}$ <11.2 nmol/g*h) while readily detected in mice treated with araC ($AUC_{0\text{-}4h}$ = 46.2 nmol/g*h). Accordingly, MB07133 exhibited a liver to bone marrow exposure (liver $AUC_{0\text{-}4h}$/bone marrow $AUC_{0\text{-}4h}$) of more than 20-fold whereas the exposure ratio for araC was ≤0.42-fold. In addition, mice treated with MB07133 showed a 45-fold lower peak plasma araC level and a 15-fold lower $AUC_{0\text{-}4h}$ than mice treated with araC. Since araCTP in the bone marrow is likely related to araC plasma levels, bone marrow araCTP may be reduced by a similar factor, which would correspond to an araCTP level in the bone marrow of mice treated with MB07133 of ≤0.75 nmol/g*h and liver to bone marrow exposure of at least 300. These extrapolated

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results correspond to a 714-fold increase in liver targeting (liver/bone marrow) for MB07133 relative to araC.

5-Day Mouse Safety Study. To assess whether differences in araCTP distribution lead to differences in safety profile, mice were treated i.p. once daily for 5 days with araC or **9A** and monitored for changes in behavior, body weight, bone marrow cell mass, circulating cell counts and serum chemistry. Both stereoisomers present in **9A** (**9** and C4 *R*-isomer of **9**) are converted to araCTP with similar kinetics (Erion et al., 2004). No effect on body weight was observed for mice treated with **9A** at araC equivalent doses as high as 1000 mg/kg whereas mice treated with 100 mg/kg araC showed a 7% reduction in weight relative to vehicle treated animals (Fig. *3C*). Nucleated bone marrow cells decreased significantly in mice treated with both 30 and 100 mg/kg doses of araC whereas reductions were observed only in mice treated with the highest dose (1000 mg/kg araC equivalents) of **9A** (Fig. *3D*). No evidence of liver toxicity was observed with either compound based on serum chemistry (total bilirubin, AST, ALT). Moreover, no histological findings were noted in liver, kidney and small intestine samples collected from animals treated with **9A**. (Comparative Biosciences). These results suggest a 10- to 30-fold difference in safety between araC and **9A**.

Byproduct Toxicity in Rat Hepatocytes. Suspended primary rat hepatocytes isolated from dexamethasone-treated rats were treated with **8A** or acetaminophen to assess the potential of the aryl vinyl ketone byproduct of HepDirect prodrugs (**5**) to deplete glutathione and cause cellular toxicity. Both stereoisomers present in **8A** (**8** and C4 *R*-

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isomer of **8**) are converted to PMEA with similar kinetics (Erion et al., 2004). After 4 h, glutathione levels were reduced ≈50% and >95% at 250 μM and 1 mM **8A,** respectively (Fig. *4A*). No significant changes were observed in cell viability relative to vehicle treated cells as determined by trypan blue exclusion or enzyme leakage (LDH or AST) (Fig. *4B*). In contrast, hepatocytes treated with acetaminophen at a concentration of 3 mM or higher resulted in significant glutathione depletion (>95%), decreased viability (4 ± 2% vs. 61% for vehicle treated cells at 4 h) and increased levels of LDH (1.8-fold) and AST (1.9-fold) in the medium (Milam and Byard, 1985). In separate studies, hepatocytes treated with **8A** (100 μM) resulted in PMEApp levels of ~30 nmoles/g cells confirming significant prodrug cleavage under these conditions.

Byproduct Toxicity in Mice. The potential for byproduct detoxification by hepatic glutathione was further evaluated in mice treated with high doses of **8A** or acetaminophen (Table 1). Glutathione levels were reduced 25% relative to vehicle treated animals following an i.p. injection of **8A** at 1000 mg/kg. Liver levels of PMEApp at 6 h reached ~117 nmoles PMEApp/g liver, which corresponds to 0.49 μmoles of byproduct produced based only on the total PMEA-related metabolites present at the 6 h time point. The corresponding glutathione conjugate (**7**) was present in serum at 6 h at a mean concentration of 2.2 ±0.3 μM. No liver toxicity was observed, including changes in serum liver enzymes (AST, ALT and LDH) and liver histology. Furthermore, no other toxicity was evident in treated animals, including changes in clinical signs, serum chemistry, or gross organ pathology. In contrast, mice treated with acetaminophen at a dose of 250 and 500 mg/kg (i.p.) showed a transient 62.5% and 85.7% decrease in

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hepatic glutathione at 1 hour, respectively, which returned to the level of the vehicle treated mice by 6 h. Severe liver toxicity was observed in animals treated with the 500 mg/kg dose, including a 50 to 200-fold elevation in serum liver enzymes and gross surface lesions (Mitchell et al., 1973).

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Discussion

Site-specific delivery of drugs to extravascular sites within specific organs remains a highly attractive, but generally elusive goal for improving drug efficacy and reducing systemic side effects. In the present study, we demonstrate that HepDirect prodrugs of two structurally-different NMP analogues result in liver-targeted production of the corresponding biologically-active nucleoside triphosphate (NTP) analogue following administration to rodents. Liver-targeting is principally attributed to the susceptibility of HepDirect prodrugs to oxidation by a liver enzyme, CYP3A, coupled with their stability in aqueous solutions, blood and most non-hepatic tissues (Erion et al., 2004). Since CYP3A is expressed predominantly in hepatocytes and is either absent or present at very low levels in other liver cells, e.g. stellate cells (Parola et al., 1997), Kupffer cells, endothelial cells and biliary epithelial cells (Lakehal et al., 1999), HepDirect prodrugs not only target the liver but more specifically target the hepatocyte. Other factors affecting the magnitude of liver targeting and overall extrahepatic drug exposure include the ability of the hepatocyte to retain prodrug cleavage products and the pathways involved in the subsequent elimination of these products.

The dependence of liver targeting on the enzyme activating the prodrug is demonstrated in studies comparing the HepDirect prodrug of PMEA, MB06866, with the esterase-sensitive prodrug, bisPOM PMEA. Esterases are ubiquitously expressed throughout the body and exist in the rodent at high levels in the blood and most tissues, including the liver and intestines. Accordingly, bisPOM PMEA is rapidly degraded to PMEA ($t_{1/2}$ = <2 min) (Benzaria et al., 1996). Esterase cleavage reduces prodrug availability to the liver and increases plasma PMEA levels (data not shown) and

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correspondingly PMEA exposure to extrahepatic tissues (e.g. kidney). In contrast, MB06866 is stable in blood and most tissues and therefore remains intact in the circulation. High stability enables more prodrug conversion by the liver to PMEApp while simultaneously limiting PMEA levels in blood and the kidneys. The net result is a large increase in the liver/kidney (12-fold) and liver/intestine (84-fold) targeting for MB06866 relative to bisPOM PMEA. The enhanced liver/intestine targeting is noteworthy given that the intestine is the only other organ that expresses CYP3A at appreciable levels (de Waziers et al., 1990). This enhancement is attributed to the lower intestinal specific activity of CYP3A relative to esterases (Benzaria et al., 1996; Williams, 1985), and consequently the larger fraction of the prodrug dose that is able to pass through the gastrointestinal tract intact and enter the portal circulation.

The HepDirect prodrug of araCMP, MB07133, further illustrates the potential of this prodrug class for liver-targeted NTP production. In this case, liver targeting is attributed to both CYP3A expression and the tissue distribution of the kinase that phosphorylates araC to araCMP, namely deoxycytidine kinase (dCK). dCK is predominantly expressed in leukemic cells, bone marrow and gut epithelial cells (Arner and Eriksson, 1995). Accordingly, systemic administration of araC (or araCMP, which is rapidly dephosphorylated in blood to araC), leads to araCTP production in these tissues and correspondingly an antileukemic effect along with concomitant bone marrow and gut toxicity. In contrast, MB07133 targets araCTP production to the liver and greatly reduces araCTP levels in bone marrow where there is no CYP3A activity. High levels of araCTP are produced in the liver because prodrug cleavage produces araCMP, which effectively bypasses the need for dCK in the liver while simultaneously avoiding

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metabolism by cytidine deaminase; an enzyme expressed at high levels in the liver and responsible for the rapid deamination of araC to an inactive metabolite (araU) (Camiener and Smith, 1965). The net effect of the HepDirect prodrug is to redirect araCTP production from bone marrow to liver and correspondingly to minimize bone marrow and gut toxicities while producing high araCTP levels in the liver.

In addition to CYP3A activity, liver NTP levels and liver targeting are dependent on the ability of the hepatocyte to retain the prodrug cleavage products and convert them to the NTP analogue. Activation of HepDirect prodrugs initially produces a ring-opened intermediate (**3**, Fig. *1*), which is negatively charged and therefore unable to passively diffuse out of the hepatocyte. Retention of **3** results in intracellular production of the nucleoside monophosphate (NMP) following either a β-elimination reaction or possibly in the case of phosphate esters a phosphodiesterase-catalyzed ester hydrolysis reaction. Conversion of the NMP to the NTP by intracellular phosphorylating enzymes results in liver-targeted NTP production which can lower the minimally effective prodrug dose for achieving therapeutic levels of NTP and therefore lead to a reduction in the total exposure of the drug to extrahepatic organs.

Efflux of the NMP or products of NMP degradation into the circulation following either prodrug cleavage or subsequent dephosphorylation of the NTP back to the NMP can diminish the safety benefits of liver targeting. Frequently the product effluxed is the nucleoside which results from intracellular dephosphorylation of the NMP. In some cases, the nucleoside is poorly phosphorylated by cellular kinases and therefore not readily converted to the NTP in tissues. In other cases, however, the nucleoside is readily taken up by tissues and phosphorylated to the corresponding NTP. Administration of

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MB07133 led to low plasma araC levels except at very high doses which were associated with high plasma araC levels and bone marrow toxicity. The low plasma araC levels are attributed in part to the high cytidine deaminase activity that exists in the liver and the associated rapid intrahepatic deamination of araC to the biologically inactive metabolite araU that consequently limits araC efflux and extrahepatic araC exposure.

In contrast to MB07133, liver activation of MB06866 may reduce extrahepatic exposure by altering the route of PMEA excretion. Negatively charged compounds such as PMEA are effluxed into the circulation via transporters on the sinusoidal membrane and then selectively taken up by tissues that express these and other transporters (e.g. kidney, liver) (Faber et al., 2003; van Montfoort et al., 2003). Negatively charged compounds are also effluxed into the bile via ATP-dependent transporters known as multidrug resistance proteins (MRPs) that exist on the biliary canalicular membrane (Borst et al., 2004). MRPs expressed in rat microglia are known to efflux PMEA (Dallas et al., 2004) suggesting that MRP-mediated efflux may account for PMEA efflux into the bile. Accordingly, the reduced kidney exposure to PMEA following administration of MB06866 relative to bisPOM PMEA may arise from the ability of MB06866 to increase hepatic PMEA exposure and consequently increase PMEA efflux into the bile.

Generation and retention of the prodrug cleavage intermediate **3** inside CYP3A-containing cells has additional benefits beyond NTP generation in that it confines production of the prodrug byproduct to cells that contain glutathione. The byproduct generated following cleavage of this class of HepDirect prodrugs is an aryl vinyl ketone (**5**). Vinyl ketones as a compound class are associated with significant toxicity, including both cytotoxicity and genetic toxicity (Neudecker et al., 1989). Toxicity is attributed to

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alkylation of essential proteins and DNA. Intracellular glutathione detoxifies vinyl ketones through a 1,4-addition reaction, which is rapid and quantitative in tissues associated with millimolar glutathione levels such as the liver and gut (Dinkova-Kostova et al., 2001). Consequently drugs that undergo metabolism to a highly reactive vinyl ketone in the liver (e.g. acetaminophen) exhibit good safety as long as glutathione levels remain above 0.5-1 mM (*ca.* 20% of normal liver levels) (Mitchell et al., 1973). Importantly, mice treated with high doses of **8A** showed only a transient 25% reduction in hepatic glutathione levels despite substantial prodrug turnover. Moreover, unlike acetaminophen, high doses of **8A** showed no evidence of liver toxicity as judged by both serum liver enzyme levels and liver histology (Table 1). While the lack of liver toxicity may reflect rapid detoxification of **5** by intracellular glutathione, it may also reflect an overall lower toxicity potential of aryl vinyl ketones as suggested by results in glutathione-depleted hepatocytes treated with **8A** and results from an embryotoxicity study with phenyl vinyl ketone (Hales et al., 1989).

Liver-targeted NTP production is expected to significantly enhance the therapeutic potential of PMEA and araC for treatment of patients with hepatitis B and hepatocellular carcinoma, respectively. The 12-fold increase in liver targeting demonstrated for MB06866 relative to bisPOM PMEA (adefovir dipivoxil) (Fig. *3*) should at minimum enable MB06866 to match the efficacy observed in patients treated with a 30 mg/day dose of adefovir dipivoxil; a dose that is more efficacious than the 3-fold lower FDA-approved dose (10 mg/day) but not used clinically due to evidence of renal toxicity (Marcellin et al., 2003). An even greater enhancement in the therapeutic potential is possible for MB07133, since araC is ineffective against solid tumors

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putatively because of its poor conversion to araCTP (Heinemann et al., 1988). In this case, the HepDirect prodrug is expected to not only limit extrahepatic exposure to araC but to also increase araCTP production in the primary liver tumor. Higher araCTP levels result because cell penetration is independent of nucleoside transporters and because prodrug cleavage generates araCMP, which both bypasses the rate-limiting kinase responsible for the conversion of araC to araCMP, i.e. dCK, and avoids deamination by cytidine deaminase. Since primary liver tumors retain high levels of CYP3A4 activity (Zhang et al., 2000), MB07133 is expected to undergo conversion in hepatocarcinoma cells to araCTP which in turn is expected to inhibit DNA polymerase activity and consequently inhibit DNA synthesis and tumor cell growth (Miura and Izuta, 2004).

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Acknowledgments

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Footnotes

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Legends for Figures

Fig. 1. Prodrug cleavage mechanism and products. A HepDirect prodrug (**1**) diffuses into hepatocytes and undergoes a CYP3A-catalyzed oxidation of the C4 methine hydrogen to produce the C4-hydroxylated product (**2**). Rapid and irreversible ring-opening leads to the intermediate monoacid (**3**), which generates the corresponding phosphate or phosphonate (**4**) and an aryl vinyl ketone (**5**) following a β-elimination reaction or possibly in the case of the phosphate a phosphodiesterase-catalyzed hydrolysis reaction. **4** is converted to the biologically active nucleoside triphosphate analogue (NTP) (**6**) by intracellular nucleotide kinases when R-$PO_3^=$ is a nucleoside monophosphate analogue (NMP) and by PRPP synthase when the NMP analogue is PMEA. **5** is trapped by intracellular glutathione (GSH) to form conjugate **7**.

Fig. 2. (*A*) Structure of MB06866 (**8**), a HepDirect prodrug of PMEA. **8A** is the racemic mixture. (*B-D*) The concentration of radioactivity (DPM/ g tissue) in terms of PMEA-related metabolites (PMEA-RM) (PMEA + PMEAp + PMEApp, nmol/g tissue) in liver, kidney and small intestine determined at 3, 8 and 24 hours after oral administration of MB06866 (□) or bisPOM PMEA (●) to normal fasted rats at a 30 mg/kg PMEA equivalent dose. (n = 3/timepoint).

Fig. 3. (*A*) Structure of MB07133 (**9**), a HepDirect prodrug of araCMP. **9A** is the 1:1 mixture of C4 *R*- and *S*-isomers. (*B*) Mean liver and bone marrow araCTP $AUC_{0\text{-}4h}$ (nmol/g*h) and plasma araC $AUC_{0\text{-}4h}$ (μM*h) determined from samples collected at 0.5,

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1, 2 and 4 hours after i.p. injection of a 100 mg/kg araC equivalent (CE) dose of MB07133 or araC to normal mice. (n = 4/timepoint). AraCTP was not detected in bone marrow in MB07133-treated mice or in liver samples of araC-treated mice >2 h after dosing. The LOQ for araCTP (3 nmoles/g) was used in the calculation of the AUC (upper limit). (*C-D*) Dose-response of **9A** (□) and araC (●) in male mice for body weight (*C*) and bone marrow nucleated cells (*D*) measured on day 5 following once daily dosing. Body weight is percent of day 0. (n = 7-8/timepoint). Untreated (▲) and vehicle treated (Δ) mice. * p<0.05 compared with vehicle treated.

Fig. 4. Cytotoxicity studies in hepatocytes freshly isolated from male rats treated one time daily with dexamethasone for 4 days (50 mg/kg, i.p.). (*A*) Glutathione concentration over 6 hours in hepatocytes treated with vehicle, acetaminophen (ACP) or compound **8A**. (*B*) Fold changes in hepatocyte viability as assessed by trypan blue exclusion and elevation of liver enzyme levels (AST and LDH) in the medium after a 6 h incubation at an equivalent glutathione-depleting concentration of ACP (3 mM) and **8A** (1 mM). * p<0.05 vs. all other groups.

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TABLE 1

Acute toxicity studies in normal rats treated with acetaminophen and **8A**.

Acetaminophen (ACP) was administered in saline at the minimum toxic dose (500 mg/kg). **8A** was administered in carboxymethyl cellulose (CMC) at a dose of 1000 mg/kg. Glutathione (GSH) was measured in liver and liver enzymes in plasma at the time indicated. Results are expressed as mean ± SEM (n = 5/timepoint). * $p<0.05$ unpaired T test, treated vs. respective control group.

	GSH (1h) (μmoles/g)	GSH (6h) (μmoles/g)	ALT (6h) (IU/L)	AST (6h) (IU/L)	LDH (6h) (IU/L)
Saline	12.1 ± 0.7	9.8 ± 0.7	48 ± 5	78 ± 8	918 ± 84
ACP	1.8 ± 0.4*	9.4 ± 1.3	10455 ± 4294*	7081 ± 2806*	78918 ± 31201*
CMC	9.7 ± 0.9	7.0 ± 1.0	94 ± 47	109 ± 35	1477 ± 325
8A	7.3 ± 0.4*	7.2 ± 0.7	54 ± 3	117 ± 8	1916 ± 296

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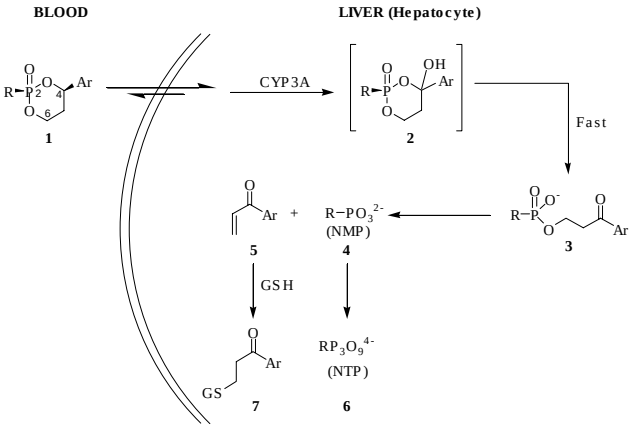

Figure 1.

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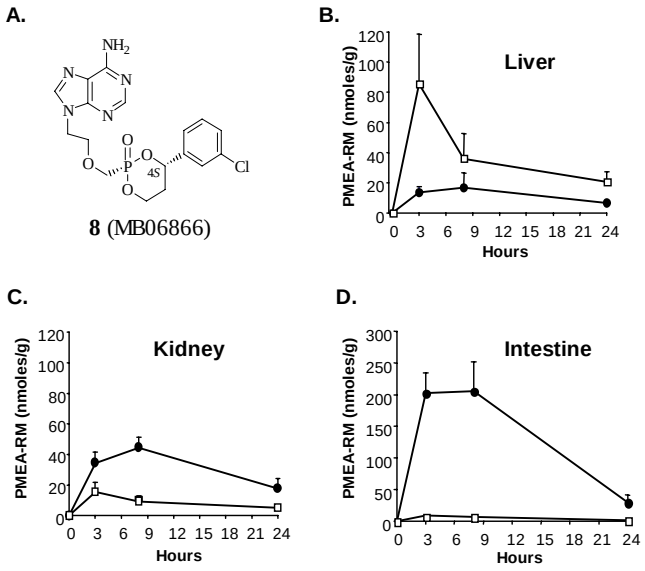

Figure 2.

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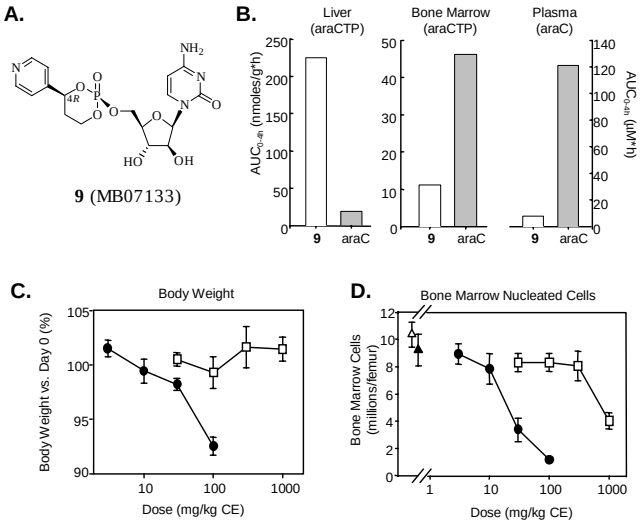

Figure 3.

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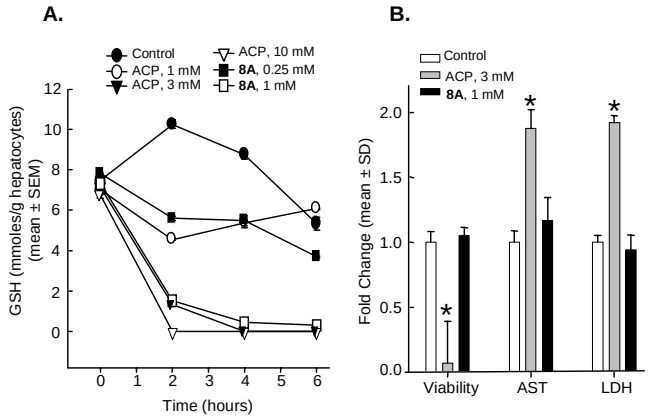

Figure 4.

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