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ORIGINAL ARTICLE

Delivery of high levels of anti-proliferative nucleoside triphosphates to CYP3A-expressing cells as a potential treatment for hepatocellular carcinoma

Deidre A. MacKenna · Annika Montag · Serge H. Boyer · David L. Linemeyer · Mark D. Erion

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Abstract
Purpose Hepatocellular carcinoma (HCC) is a life-threatening condition with only one drug treatment regimen approved for use. Oncolytic nucleosides are minimally effective against HCC putatively because of their inability to achieve cytotoxic levels of the active metabolite [nucleoside triphosphate (NTP)] in tumor cells at doses that are well tolerated. The aim of our studies was to explore the utility of CYP3A-activated prodrugs of cytarabine and fludarabine monophosphate for the treatment of HCC.
Methods Prodrugs of cytarabine and fludarabine monophosphates were evaluated for their ability to safely achieve NTP levels in the liver of normal mice that are cytotoxic to hepatoma cells.
Results While therapeutic levels of NTPs are achieved in the livers of normal rodents after administration of the prodrugs, only MB07133 achieved these levels whithout exhibiting signs of liver toxicity or myelosuppression.
Conclusions As the levels of araCTP achieved in the liver at therapeutic doses are only toxic to proliferating cells (such as those in HCC tumors), but not the non-proliferative adjacent tissue, MB07133 treatment has the potential to be both efficacious and well tolerated in HCC patients.

D. A. MacKenna (✉) · A. Montag · S. H. Boyer · D. L. Linemeyer · M. D. Erion
Departments of Biological Sciences and Medicinal Chemistry, Metabasis Therapeutics Inc., 11119 North Torrey Pines Road, La Jolla, CA 92037, USA
e-mail: dmackenna@ymail.com

Keywords Hepatocellular carcinoma · Nucleosides · Cytarabine · Liver targeting · Prodrug · P450 CYP3A4 · Chemotherapy

Introduction

Hepatocellular carcinoma (HCC) is a serious life-threatening disease with 0.5–1 million new cases diagnosed worldwide each year [28]. Limited treatment options are the cause of a poor prognosis with median survival times of <1 year for untreated, non-resectable HCC [41]. HCC is generally resistant to chemotherapy, although the multi-kinase inhibitor, sorafenib, has recently been approved by the FDA following positive Phase III studies where extension of survival times was the primary endpoint [1, 25, 26]. Surgical resection has also been used with some success [30], but clinical outcomes vary as HCC tumors are often multifocal and/or have undetectable micrometastases [4, 30] resulting in high recurrence rates. Liver transplantation remains the most successful treatment regimen, but the lack of organ availability and regrowth of extrahepatic metastases limits success [3, 4].

Therapies utilizing nucleoside oncolytic agents are the standard of care for many hematopoietic-derived cancers. Intracellular generation of nucleoside triphosphates (NTPs) inhibit DNA polymerases and cause DNA chain termination after incorporation. Unfortunately, therapies based on nucleoside oncolytics have limited efficacy against solid tumors due to the low abundance of nucleoside transporters and narrow substrate specificity of nucleoside kinases, resulting in non-cytotoxic levels of oncolytic NTPs at tolerable doses [11, 38, 40]. Hence, bypassing the need for nucleoside transporters and delivering high concentrations of the oncolytic nucleoside

monophosphates (NMPs) directly into tumor cells could be an effective approach. HepDirect nucleoside monophosphate prodrugs passively diffuse across cell membranes and are acted on by cytochrome P450 3A (CYP3A) to generate NMPs [14] that are converted to NTPs by nucleotide kinases. HepDirect prodrugs are stable in plasma and not activated in cells that do not express CYP3A [13]. As such, HepDirect prodrugs should enable NTP production in the liver and limit extrahepatic exposure to the cytotoxic metabolites assuming that the circulating nucleoside levels remain low following dephosphorylation in the liver. Only the tumor cells should be susceptible to the toxicity of the NTPs given that the liver is typically relatively quiescent and only the tumor cells should be replicating at a significant rate [18]. Therefore targeting of the HCC tumor is achieved through both tissue (liver) targeting and functional (anti-proliferative) targeting giving rise to an expectation of anti-tumor activity with good therapeutic safety window.

Our goal was to explore the utility of these prodrugs of known oncolytic nucleosides for use in primary HCC. Although several nucleosides were evaluated, studies of cytarabine (araC) and fludarabine (FaraA) demonstrated the most promising results and are described here. We provide experimental evidence that potentially therapeutic concentrations of araCTP can be achieved in the livers of normal rodents with minimal extra-hepatic effects after administration of MB07133, a HepDirect prodrug of araCMP. Compounds could not be evaluated in animal models of HCC due to experimental limitations, so tissue distribution and safety studies were used to estimate utility. These studies suggest that a treatment regimen based on MB07133 may provide an improved therapeutic strategy over those based on araCMP [6, 13, 14].

Methods

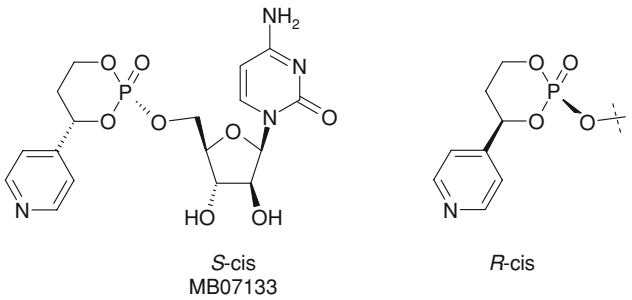
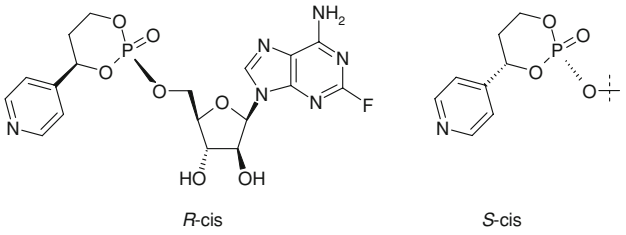
Compounds

MB07133, MB07133(R/S) and MB07191 (a HepDirect prodrug of FaraAMP) were synthesized at Metabasis Therapeutics (Table 1) [6]. AraC, FaraA, fludarabine monophosphate (FaraAMP), 2-fluoroadenine (2FA), and araCTP and FaraATP standards were purchased from commercial sources. FaraAMP, a water soluble form of FaraA that is rapidly converted to FaraA in plasma, was used for in vivo administration [16].

Cellular assays

Cell viability and proliferation rates were measured in HepG2 cells using Alamar Blue (Biosource International, Camarillo, CA) and ^{3}H-thymidine incorporation [19] assays, respectively. Cells were evaluated after a 6-day treatment with araC or FaraA with media changed and fresh compound added on Day 3. To estimate the therapeutically relevant concentrations of NTPs, HepG2 cells were grown to confluence, treated with araC or FaraA for indicated times, trypsinized, separated from medium by centrifugation through an oil layer, and intracellular NTP levels measured as described [6].

Table 1 Structures of HepDirect prodrugs of araCMP and FaraAMP

Compound[a]	Structure
MB07133(R/S)	 *S*-cis MB07133 *R*-cis
MB07191	 *R*-cis *S*-cis

[a] MB07133(R/S) is a mixture of MB07133 and its similarly active diastereomer at the C4-position and phosphorus-nucleoside bond of the dioxaphosphorinane [6]. MB07191 is also a mixture of two diastereomers at the same positions

Animal studies

All animal studies were approved by the Metabasis Institutional Animal Care and Use Committee and performed according to the NIH Guide for the Care and Use of Laboratory Animals. MB07133 and MB07191 doses are reported based on the molar equivalents of araC and FaraA, respectively. This allows comparison of drug levels based on equivalent doses.

Tissue distribution in rodents

Non-fasted, male NIH Swiss Webster mice (Harlan Sprague Dawley, Indianapolis, IN) or Simonsen Albino rats (Simonsen Laboratories, Inc., Gilroy, CA) were injected i.p. with 100 mg/kg of nucleoside or prodrug. Blood, liver and other organs were harvested at 0.5, 1, 2 and 4 h after dosing and processed for metabolite analysis as described [6]. For MB07191, additional samples were obtained at 8, 12 and 24 h after dosing due to the long half-life of FaraATP.

5-day safety study

Male NIH Swiss Webster mice were injected i.p. with the prodrugs, nucleosides or vehicle (0.9% saline) once daily for 4 days and sacrificed ca. 24 h after the final dose [14]. MB07133(R/S), a racemic mixture of MB07133 and its diastereomer that has similar activation kinetics [6], was used for these studies. Blood was collected for hemogram, white blood cell differential, and plasma clinical chemistry analyses. Livers from MB07133(R/S)- and araC-treated animals were fixed in formalin and evaluated by a histopathologist (Comparative Biosciences, Mountain View, CA, USA). For the MB07191/FaraAMP study, NTP levels were measured in the liver, kidney, and red blood cells. In both studies, bone marrow flushes were stained with crystal violet to quantify nucleated bone marrow cells [14].

Bolus IV administration in rats

MB07133(R/S) was administered to male Simonsen rats via tail vein catheters as a bolus dose of 100 mg/kg. Heparinized blood samples were obtained from arterial catheters and plasma samples extracted using methanol. Clarified supernatants were analyzed for prodrug, araC and araU by HPLC.

Continuous IV infusion in rats

Male Simonsen rats were instrumented with indwelling femoral vein catheters 3–5 days prior to the study. Catheters were exteriorized between the scapulae. MB07133 [200 mg/kg/day] or araC [400, 1,000 or 2,000 mg/kg/day] were infused for up to 48 h.

Tissue processing and HPLC

Nucleosides and NTPs were analyzed by HPLC as described [6, 17]. Briefly, frozen tissue (liver, kidney, intestine) samples were homogenized using in 3 volumes of ice-cold 10% (v/v) perchloric acid (PCA). One ml of supernatant was neutralized using 0.3 ml 3 M KOH/3 M $KHCO_3$, mixed thoroughly. For bone marrow, the cellular content was dissolved in 12 volumes of 3% PCA (v/v) and 90 μL of extracted supernatant was neutralized to pH 7–8 using 30 μL 1 M KOH/1 M $KHCO_3$ and again centrifuged. For ara-CTP containing samples, clarified supernatants were treated with periodate to remove endogenous ribonucleotides which otherwise might interfere with ara-CTP detection [17].

For FaraA, the acetonitrile gradient was 15% over 6 min followed by a washout using 50% acetonitrile over 10 min. AraC and FaraA eluted at ~5.0 and 5.1 min, respectively. For the NTPs, UV absorbance was monitored at 254 and 270 nm for FaraATP and araCTP, respectively. AraCTP, FaraATP and FATP eluted at 12, 17 and 15 min, respectively.

Data analysis

Unless otherwise specified, data are expressed as mean ± standard error of the mean. Areas under the curves (AUC) were calculated using trapezoidal summation from time zero to the last time point with the limit of quantitation (LOQ) used if values were below LOQ (generally 3 nmol/g for liver and bone marrow araCTP, 0.5 μM for plasma araC; 0.7 nmol/g for liver and kidney FaraATP; and 0.3 μM for plasma FaraA.). Liver targeting indices (LTI) were defined as the ratio of the NTP AUC values in the liver to those in the target organs of toxicity or to the nucleoside levels in the plasma. For the safety studies, data were analyzed using a one-way ANOVA with a Dunnett's post hoc test. For each parameter, the Bartlett statistic of normality was evaluated and, if necessary, the data were transformed using a Box-Cox transformation in JMP6.0.

Results

Cytotoxicity and target NTP levels

Cytotoxicity of HepDirect prodrugs could not be evaluated in vitro because proliferating hepatoma cell lines and plated rat hepatocytes do not retain CYP3A activity required for HepDirect prodrug activation [32, 35]. Therefore, the intracellular concentrations of NTPs required for cytotoxicity were measured in HepG2 cells incubated with high concentrations of the nucleosides araC and FaraA (Fig. 1). These

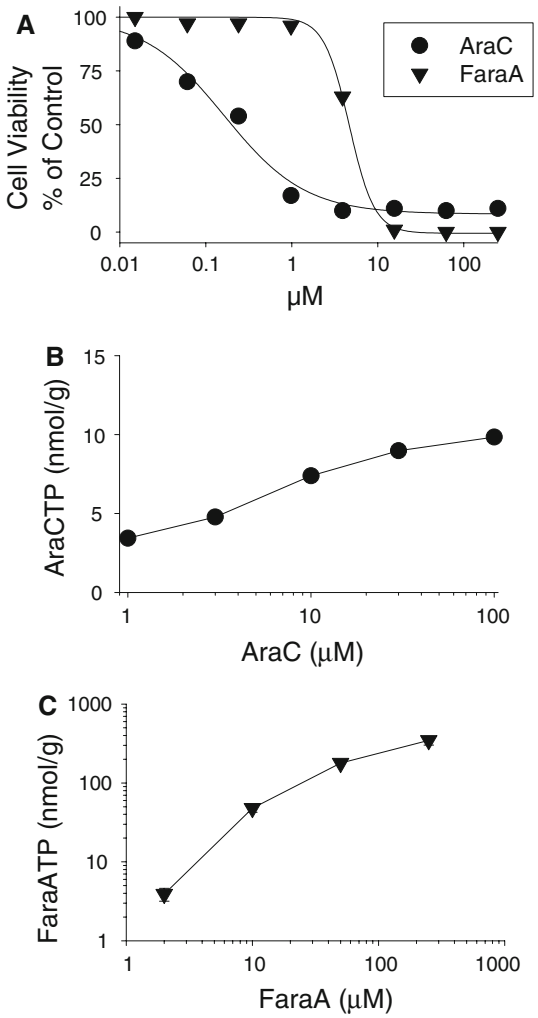


Fig. 1 Relationship between cytotoxicity and intracellular araC and FaraA NTP levels in HepG2 cells. **a** Cell viability measured using Alamar Blue after 6 days of incubation with nucleosides. **b**, **c** Quantitation of intracellular NTP levels in HepG2 cells treated with increasing concentrations of nucleosides at a time when steady state levels were achieved (**b** 5 h for araC and **c** 24 h for FaraA)

cytotoxic NTP concentrations were used as target levels in tissues of animals treated with the prodrugs. Both araCTP and FaraATP were toxic, causing cytotoxicity of >80% of the HepG2 cells at steady state intracellular concentrations of 5 and 190 nmol/g, respectively. AraC also inhibited the rate of ^{3}H-thymidine incorporation in HepG2 cells with a comparable intracellular araCTP concentration (data not shown).

Liver targeting in rodents

Liver targeting profiles for MB07133 and araC in mice are published elsewhere [6, 14] with key parameters presented in Table 2 for comparison with FaraAMP and MB07191. Notably, at comparable doses, liver araCTP levels were >8-fold higher after treatment with MB07133 than after treatment with araC. Similarly, MB07191 enhanced liver FaraATP exposure by 31-fold in comparison with FaraAMP in mice. AraCTP levels in the bone marrow were <3 nmol/g at all time points. Kidney and spleen were selected as target organs to estimate the therapeutic window for MB07191 and FaraA. FaraATP levels were detectable at all time points in both organs after dosing. The LTI's were 3- and 79-fold higher for MB07191 than for FaraAMP in the kidney and spleen, respectively.

Liver targeting of MB07133(R/S) in normal rats was similar to that observed in mice. As shown in Fig. 2, MB07133(R/S)-treated rats had liver araCTP levels that peaked at 81.4 ± 11.8 nmol/g with an $AUC_{0-4\,h}$ value of 135.1 nmol/g h. Levels of araCTP in the bone marrow were below LOQ at all time points. Using these LOQ values to calculate the LTI, MB07133(R/S) achieved more than a 7.2-fold greater liver araCTP exposure than that found in the bone marrow. As observed in mice, plasma araC levels were low in the rats. The levels peaked at 3.73 μM with an $AUC_{0-4\,h}$ value of 10.4 μM h giving a 13-fold lower exposure than the araCTP levels in the liver.

Mouse 5-day safety

To assess dose-limiting toxicity, MB07191, MB07133(R/S) and the corresponding nucleosides were administered i.p. to normal mice for 5 days. As shown in Tables 3, 4, MB07133(R/S) treatment did not affect body weight, while araC, FaraAMP, and MB07191 all decreased body weight at doses at or above 100, 300 or 30 mg/kg/day, respectively. For araC, lowering of hematocrit was observed at or above 10 mg/kg/day and lowering of other hematopoietic parameters occurred at doses of 30 and 100 mg/kg/day (Fig. 3, Table 3). No effect was observed on platelets (data not shown). Liver enzymes (AST, ALT, alkaline phosphatase and bilirubin) and other clinical chemistry parameters (BUN) were within normal limits [20] at the doses tested (data not shown), with the exception of a 30% decrease in alkaline phosphatase and a 2-fold increase in creatinine at 100 mg/kg/day (Table 3). Similar effects were observed for MB07133(R/S), but at doses 33-fold higher than for araC. MB07133(R/S) treatment decreased hematocrit and nucleated bone marrow cells at doses of 300 and 1000 mg/kg/day, respectively (Table 3, Fig. 3). Modest reductions in white blood cell (WBC) count were observed that paralleled the effects observed with araC (Fig. 3). No effects were observed on liver enzyme levels or liver histology (data not shown).

FaraAMP treatment decreased mononuclear WBC count and nucleated bone marrow cells at 100 and 300 mg/kg/day (Table 4). Similar effects were observed only at the

Table 2 Summary of tissue distribution data in male NIH Swiss mice

Analyte	AraC			MB07133			$t_{½}$ (min)
	C_{max} (nmol/g or μM)	$AUC_{0–4\ h}$ (nmol/g h or μM h)	LTI	C_{max} (nmol/g or μM)	$AUC_{0–4\ h}$ (nmol/g h or μM h)	LTI	
AraCTP							
Liver	7.6	<19.2[b]		69.9	148.8[b]		70
Bone marrow	19.0	46.2[b]	<0.4	<LOQ (<3)	<3.8[b,c]	>13.2[d]	
AraC							
Plasma	149.5	121.2	<0.16	3.0	7.6	19.5	
	FaraAMP			MB07191			$t_{½}$ (min)
	C_{max} (nmol/g or μM)	$AUC_{0–4\ h}$ (nmol/g h or μM h)	LTI	C_{max} (nmol/g or μM)	$AUC_{0–4\ h}$ (nmol/g h or μM h)	LTI	
FaraATP							
Liver	27.9	68.6		638.7	2,122.8/4,048.8[a]		180
Kidney	2.0	<4.8	14.3	16.6	52.0/88.0[a]	40.8/40.6[a]	
Spleen	23.0	56.2	1.2	10.2	23.2/141.2[a]	94.4/28.7[a]	
FaraA							
Plasma	55.4	158.4	0.43	18.6	45.4/57.4[a]	46.7/70.6[a]	

Compounds were administered at of 100 mg/kg of nucleosides or molar equivalents of prodrugs. $AUC_{0–4\ h}$, Area under the curve of NTP or nucleoside exposure from 0 to 4 h

[a] Exposure from 0 to 24 h, *LTI* liver targeting index

[b] Previously published data presented for comparison purposes [6]

[c] Bone marrow araCTP levels following administration of MB07133 were below LOQ, but are estimated to peak at 1.2 nmol/g using the ratio of plasma araC to liver araCTP after araC administration

[d] May be as high as 51 if bone marrow araCTP is estimated using the plasma araC levels

1,000 mg/kg/day dose of MB07191. Treatment with MB07191, but not FaraAMP, elevated transaminase levels at doses above 30 mg/kg/day (Table 4). Moreover, total bilirubin and alkaline phosphatase were increases at the highest dose of MB07191 (Table 4). BUN levels were significantly lower following treatment at 300 mg/kg/day of FaraAMP and at both 300 and 1,000 mg/kg/day of MB07191.

Metabolism

AraU, an inactive catabolite of araC [33], was detected in rodent plasma at levels similar to or slightly higher than araC levels following treatment with araC or MB07133. Figure 4a shows example data from an i.v. pharmacokinetic study performed in rats.

Samples obtained from cells or animals treated with MB07191 had an additional NTP peak that eluted between ATP and FaraATP during the HPLC analysis. This peak is presumed to be FATP based on a variety of data including its co-elution with the triphosphate generated after incubation of hepatocytes with 2FA. Liver, kidney and red blood cell NTP levels were measured 24 h after the final treatment in the MB07191/FaraAMP safety study. FaraATP was undetectable in all tissues, but FATP levels were measurable and higher than they would have been after a single dose (Fig. 4b).

Continuous infusion

AraC is administered to leukemia patients by continuous intravenous infusion due to its short half-life [2, 34]. Pilot studies suggested that araCTP also has a short half life in rat hepatocytes and HepG2 cells (MacKenna, unpublished observations), thus, MB07133 was administered using a similar dosing regimen. To estimate the dose required to maintain the concentration of araCTP in the liver at or above the concentrations that caused cytotoxicity in hepatoma cells, MB07133 and araC were administered to rats by intravenous infusion for up to 48 h. MB07133, at a dose of 200 mg/kg/day, reached a steady state araCTP level in the liver that was 2–3-fold higher than the concentrations that caused cytotoxicity in hepatoma cells (Fig. 5). Bone marrow araCTP levels were below LOQ after treatment with MB07133, but were high and increased in a dose-related manner with araC infusion (Fig. 5). Since the plasma araC levels were 120-fold higher for rats infused with araC at 1,000 mg/kg/day than with MB07133 infused

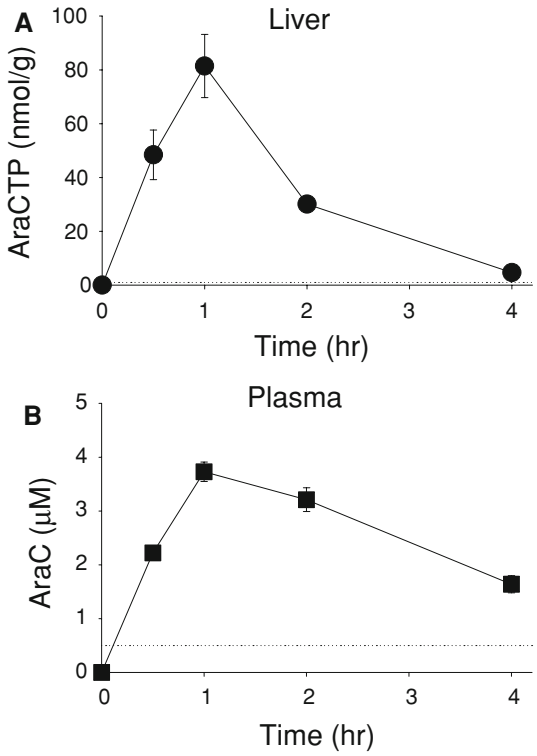


Fig. 2 Tissue distribution studies in rats after i.p. administration of MB07133(R/S) at a dose equivalent to 100 mg/kg of araC. **a** Liver araCTP and **b** plasma araC levels at 0.5, 1, 2 and 4 h after dosing. AraCTP levels were also measured in bone marrow, but were below the LOQ for all time points (5 nmol/g)

at 200 mg/kg/day, the LTI could be as much as 600-fold greater for MB07133 than for araC.

Discussion

Therapeutic strategies based on nucleoside oncolytics such as araC and FaraA have limited utility for the treatment of solid tumors including HCC because they fail to generate therapeutic levels of oncolytic NTPs at non-toxic doses [21, 38]. Our studies demonstrated that both araCTP and FaraATP are toxic to proliferating human hepatoma cells at concentrations comparable to those observed for in vitro models of leukemia [7, 10, 15, 24]. Moreover, the half-lives of the NTPs in rat hepatocytes, intact liver in both mice and rats, and HepG2 cells (MacKenna, unpublished observations) are comparable to those generated by the parent nucleosides in leukemic cells and xenograft models [2, 34]. In normal liver, concentrations that would be expected to cause toxicity in HCC cells could not be achieved when rats were dosed with araC by continuous infusion at doses up to 2,000 mg/kg/day but were slightly above this threshold when administered to mice as a bolus of 100 mg/kg/day. High NTP levels could be achieved in liver following administration of MB07133 or MB07191, which suggests that it is the initial nucleoside kinase, responsible for conversion to the NMP, that is rate limiting rather than defects in the subsequent nucleotide kinases.

HepDirect prodrugs of NMPs were generated to differentially deliver and maintain high concentrations of NTPs in hepatic cells while having low levels of these NTPs in healthy peripheral proliferative tissues [38, 40]. NTP production is limited to CYP3A-expressing cells (Fig. 6), which include normal liver and primary liver tumor, but not hepatic metastases derived from non-hepatic tissues [13]. Unlike ifosfamide, an oncolytic prodrug also activated by CYP3A, HepDirect prodrugs of NMPs generate charged metabolites following prodrug activation which confines the active metabolite, and therefore the anti-proliferative activity, to the cells catalyzing prodrug cleavage. Therefore, NTP levels are high in the liver, but low elsewhere.

Liver targeting was applied to nucleoside oncolytic agents to further enhance tumor action by harnessing the mechanistic specificity of these agents. Cytarabine and fludarabine are anti-proliferative nucleoside oncolytics,

Table 3 Effects of araC and MB07133(R/S) on NIH Swiss mice

Parameter	Vehicle	Drug	Dose [mg/kg/day]					
			3	10	30	100	300	1,000
Body weight on day 5[b] (% of day 0)	0.22 ± 1.09	AraC	1.30 ± 0.69	−0.46 ± 0.95	−1.85 ± 0.48	−7.32 ± 0.73[a]	ND	ND
		MB07133(R/S)	ND	ND	0.65 ± 0.56	−0.01 ± 1.28	1.32 ± 0.86	1.43 ± 1.10
Hematocrit (%)	43.8 ± 1.2	AraC	41.6 ± 0.6	40.3 ± 0.6[a]	35.2 ± 1.0[a]	36.5 ± 0.7[a]	ND	ND
		MB07133(R/S)	ND	ND	40.5 ± 0.7	39.5 ± 0.6	37.9 ± 0.7[a]	32.7 ± 1.3[a]
Alkaline phosphatase (IU/l)	87.6 ± 5.6	AraC	91.4 ± 4.4	85.5 ± 3.8	83.9 ± 6.1	61.6 ± 7.5[a]	ND	ND
		MB07133(R/S)	ND	ND	91.9 ± 2.2	94.3 ± 1.4	100.3 ± 4.6	92.4 ± 8.2
Creatinine (mg/dl)	0.14 ± 0.02	AraC	0.10 ± 0.00	0.11 ± 0.01	0.14 ± 0.02	0.27 ± 0.05[a]	ND	ND
		MB07133(R/S)	ND	ND	0.11 ± 0.01	0.13 ± 0.02	0.13 ± 0.02	0.10 ± 0.00

Compounds administered at doses equivalent to parent nucleoside for 5 day i.p. *ND* Not determined

[a] $P < 0.05$ to vehicle by Dunnett's post hoc test)

[b] Data previously published or described, respectively [14], included for comparison purposes

Table 4 Effects of FaraAMP and MB07191 on NIH Swiss mice

Parameter	Vehicle	Drug	Dose [mg/kg/day]			
			30	100	300	1,000
Body weight on day 5 (% of day 0)	7.64 ± 1.67	FaraAMP	4.45 ± 1.16	3.85 ± 0.95	−13.08 ± 1.63[a]	ND
		MB07191	1.51 ± 0.85[a]	1.55 ± 1.03[a]	−3.11 ± 1.67[a]	−15.59 ± 1.65[a]
AST (IU/l)	54 ± 13	FaraAMP	70 ± 16	49 ± 4	98 ± 28	ND
		MB07191	78 ± 17	113 ± 25[b]	199 ± 48[b]	730 ± 161[a,b]
ALT (IU/l)	28 ± 3	FaraAMP	25 ± 2	24 ± 2	44 ± 12	ND
		MB07191	30 ± 3	76 ± 18[b]	194 ± 62[a,b]	949 ± 200[a,b]
Alkaline phosphatase (IU/l)	129 ± 11	FaraAMP	108 ± 9	105 ± 7	115 ± 15	ND
		MB07191	94 ± 11	129 ± 8	175 ± 12	604 ± 12[a,b]
Total bilirubin (mg/dl)	1.9 ± 0.1	FaraAMP	1.7 ± 0.1	1.6 ± 0.1	2.5 ± 0.6	ND
		MB07191	1.7 ± 0.2	1.6 ± 0.1	2.2 ± 0.2	5.0 ± 0.6[a,b]
BUN (mg/dl)	21 ± 1	FaraAMP	20 ± 1	19 ± 1	13 ± 1[a]	ND
		MB07191	18 ± 1	18 ± 1	15 ± 1[a]	11 ± 1[a]

Compounds administered at doses equivalent to parent nucleoside for 5 day i.p

ND not determined

[a] $P < 0.05$ to vehicle by Dunnett's post hoc test

[b] Data transformed to improve normality and $P < 0.05$ to vehicle by Dunnett's post hoc test on transformed data

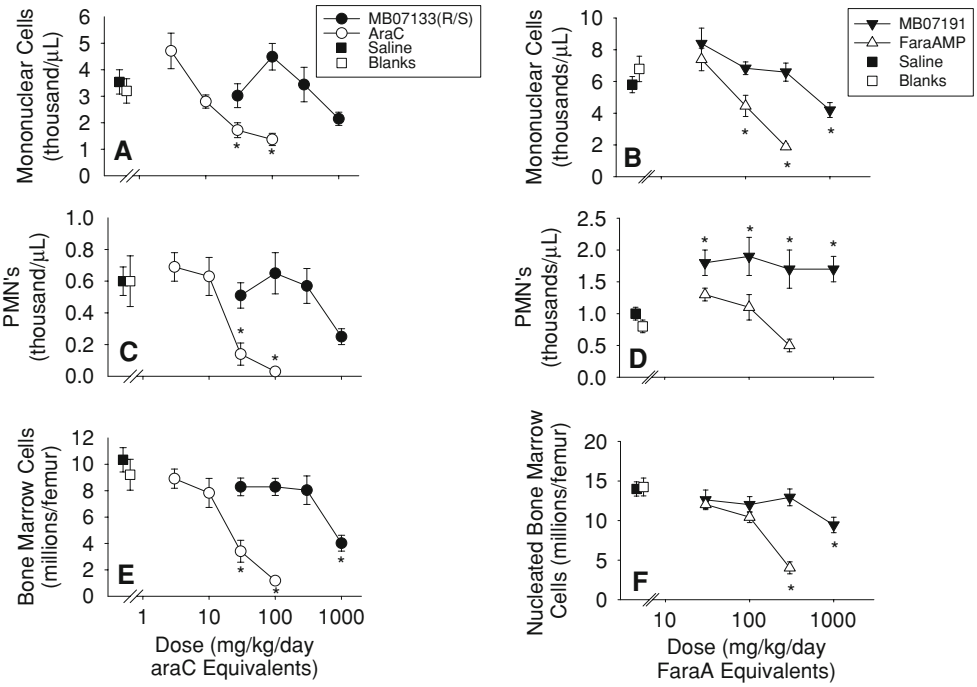


Fig. 3 Key hematology parameters in 5-day safety studies in NIH Swiss mice treated with MB07133(R/S) (**a**, **c**, **e**), MB07191 (**b**, **d**, **f**), or corresponding nucleosides. **a**, **b** Mononuclear and **c**, **d** polymorphonuclear cells (PMN's) from whole blood cell differential count. **e**, **f** Nucleated cells from a bone marrow flush. *Panel E* represents previously published data [14]. * $P < 0.05$ compared with saline-treated animals

incorporating into the DNA and thereby killing only replicating cells. Given that the healthy liver should not have a high rate of replication, we anticipate that these agents should only be toxic to the replicating hepatoma cell. As such, additional targeting, safety and ultimately efficacy should be achievable. We could not test the concept that this approach would be efficacious in animal models of HCC due to experimental limitations. In particular, hepatoma cell lines do not retain CYP3A activity [35], therefore, MB07133 and MB07191 could not be tested in xenograft models. Chemically induced and transgenic models of HCC have incomplete penetration and are asynchronous with respect to tumor accumulation [31, 39, 42]. Thus, pharmacokinetic, targeting, and safety profiles were used to predict utility of the prodrugs described here. It should be noted that the levels of cytidine deaminase are lower in rats than humans or mice and expressed in different tissues in different species [8, 37], so data generated in the rodents may represent an over estimation of the active metabolite at any specific dose. However, the metabolism is

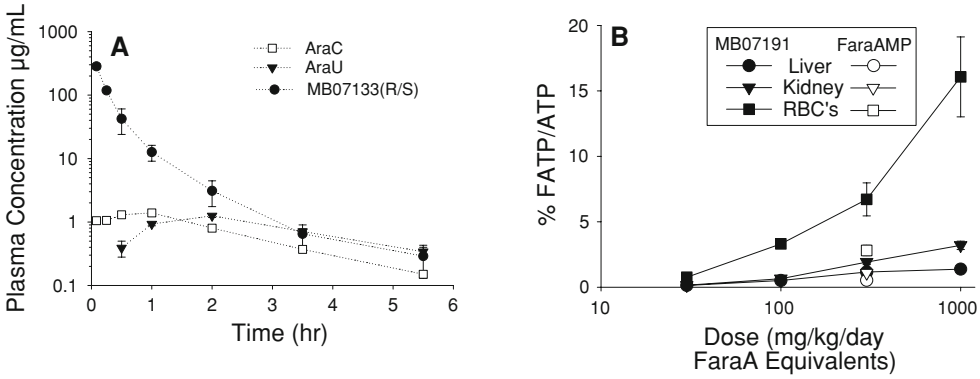


Fig. 4 **a** Plasma concentrations of araC, araU, and prodrug after a bolus intravenous administration of MB07133(R/S) to male Sprague Dawley rats at a dose equivalent to 100 mg/kg of araC. **b** FATP levels as % of ATP concentration (by peak area) in liver, kidney and red blood cells 24 h after the final dose of MB07191 or FaraAMP in the 5-day safety study. ATP levels did not change with treatment (data not shown)

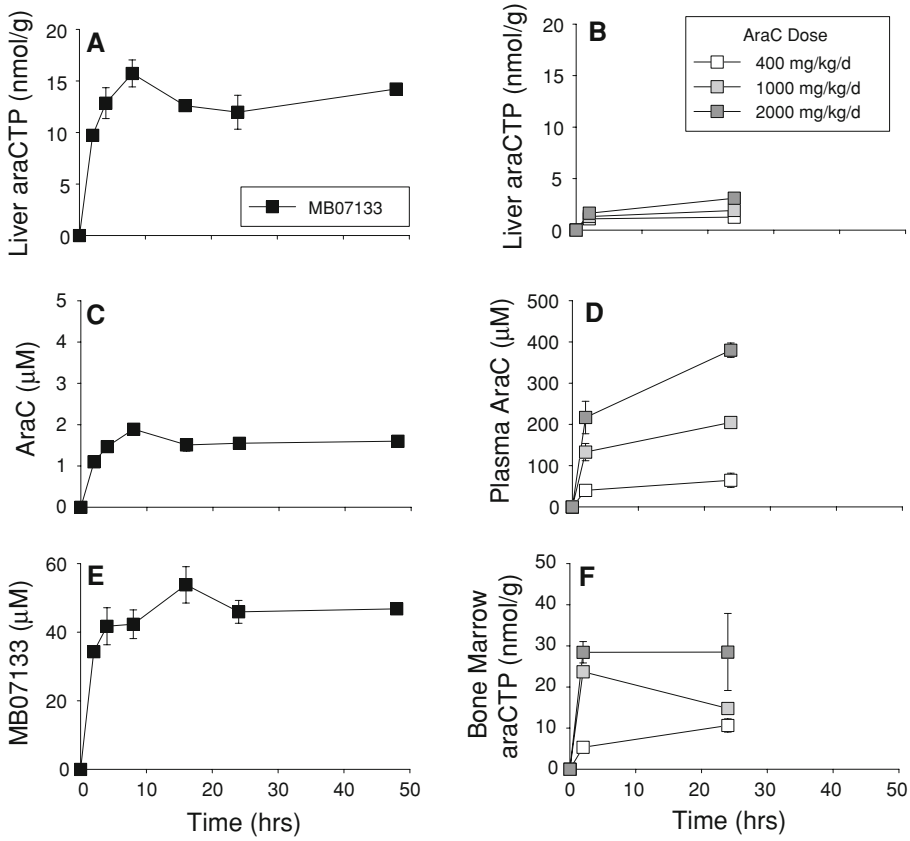


Fig. 5 Continuous infusion of MB07133 or araC in Sprague Dawley rats for up to 48 h. MB07133 was administered at a dose equivalent to 200 mg/kg/day of araC and tissues harvested to measure **a** liver araCTP, **c** plasma araC, and **e** plasma MB07133 levels. AraC was administered for 24 h at doses ranging from 400 to 2,000 mg/kg/day to assess **b** liver araCTP, **d** plasma araC, and **f** bone marrow araCTP levels

expected to be comparable for both the prodrugs and their parent nucleosides, so the relative targeting data presented here should translate across species and does so for the rat and mice data shown here.

MB07133 was well-tolerated by normal mice when administered daily for 5 days at doses that generated levels of araCTP in the liver that are expected to be cytotoxic to >80% of tumor cells [14]. The hematopoietic effects that occurred at doses ≥300 mg/kg/day of MB07133 were similar to the effects observed with araC at doses of 10–30 mg/kg/day [5]. As MB07133 is stable in plasma and not activated in cells that do not express CYP3A, the hematopoietic effects are presumed to be associated with dephosphorylation of liver araCMP/araCTP and reverse transport of araC into the plasma (Fig. 6). AraC is also likely to be deaminated to araU in the liver before release into the bloodstream (Fig. 6). AraU, a non-toxic metabolite [33], was detected at similar or slightly greater levels than araC in the in rodent studies performed with MB07133, thus further limiting peripheral exposure to araC. It should be noted that these studies were carried out in mice because rats are relatively resistant to the toxic effects of araC [9].

Prodrugs of FaraAMP were more toxic than MB07133, as demonstrated by MB07191, which elevated liver enzyme levels at doses predicted to be required for efficacy. While not completely understood, the toxicity of FaraAMP prodrugs is likely linked to unfavorable drug metabolism and accumulation of FATP (Fig. 6). FATP has

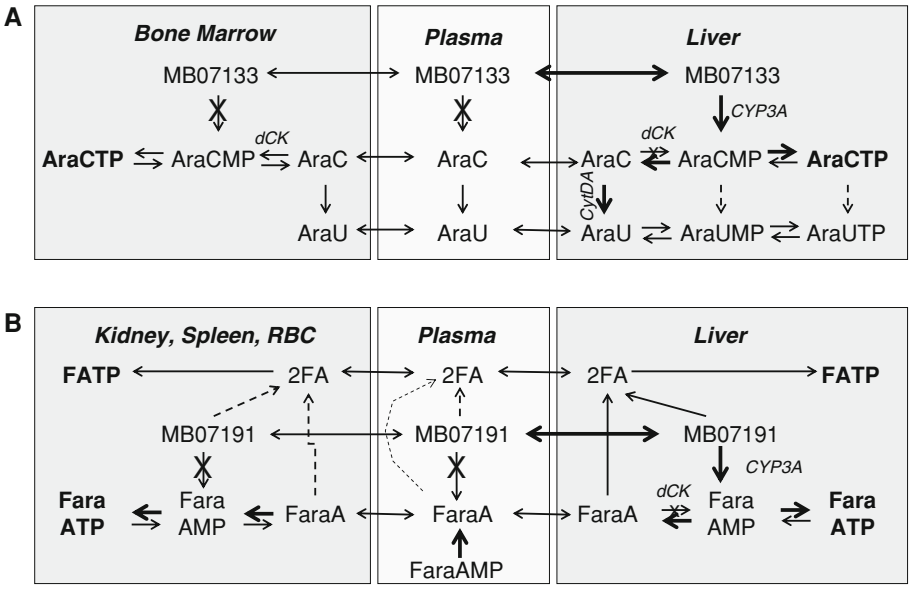


Fig. 6 Activation schema for **a** MB07133/araC and **b** MB07191/FaraA. **a** MB07133 is activated to araCMP in hepatocytes via a CYP3A mechanism; this does not occur in plasma or non-CYP3A expressing tissues such as bone marrow. AraCMP is converted to araCTP by nucleotide kinases. Catabolism of araCTP and araCMP results in araC which can be released into the circulation and/or further deaminated to araU. AraU and its metabolites are non-toxic. Plasma araC is not readily converted to araCMP in liver due to low deoxycytidine kinase (dCK) levels in the liver, but is converted to araCTP in bone marrow. **b** Activation pathways of MB07191/FaraA are similar to those for MB07133/araC. Additional metabolism pathways; both MB07191 and FaraA are metabolized to 2FA in liver and possibly elsewhere. 2FA is converted to FATP, a highly cytotoxic agent. FaraAMP is used in vivo to enhance solubility for formulation, but is rapidly dephosphorylated to FaraA in the plasma. *Solid lines* indicate reactions that have been confirmed here or in the literature. *Dashed lines* indicate reactions that may occur but data neither support nor deny. *Bold lines* indicate primary metabolic pathways

a long half-life and has been linked to toxicity of FaraAMP in other models [2].

The activation of HepDirect prodrugs shows accumulation of active metabolite correlating with CYP3A activity [27]. HCC tumors retain approximately half the CYP3A4 levels as surrounding, non-tumor liver tissue [12, 29, 43]. Some suggest that cirrhotic livers are repopulated by a unique stem-cell derived population [22]; if that is the case, MB07133 should not be toxic to those cells because they do not yet express CYP3A4 [36]. Of the 28 patients that received MB07133 in a dose escalation protocol, only 7 demonstrated treatment-related dose-limiting toxicities [23]. The majority of those were mild-to-moderate in severity, were reversible and were primarily related to the hematopoetic effects of araC. The few hepatic events that were noted involved patients with previously diagnosted liver cirrhosis and were difficult to distinguish from the normal progression of the disease. The exellent tolerability of MB07133 by patients with HCC suggests that liver toxicity may not be a significant risk in the regenerating liver.

In conclusion, MB07133, a HepDirect prodrug of araCMP, efficiently delivered the cytotoxic agent araCTP to cells expressing CYP3A. As an anti-proliferative agent, it was well tolerated by non-tumorous liver tissue, which has a low proliferation index. Hepatoma cells were susceptible to the cytotoxic effects of araCTP at intracellular levels comparable to those required to treat hematopoietic cancers. Taken together, MB07133 has excellent potential as an anti-cancer agent in primary hepatocellular carcinoma, a disease with bleak prognosis and limited treatment options. MB07133 has recently completed a Phase Ib clinical trial for the treatment of HCC [23]. Initial data suggest that MB07133 was well-tolerated in patients with no overt effects on liver function. Moreover, preliminary efficacy endpoints suggest that MB07133 could have anti-tumor activity and therefore could prove to be an important new treatment of HCC patients.

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