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Hepatic Membrane Transport of Organic Cations Using Isolated Rat Hepatocyte Membrane Vesicles: Structure–Transport Relationships[1]

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INTRODUCTION

Extensive first-pass hepatic drug extraction results in the decreased bioavailability and pharmacologic activity of therapeutic agents. Vectorial hepatobiliary excretion of drugs from blood to bile involves three separate but sequential transport processes: 1) hepatic uptake from sinusoidal blood, 2) transcellular transport, and 3) canalicular secretion. Recent studies have demonstrated that both the hepatocyte uptake and biliary secretion processes for organic anions and cations may involve carrier-mediated facilitated transport systems. Therefore, potential approaches for minimizing the extent of the hepatobiliary extraction of drugs may include chemical modifications of the molecule, once structure-transport relationships are known, or co-administration of transport inhibitors, once the mechanism(s) involved in hepatic membrane transport are elucidated.

The concept of a molecular weight threshold for the biliary excretion of xenobiotics is widely accepted; however, for organic cations, this relationship is supported by only sparse data. For monoquaternary ammonium compounds, elimination of greater than 10 % of the dose by biliary excretion occurs with compounds with molecular weights of 200 ± 50 in the rat, rabbit, and guinea pig (1, 2). For the monoquaternary cations examined by Hirom et al. (2), the molecular weights of the compounds were closely related to their lipid solubilities, resulting in a log P threshold value of about - 2. Diquaternary ammonium compounds require a molecular weight of about 500 - 600 before any substantial (> 10 %) biliary excretion is seen (3, 4).

Molecular weight (MW) or the extent of lipophilicity, though, cannot fully explain the extent of biliary elimination for monoquaternary ammoniums. For instance, tripropylmethyl ammonium (MW = 158, log P = - 2.77, estimated value) was excreted up to 20 % of the dose into bile after an intravenous bolus injection into rats, whereas the more lipophilic benzyltrimethylammonium (MW = 150, log P = - 1.56, estimated value), with a similar molecular weight, exhibited very low biliary excretion, less than 1 % of dose (5). Additionally, even hydrophilic low molecular weight monoquaternary ammoniums (MW < 156) can be substantially accumulated in the liver within 10 minutes after intravenous injection into rats, indicating that there is no clear relationship between lipophilicity and hepatic membrane uptake of those cations. In fact, studies on the hepatobiliary excretion of monoquaternary ammoniums (5) have indicated that for the high molecular weight monoquaternary ammonium compounds (MW >200), bile to liver concentration ratios exceeded 10 and bile to plasma ratios were often even higher than that, with apparent bile to plasma concentration ratios ranging from 13 to 830. These studies highlight a major difficulty in studying hepatobiliary excretion: two membrane transport steps are involved and the transport of substrates may be fundamentally different at these two domains with either step being rate-limiting.

Recent studies have used rat liver basolateral (blLPM) and canalicular (cLPM) plasma membrane vesicle preparations to characterize the hepatic transport of organic cations, such as thiamine (6), tetraethylammonium (TEA) (7), N^1-methylnicotinamide (8), choline (9), and daunomycin (10). These studies have elucidated several distinct transport systems for endo/exogenous organic cations. TEA, a monoquaternary amine, undergoes carrier-mediated transport across the blLPM and cLPM, transport processes which appear to be shared by a number of monoquaternary amine xenobiotics (7). In the present investigation we examined the inhibitory effects of a series of 22 aliphatic amine compounds on the transport of the monovalent organic cation, TEA, using rat liver blLPM and cLPM vesicle preparations. These data were then used to examine correlations between physicochemical properties (log P and molar refractivity) of inhibitors and their inhibitory effects on TEA transport.

MATERIALS AND METHODS

Materials. [^{14}C]tetraethylammonium (50 mCi/mmol) was purchased from Wizard Laboratory (Davis, CA). [^{3}H]taurocholate (2.1 Ci/mmol) and [^{3}H]daunomycin (3.3 Ci/mmol) were purchased from NEN Research Products (Boston, MA). Diethylamine hydrochloride, triethylamine hydrochloride, tetraethylammonium chloride, and tetrabutylammonium chloride were purchased from Sigma Chemical Co. (St. Louis, MO) and all other amines were purchased from Aldrich Chemical Co. (Milwaukee, WS). 2-Methoxyethanol was purchased from EM Science (Gibbstown, NJ) and the Biodegradable Counting Scintillant was purchased from Amersham Corp. (Arlington Heights, IL).

Preparation of Rat Liver Plasma Membrane Vesicles. Basolateral (blLPM) and canalicular (cLPM) rat liver plasma membrane vesicles were isolated from male Sprague-Dawley rats (Harlan Sprague Dawley Inc., Indianapolis, IN) weigh-

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ing 155 - 180 g and 200 - 250 g using the procedures of Blitzer et al. (11) and Inoue et al. (12), respectively. The purities of the vesicle preparations were routinely assessed by determining the ratios of the activities of the membrane marker enzymes, Na^+-K^+-ATPase, Mg^{+2}-ATPase, and alkaline phosphatase, in vesicle preparations compared with that in crude homogenates (11). The functionalities of vesicle preparations were confirmed by replicating the results of previous transport studies that examined Na^+-dependent [^{3}H]taurocholate uptake (11) in blLPM vesicles and *ATP*-dependent [^{3}H]taurocholate uptake (13) and [^{3}H]daunomycin uptake (10) in cLPM vesicles. Protein concentrations of the vesicle preparations were measured using the Micro Protein Determination Kit from Sigma Chemical Co. (St. Louis, MO) with bovine serum albumin as a standard. Immediately after isolation, membranes were suspended in the desired membrane suspension buffer (exact composition is designated in the legend for Table 1) at a protein concentration of 40 - 80 μg / 20μl and stored at -70 °C for up to one week before transport studies.

Uptake Experiments. The uptake of 50 μM [^{14}C]TEA was measured by a rapid filtration technique according to the procedures of Blitzer et al. (11). Frozen vesicles were quickly thawed by immersing in a 37 °C water bath and revesiculized by passing the suspension 20 times through a 25-gauge needle. Twenty μl of vesicle suspension containing 40 - 80 μg of protein were preincubated in a test tube at 37 °C for 4 minutes. Uptake experiments of [^{14}C]TEA into both blLPM and cLPM were initiated by adding 80 μl of desired incubation buffer (exact composition is given in the figure and table legends) containing 50 μM [^{14}C]TEA without (control) or with 500 μM or 5 mM of an organic cation. The tube was vortexed and returned to the water bath. After incubating for 30 seconds, the uptake of [^{14}C]TEA into the vesicles was stopped by adding 3.5 ml of ice-cold stop solution, (in mM: 100 sucrose, 100 NaCl, 10 Hepes/Tris, 0.2 $CaCl_2$, 5 $MgCl_2$ (pH 7.4) for blLPM study, and 70 sucrose, 100 K-gluconate, 76 Hepes, 70 Tris, 0.2 Ca-gluconate, 5 Mg-gluconate (pH 7.9) for cLPM study). The entire contents were rapidly filtered through a 0.45 μm HAWP 25 mm filter (Millipore Corp., Bedford, MA) presoaked in stop solution. The tube was washed one more time with 3.5 ml of ice-cold stop solution and filtered. The filter was rinsed an additional two more times with cold stop solution. The filter was dissolved in 1 ml of 2-methoxyethanol, mixed with 4 ml of scintillant and counted in a Packard Tri-Carb 1900CA Liquid Scintillation Analyzer (Packard Instrument Co., Downers Grove, IL). Nonspecific binding of [^{14}C]TEA to the filter paper and vesicle membranes was corrected by subtracting uptake values obtained from a blank in which 3.5 ml of ice-cold stop solution was mixed with 20 μl of vesicles prior to the addition of incubation buffer containing radiolabelled TEA, followd by the above same procedure.

Data Analysis. The data was compared by the Student's t-test and differences were considered to be statistically significant when $P < 0.05$. Log P and molar refractivity (MR) values for amines were calculated by the fragment method and the additive substituent constant method, respectively (14). The correlation coefficients were calculated using a computer program (QSAR-PC: PAR, Biosoft, Cambridge, U.K.).

RESULTS

The purities of both vesicle preparations were assessed based on the enrichment ratios of marker enzyme activities. In blLPM, Na^+-K^+-ATPase, Mg^{+2}-ATPase and alkaline phosphatase activities were increased 15.2 ± 1.25, 4.1 ± 0.2 and 2.8 ± 0.2 fold (mean ± SE, n = 16), respectively, with reference to those of the initial crude homogenate. These values are similar to those reported by Blitzer and Donovan (11); however, there is some enrichment in cLPM enzyme activity (2.8-4.4 fold). Enzyme activities for Na^+-K^+-ATPase, Mg^{+2}-ATPase and alkaline phosphatase in cLPM were increased 1.3 ± 0.3, 14.7 ± 2.5, and 18.1 ± 2.1 fold (n = 6), respectively, compared with those in the crude homogenate. To confirm the transport-functionalities of blLPM and cLPM, [^{3}H]taurocholate uptake in the presence and absence of an inwardly directed sodium gradient into blLPM, and [^{3}H]taurocholate and [^{3}H]daunomycin uptake in the presence and absence of *ATP* into cLPM were measured. The initial uptake rates of those substrates into both blLPM and cLPM were markedly enhanced in the presence of the corresponding driving forces (11, 13, 10), indicating that the functionalities of vesicles were well maintained (data not shown). The inside negative membrane electric potential dependent transport of [^{14}C]TEA into blLPM and the pH dependent transport of [^{14}C]TEA into cLPM were also replicated, and similar transport characteristics were observed, as described by Moseley et al. (7).

The inhibitory effects of a series of amines on the uptake of [^{14}C] TEA (50 μM) into blLPM vesicles 30 seconds after incubation with the amines (500 μM or 5 mM), with an inside negative membrane potential as the driving force, are presented in Table I, along with the physicochemical properties of the organic amines, including molecular weight (MW), log P, and MR. Primary amines (MW < 73) did not show any statistically significant inhibition at either 500 μM or 5 mM concentrations (10- and 100- fold higher than TEA). Secondary and tertiary amines with butyl-, pentyl- and hexyl-substituents and quaternary ammoniums except a tetramethyl derivative produced statistically significant inhibition on TEA transport at both concentrations examined. The correlation coefficients (r) for log P and MR, with % control values of those amines (500 μM) showing statistically significant inhibitory effects (total 11 cations), were 0.69 and 0.80, respectively. Using 5 mM concentrations of amines, the same r value (0.87) was estimated for the relationship between log P or MR with % control values. Percent control values for the 22 amines estimated at a 5 mM concentration in both vesicle preparations were plotted against their MW, log P and MR (Fig. 1).

In cLPM (Table I), the H^+ exchange transport system for TEA was inhibited by only quaternary ammoniums (MW > 130) at a concentration of 500 μM, with r values of 0.85 and 0.83 for the relationship between log P values and % control values, and MR and % control values, respectively. However, these correlations did not reach statistical significance (as assessed by a t-test), due to the fact that only 5 quaternary ammoniums significantly inhibited TEA transport. At the higher concentration (5 mM), dihexyl-, tributyl-, tripentyl-, and trihexylamines also showed significant inhibition of TEA transport (all molecular weights > 185), in ad-

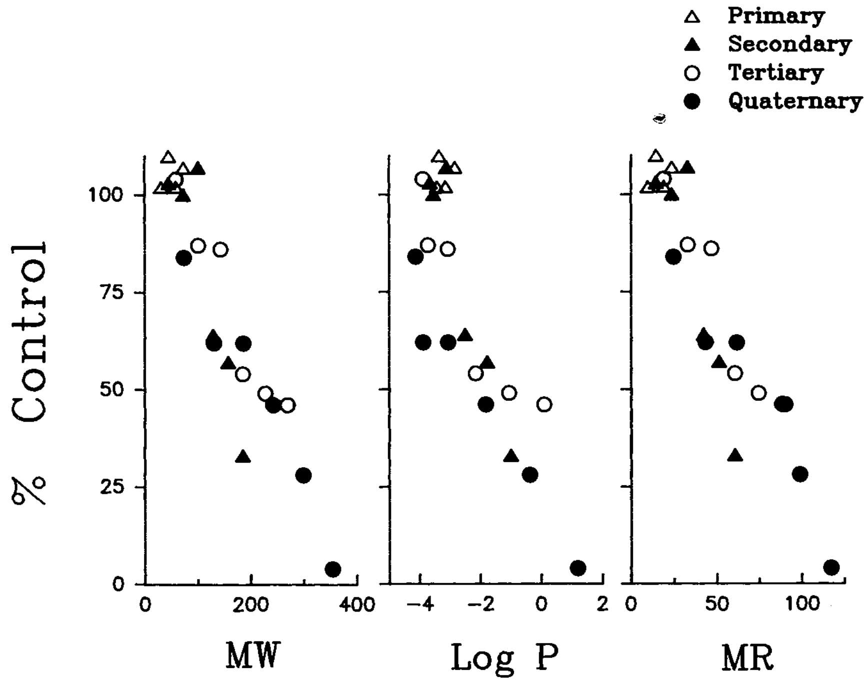

Fig. 1. Relationship between inhibition of [^{14}C]TEA uptake in blLPM by a series of 22 aliphatic amines and their physicochemical properties, molecular weight (MW), log P, and molar refractivity (MR). Data is presented as % control values determined at 5 mM inhibitor concentration and represents the mean values obtained from experiments in 2 - 4 separate blLPM vesicle preparations. The correlation coefficients (r) for the relationship between MW and % control values, log P and % control values, and MR and % control values for those amines showing statistically significant inhibitory effects (total of 11 cations) were all 0.87 ($p < 0.001$).

dition to the previous 5 quaternary ammoniums. At this higher inhibitor concentration, the r value for the relationship between MR and % control values was 0.90, while that for log P and % control values was 0.81. A plot of the relationship between cation inhibitory potency (at 5 mM concentration) and MW, log P and MR is given in Fig. 2. Molecular weights were highly correlated with the calculated log P and MR values for aliphatic amines investigated in the present study ($r = 0.90$ and 1.00, respectively).

Significant correlations between cation inhibitory potency (% control values) determined in the two membrane domains were observed ($r = 0.76$ for 500 μM conc. and $r = 0.88$ for 5 mM conc.) (Fig. 3).

DISCUSSION

A large number of drugs contain one or more secondary-, tertiary-, or quaternary- amine moieties. Quaternary ammonium moieties are positively charged at all pH's, while secondary- and tertiary- amines with pKa values of 9.4 or greater will also be protonated more than 99 % at physiological pH. Carrier-mediated systems for the transport of such ionized compounds across both the sinusoidal and canalicular membranes of hepatocytes have been characterized for a number of substrates, including thiamine (6), TEA (7), N^1-methylnicotinamide (8), and choline (9). TEA, a model monoquaternary amine, is transported across the blLPM by a carrier-mediated system stimulated by an inside-negative membrane potential and across the cLPM by a process driven by an electroneutral organic cation: H$^+$ exchange. Transport of TEA into both blLPM and cLPM vesicles can be inhibited by tributylmethylammonium, procainamide ethobromide (both monoquaternary amines) and vecuronium (a diquaternary amine), suggesting that the TEA carrier-mediated transport systems may be shared by a range of mono- and di- quaternary ammonium xenobiotics (7).

In the present investigation, we examined the relationships between molecular weight, log P, and MR and inhibition of TEA transport across rat liver blLPM and cLPM for a series of monovalent primary, secondary, tertiary, and quaternary amines. To our knowledge, this study represents the first attempt to evaluate the physicochemical determinants of hepatic transport in the two hepatocyte membrane domains, namely, the sinusoidal (basolateral) and canalicular membranes. In blLPM vesicle preparations, molecular weight, lipophilicity (log P) and MR were all highly correlated with inhibition of TEA transport. The relationship between MW and transport is likely due to the strong correlations seen between MW and various physicochemical properties of molecules, such as log P, MR, Van der Waals volume or surface area. Secondary, tertiary, and quaternary amines with molecular weights ≥ 129 (and MR ≥ 42) all significantly inhibited the transport of TEA in blLPM vesicles. Tripropylamine represented an outlier in this series since it did not inhibit transport even at a 5 mM concentration (100- fold higher concentration than TEA), despite the

Table I. Inhibitory Effects of a Series of Aliphatic Amines on $[^{14}C]$Tetraethylammonium Uptake into Rat Liver blLPM and cLPM Vesicle Preparations at Two Different Concentrations[a]

				% Control of $[^{14}C]$TEA uptake			
				blLPM		cLPM	
Cation	MW[b]	logP	MR[c]	500 μM	5 mM	500 μM	5 mM
Primary amines							
Methylamine	31.1	−3.41	10.02	101	102	97	102
Ethylamine	45.1	−3.35	14.64	105	110	101	97
Propylamine	59.1	−3.14	19.26	96	102	95	102
Butylamine	73.1	−2.83	23.88	106	107	103	84
Secondary amines							
Dimethylamine	45.1	−3.65	14.64	101	103	103	103
Diethylamine	73.1	−3.53	23.88	99	100	97	108
Dipropylamine	101.2	−3.11	33.12	93	107	106	89
Dibutylamine	129.2	−2.49	42.36	72*	64*	102	96
Dipentylamine	157.3	−1.77	51.60	65*	57*	101	81
Dihexylamine	185.4	−0.99	60.84	62*	33*	91	65*
Tertiary amines							
Trimethylamine	59.1	−3.89	19.26	101	104	92	94
Triethylamine	101.2	−3.71	33.12	81	87	91	89
Tripropylamine	143.3	−3.08	46.98	90	86	100	103
Tributylamine	185.4	−2.15	60.84	87*	54*	103	62*
Tripentylamine	227.4	−1.07	74.70	62*	49*	100	68*
Trihexylamine	269.5	0.10	88.56	60*	46*	81	42*
Quaternary amines							
Tetramethylammonium	74.2	−4.13	24.91	97	84	84	86
Tetraethylammonium	130.3	−3.89	43.39	72*	62*	76*	71*
Tetrapropylammonium	186.4	−3.05	61.87	57*	62*	63*	63*
Tetrabutylammonium	242.5	−1.81	80.35	52*	46*	78*	66*
Tetrapentylammonium	298.6	−0.37	98.83	54*	28*	46*	34*
Tetrahexylammonium	354.7	1.19	117.31	14*	4*	35*	21*

[a] Membrane vesicles were suspended in media either containing (in mM) 100 sucrose, 100 K-gluconate, 10 Hepes/Tris and 0.2 Ca-gluconate (pH 7.4) for blLPM studies, or containing (in mM) 82 sucrose, 100 K-gluconate, 91 MES, 29 Tris, 14 Hepes and 0.2 Ca-gluconate (pH 5.9) for cLPM studies, and all vesicles were preincubated with valinomycin (5 μg/mg protein) for 20 minutes at 37°C. Uptake of 50 μM $[^{14}C]$TEA at 37°C was measured in media either containing (in mM) 100 sucrose, 100 Na-gluconate, 10 Hepes/Tris and 0.2 Ca-gluconate (pH 7.4) for blLPM studies, or containing (in mM) 70 sucrose, 100 K-gluconate, 76 Hepes, 70 Tris and 0.2 Ca-gluconate (pH 7.9) for cLPM studies, in the presence of 500 μM or 5 mM cations. Data are the % control values and presented as mean values. Each uptake experiment was generally performed in quadruplicate in 2 to 4 different vesicle preparations; in a few cases with the 5 mM concentration of inhibitors, only one study was performed. The mean coefficient of variability for replicate control uptake data was 6.6%, while that for the replicate data for the 500 μM concentration of inhibitors was 17%. Similar variability was seen for the 5 mM concentration of inhibitors.

[b] Molecular weight.

[c] Molar refractivity.

* $p < 0.05$.

fact that its molecular weight was 143.3, log P = - 3.08, and MR = 46.98.

Our studies examining inhibition of TEA transport in cLPM vesicle preparations demonstrated differences from that observed in blLPM vesicle preparations. At the lower concentration of inhibitors examined, only quaternary ammoniums inhibited TEA transport. Tetraethylammonium with a molecular weight of 130.3, log P of - 3.89, and MR of 43.49 represented the substrate with the smallest MW, log P and MR values that significantly inhibited uptake. However, tertiary amines with molecular weight, log P or MR values greater than those of tetraethylammonium (such as tripentylamine or trihexylamine) did not inhibit TEA transport. Although significant relationships between molecular weight, log P and MR with inhibitory effects are evident when examining quaternary ammonium compounds, such relationships are poorer if the data from secondary-, tertiary- and quaternary- amines are evaluated together and suggest higher MW and MR thresholds for secondary and tertiary amines compared with quaternary amines.

The strongest correlations between physicochemical properties and inhibition of TEA transport in cLPM were observed with MR. The MR of a substance reflects the fundamental property of molecular volume. Studies on physicochemical properties of compounds that modulate P-glycoprotein mediated multidrug resistance in human leukemic

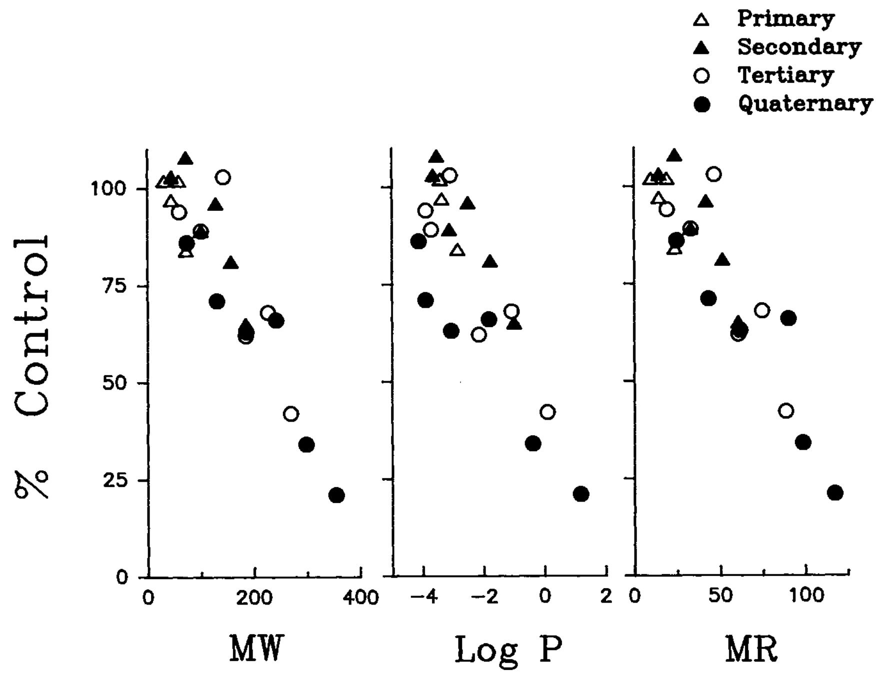

Fig. 2. Relationship between inhibition of [^{14}C]TEA uptake in cLPM by a series of 22 aliphatic amines and their physicochemical properties, molecular weight (MW), log P, and molar refractivity (MR). Data is presented as % control values determined at 5 mM inhibitor concentrations and represents the mean values obtained from experiments in 2 - 4 separate cLPM vesicle preparations. The r values for the relationship between MW and % control values, log P and % control values, and MR and % control values for those cations showing statistically significant inhibition (total of 9 cations) were 0.90 ($p<0.001$), 0.81 ($p<0.005$) and 0.90 ($p<0.001$), respectively.

cells (15, 16) indicated that, in addition to lipid solubility, effective modulators have similar MR values. However, since log P and MR were highly correlated with each other ($r = 0.90$) for the present series of alkyl amines, a broader range of amine compounds with more structural diversity needs to be investigated, in order to distinguish the influence of log P and MR.

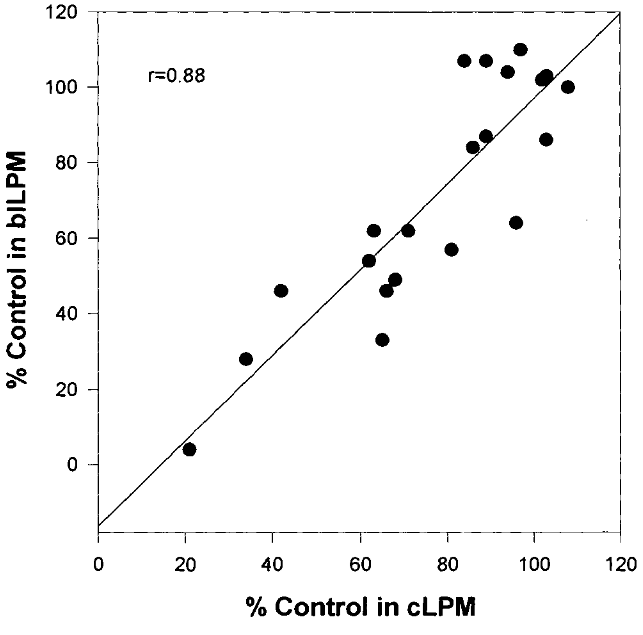

Fig. 3. Relationship between % control values in blLPM and % control values in cLPM for the 22 organic cations evaluated at a 5 mM concentration ($r=0.88$, $p<0.001$).

In conclusion, the results of the present investigation indicate the importance of lipophilicity and MR as physicochemical determinants for substrates that inhibit the transport of the model monovalent aliphatic amine TEA, across both the blLPM and cLPM. Further studies are necessary to elucidate the importance of steric properties of amines, as suggested by the high correlations between MR and inhibitory potency on hepatic membrane transport, and to further evaluate the differences in transport across the two liver membrane domains.

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