Arch Pharm Res Vol 25, No 6, 984-988, 2002

Archives of Pharmacal Research
http://apr.psk.or.kr

Effect of Nitric Oxide on the Sinusoidal Uptake of Organic Cations and Anions by Isolated Hepatocytes

Im-Sook Song[1], In-Kyoung Lee[1], Suk-Jae Chung[1], Sang-Geon Kim[2], Myung-Gull Lee[2], and Chang-Koo Shim[1]

[1]Department of Pharmaceutics and [2]Department of Pharmacology, College of Pharmacy, Seoul National University, Seoul 151-742, Korea

(Received September 10, 2002)

The issue of whether or not the presence NOx (NO and oxidized metabolites) in the hepatocytes at pathological levels affects the functional activity of transport systems within the sinusoidal membrane was investigated. For this purpose, the effect of the pretreatment of isolated hepatocytes with sodium nitroprusside (SNP), a spontaneous NO donor, on the sinusoidal uptake of tributylmethylammonium (TBuMA) and triethylmethyl ammonium (TEMA), representative substrates of the organic cation transporter (OCT), and taurocholate, a representative substrate of the Na^+/taurocholate cotransporting polypeptide (NTCP), was measured. The uptake of TBuMA and TEMA was not affected by the pretreatment, as demonstrated by the nearly identical kinetic parameters for the uptake (i.e., V_{max}, K_m and CL_{linear}). The uptake of mannitol into hepatocytes was not affected, demonstrating that the membrane integrity remained constant, irregardless of the SNP pretreatment. On the contrary, the uptake of taurocholate was significantly inhibited by the pretreatment, resulting in a significant decrease in V_{max}, thus providing a clear demonstration that NOx preferentially affects the function of NTCP rather than OCT on the sinusoidal membrane. A direct interaction between NOx and NTCP or a decrease in Na^+/K^+ ATPase activity as the result of SNP pretreatment might be responsible for this selective effect of NOx.

Key words: SNP, NOx, Isolated hepatocytes, Taurocholate, Na^+/K^+ ATPase

INTRODUCTION

Hepatobiliary excretion is one of the major routes for drug elimination in the body. The first step in the excretion process involves the hepatic uptake across the sinusoidal membrane. Na-independent [e.g., an organic cation transporter (OCT) and an organic anion transporter (OAT)] and Na-dependent carrier-mediated transport processes [e.g., Na^+/taurocholate cotransporting polypeptide (NTCP)] are responsible for the hepatic uptake of various organic cations and organic anions (Trauner *et al.*, 1999; Hooiveld *et al.*, 2001).

NO, a chemically reactive free radical, is endogenously synthesized from L-arginine by NO synthases, and immediately interacts with superoxide to form oxidized NO derivatives, including peroxynitrites, a more reactive one. These NOx (NO and oxidized metabolites) further interact with a variety of DNA molecules, lipids, thiol and tyrosine residues, aromatic amino acids and transition metals (Geng *et al.*, 1994; Hess *et al.*, 1994; McDonald and Moss, 1994), leading to the pertubation of the biological functions of numerous receptors and enzymes (Dimmeler *et al.*, 1994; Kuhn and Arthur, 1996; Mohr *et al.*, 1996). Functional impairment by these NOx of the transport of glutamate, serotonin and reduced folates has also been reported (Pogun *et al.*, 1994; Trotti *et al.*, 1996; Smith *et al.*, 1999), suggesting that functional impairment of transporters in phathological states likely involve massive NOx production. Recently, a variety of hepatic dysfunctions such as inflammation, liver cirrhosis and chronic exposure to cholesterol and alcohol (Vos *et al.*, 1997; Wei *et al.*, 2002; Kim *et al.*, 2002; Baraona *et al.*, 2002) have been reported to induce the massive production of NOx in heptaocytes via the induction of NO synthetases.

Despite frequent exposure of NOx in both normal and

Correspondence to: Chang-Koo Shim, Department of Pharmaceutics, College of Pharmacy, Seoul National University, San 56-1, Shinlim-dong, Kwanak-gu, Seoul, 151-742, Korea
E-mail: shimck@plaza.snu.ac.kr

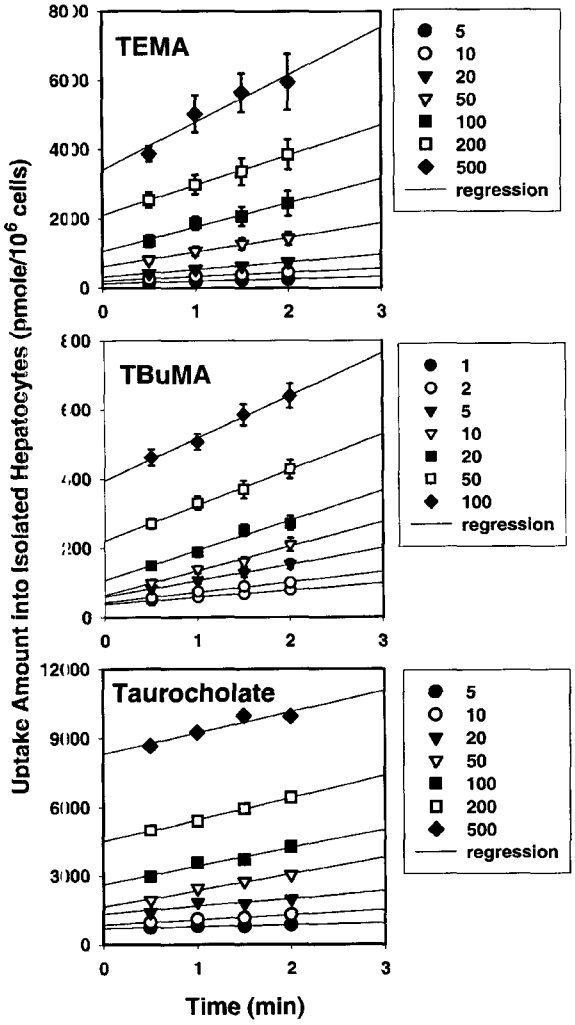

Fig. 1 Temporal uptake of TEMA, TBuMA and taurocholate into hepatocytes across the sinusoidal membrane for various concentration ranges (5-500 μM for TEMA and taurocholate and 1-100 μM for TBuMA). Each data point represents the mean ± S.D. of triplicate measurements from six different hepatocyte preparations.

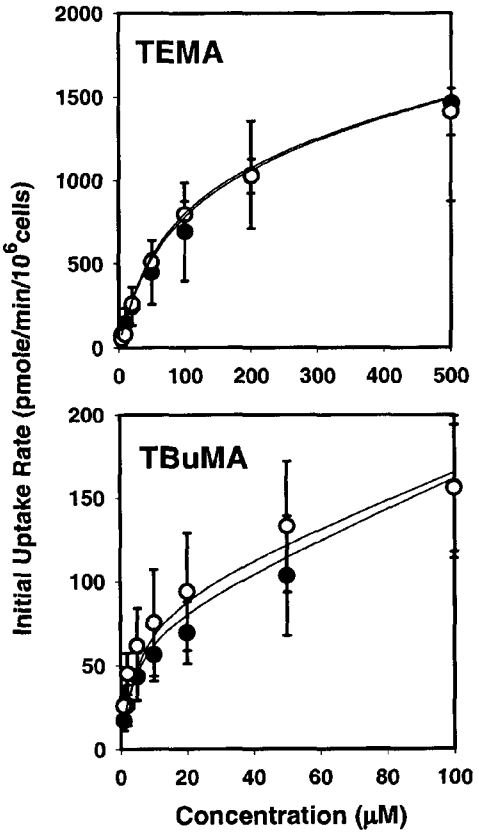

Fig. 2. Effect of 0.4 mM ascorbic acid on the initial rate of TEMA (5-500 μM) and TBuMA (1-100 μM) across the sinusoidal membrane of hepatocytes. The initial uptake rate was obtained from the slope of the plot for the first 3 min period using a linear regression analysis. Each data point represents the mean ± S.D. of triplicate measurements from three different hepatocyte preparations. ○: control, ●: in the presence of 0.4 mM ascorbic acid.

diseased states, the issue of whether or not NOx affects the functional activity of transport systems at its pathological level is not known. The objective of this study, therefore, was to investigate the influence of NOx on the sinusoidal uptake of triethylmethylammonium (TEMA) and tributylmethylammonium (TBuMA), representative substrates for OCT (Han *et al.*, 1999), and taurocholate, a representative substrate for NTCP (Trauner *et al.*, 1999), in isolated hepatocytes.

MATERIALS AND METHODS

Materials

[^{3}H TBuMA (0.5 Ci/mmole) and [^{3}H]TEMA (0.5 Ci/mmole) were synthesized as previously described (Song *et al.*, 1999). [^{3}H]Taurocholate (2 Ci/mmole) and [^{14}C]mannitol (50 mCi/mmole) were purchased from NEN Life Science Inc. (Boston, MA). All other reagents including sodium nitroprusside (SNP) and ascorbic acid were purchased from Sigma Chemical Co. (St. Louis, MO)

Preparation of isolated hepatocytes

Isolated hepatocytes were freshly prepared using the procedure described by Han *et al.* (1999) with minor modifications. Briefly, male SD rats (200-250 g, Dae-Han Biolink, Taejon, Korea) were anesthetized with ketamine (Ketalar®, Yuhan Co., Kyounggi-do, Korea) and acepromazine (Sedaject®, Samu Chemical Co., Kyounggi-do, Korea). After catheterization of the portal vein, the liver was perfused for 10 min with an oxygenated, Ca-free buffer (137 mM NaCl, 5.4 mM KCl, 0.5 mM NaH_2PO_4, 0.4 mM Na_2HPO_4, 4.2 mM $NaHCO_3$, 5 mM glucose, 0.5 mM EDTA, 10 mM HEPES, pH 7.2, 37°C) at a flow rate of 30 ml/min. The perfusion was then switched to a recir-

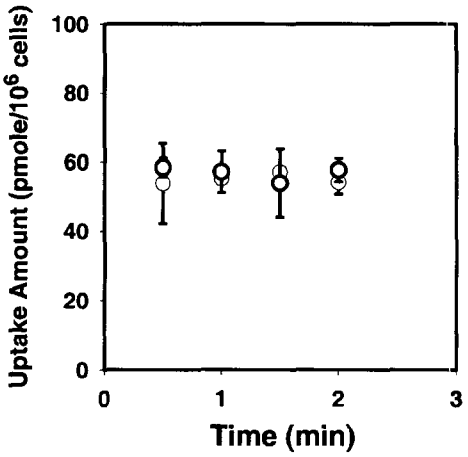

Fig. 3. Effect of SNP pretreatment (10 mM for 20 min) on the sinusoidal uptake of 50 μM mannitol. ○: control, ●: SNP pretreatment. Each data point represents the mean ± S.D. of triplicate measurements from three different hepatocyte preparations.

culating system with an oxygenated, calcium and collagenase containing buffer (137 mM NaCl, 5.4 mM KCl, 5 mM $CaCl_2$, 0.5 mM NaH_2PO_4, 0.4 mM Na_2HPO_4, 4.2 mM $NaHCO_3$, 10 mM HEPES, 0.05% collagenase, pH 7.2, 37°C). The perfusion was continued for 15 min, and the liver was then transferred to a beaker filled with the perfusion buffer (80 ml) and gently disrupted. After filtration through a nylon filter (50 μm pore size) and cooling on ice, the resulting cell preparation was washed by the addition of an ice-cold incubation medium, and centrifuged (50 g, 2 min, 4°C). This procedure was repeated three times. The resulting pellet was resuspended in ice-cold HEPES supplemented Hank's buffer (137 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.8 mM $MgSO_4$, 0.5 mM NaH_2PO_4, 0.4 mM Na_2HPO_4, 4.2 mM $NaHCO_3$, 5 mM glucose, 10 mM HEPES, oxygenated, pH 7.4) to give a typical concentration of 3×10^6 cells/ml. The viability of hepatocytes was in excess of 90% as measured by trypan blue exclusion.

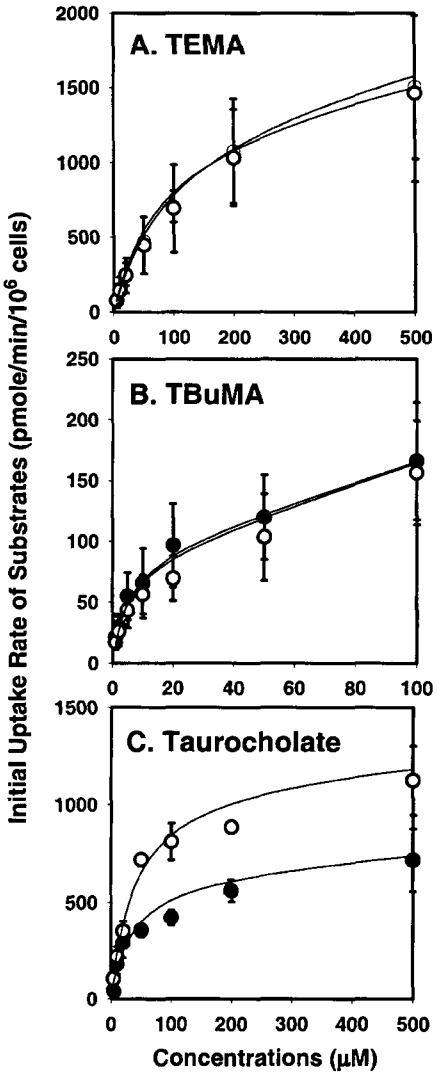

Fig. 4. Effect of SNP pretreatment (10 mM for 20 min) on the concentration dependent sinusoidal uptake of TEMA (5-500 μM), TBuMA (1-100 μM) and taurocholate (5-500 μM) in isolated hepatocytes. ○: control, ●: SNP pretreatment. Each data point represents the mean ± S.D. of triplicate measurements from six different hepatocyte preparations.

Estimation of uptake clearance

The cell suspension was incubated in a medium containing 10 mM SNP, a NO donor, and 0.4 mM ascorbic acid for 20 min at 37°C. After the incubation, SNP and ascorbic acid were removed by centrifugation, and the cell suspension (2 ml, 3.0×10^6 cells/ml) was further incubated in the medium for 5 min at 37°C. An 20 μl aliquot of various concentrations of [^{3}H]TEMA, [^{3}H]TBuMA and [^{3}H]taurocholate was added to the suspension to give medium concentrations of 5-500 μM for TEMA and taurocholate, and 1-100 μM for TBuMA. Aliquots (200 μl) of the suspension were removed at 30, 60, 90 and 120 sec, and placed in a centrifuge tube (0.4 ml) containing KOH (50 μl, 3 M) and silicone/mineral oil (100 μl, density 1.015). Subsequent centrifugation at 50 g for 5 sec resulted in the sedimentation of hepatocytes through the oil layer into the KOH layer. The bottom KOH layer, containing the hepatocytes, was collected and the radioactivity determined and the amount of substrates in the hepatocytes (expressed in pmole/10^6 cells) was plotted against time. The initial uptake rate of substrates into the hepatocyte was calculated from the linear portion (i.e., generally up to 2 min) of the plot using a linear regression analysis. The rate of initial uptake was then plotted against the initial concentration of the substrate in the medium. A nonlinear regression analysis was performed to the following equation using WINNONLIN (version 3.1, Pharsight Co., Mountainview,

Table I. Effect of SNP Pretreatment on the Sinusoidal Uptake of TEMA, TBuMA and Taurocholate in Isolated Hepatocytes[a]

		Control	SNP
TEMA	V_{max} (pmole/min/10^6 cells)	1256 ± 341	1344 ± 269
	K_m (μM)	75.05 ± 29.0	94.72 ± 36.7
	CL_{int} (μl/min/10^6 cells)[b]	18.89 ± 3.96	16.27 ± 2.96
	CL_{linear} (μl/min/10^6 cells)	0.8207 ± 0.227	0.8976 ± 0.172
TBuMA	V_{max} (pmole/min/10^6 cells)	90.33 ± 15.9	84.18 ± 10.28
	K_m (μM)	5.124 ± 0.819	4.653 ± 1.48
	CL_{int} (μl/min/10^6 cells)	16.75 ± 4.91	17.62 ± 2.76
	CL_{linear} (μl/min/10^6 cells)	0.7942 ± 0.358	0.8402 ± 0.313
Taurocholate	V_{max} (pmole/min/10^6 cells)	1137 ± 101	651.8 ± 153*
	K_m (μM)	39.24 ± 8.37	34.06 ± 8.94
	CL_{int} (μl/min/10^6 cells)	28.98 ± 5.10	19.13 ± 6.78*
	CL_{linear} (μl/min/10^6 cells)	0.2559 ± 0.117	0.2550 ± 0.139

[a]Each data point represents the mean ± S.D.
[b]CL_{int} was calculated from V_{max}/K_m
*Statistically different from the control ($p<0.01$)

CA).

$$V_o = V_{max} \cdot S/(K_m + S) + CL_{linear} \cdot S \quad \text{(equation 1)}$$

Where V_o is the initial rate of uptake of the substrates (pmole/min/10^6 cells), and S the initial concentration of substrates in the medium (μM). V_{max} and K_m represent the maximum uptake rate and the medium concentration at half of the maximal uptake rate, respectively, and CL_{linear} represents the linear uptake clearance. The intrinsic clearance for the uptake (CL_{int}) was obtained from V_{max}/K_m.

Data analysis

All data are expressed as means ± S.D. The Student's unpaired t-test was used to test the difference between treatments. In all cases, $p < 0.01$ was accepted as representing a statistical difference.

RESULTS AND DISCUSSION

Sequential unit transport processes (e.g., sinusoidal uptake into hepatocytes, sinusoidal efflux, intracellular transport, binding to intracellular proteins and canalicular excretion) are involved in the hepatobiliary excretion of substrates. In order to exclude the effect of other sequential unit processes from sinusoidal uptake, the initial rates of uptake of substrates into hepatocytes were measured. The uptake of TEMA, TBuMA and taurocholate into hepatocytes increased linearly with time up to 2 min for all concentrations examined (Fig. 1).

Because NO is relatively unstable and reactive, it is impossible to determine the exact concentration of NO in biological fluids. For this reason, we used SNP as a NO donor, which spontaneously releases NO in biological fluids and tissues. For maximal NO production, ascorbic acid was also used as a reducing agent (Bates *et al.*, 1991). To investigate the issue of whether the presence of ascorbic acid affects the sinusoidal uptake of substrates or not, the sinusoidal uptake of TEMA, TBuMA and taurocholate (data not shown) in the presence and absence of 0.4 mM ascorbic acid was measured. In all cases, the presence of ascorbic acid had no effect on the data (Fig. 2). Pretreatment with 10 mM SNP for 20 min had no influence on the hepatic uptake of mannitol (50 μM) (Fig. 3), indicating that the treatment has no effect on membrane leakage.

The initial rates of uptake of TEMA, TBuMA and taurocholate, showed a concentration dependency in the concentration range of 5-500 μM, 1-100 μM and 5-500 μM, respectively, consistent with the OCT mediated transport for TEMA and TBuMA (Han *et al.*, 1999) and NTCP mediated transport for taurocholate (Hooiveld *et al.*, 2001) (Open circles in Fig. 4A and B). SNP pretreatment had no apparent effect on the concentration-initial uptake rate profiles of TEMA and TBuMA (Closed circles in Fig. 4A and B). As a result, similar kinetic parameters (V_{max}, K_m, CL_{int} and CL_{linear}) were obtained for the control and SNP treated groups in the sinusoidal uptake of both compounds (Table I). On the other hand, the concentration-initial uptake rate profiles for taurocholate (5-500 μM) were significantly altered by the SNP pretreatment (Closed circles in Fig. 4C), resulting in a 43 % decrease in the value of V_{max} (1137 ± 101 vs 651.8 ± 153 pmole/min/10^6 cells) without influencing the value of K_m. As a result, a 34% decrease in the value of CL_{int} (28.98 ± 5.10 vs 19.13

± 6.78 μl/min/10^6 cells) was observed. Similar to the cases for TEMA and TBuMA, the CL_{linear} for taurocholate was not changed by the SNP pretreatment (Table I).

Collectively, Na^+-dependent taurocholate uptake was decreased, while Na^+-independent TEMA and TBuMA uptake remained unchanged, by the SNP pretreatment. It has been reported that the incubation of brain and kidney cells with SNP results in a decrease in the activity of membrane associated Na^+/K^+ ATPase in the cells (Qayyum *et al.*, 2001; Liang and Knox, 1999). Such a decrease may lead to a decrease in the Na^+ gradient in the cells, a driving force for the NTCP-mediated transport of substrates. Thus, the decrease in Na^+ gradient due to a decrease in the activity of Na^+/K^+ ATPase appears to be responsible for the NPCP selective effect of the SNP pretreatment. The fact that V_{max} was decreased but K_m was not for the uptake of taurocholate provides support for this hypothesis. However, a direct interaction between NO and NTCP cannot be excluded from the possible mechanism.

ACKNOWLEDGEMENT

This work was supported by a grant (R01-2000-00180) from the Basic Research Program of the Korea Science and Engineering Foundation.

REFERENCES

Baraona, E., Zeballos, G. A., Shoichet, L., Mak, K. M., and Lieber, C.S., Ethanol consumption increases nitric oxide production in rats, and its peroxynitrite-mediated toxicity is attenuated by polyenylphosphatidylcholine. *Alcohol Clin. Exp. Res.*, 26, 883-889 (2002).

Bates, N. J., Baker, M. T., Guerra, R. Jr., and Harrison, D. G., Nitric oxide generation from nitroprusside by vascular tissue. Evidence that reduction of the nitroprusside anion and cyanide loss are required. *Biochem. Pharmacol.*, 11, S157-165 (1991).

Dimmeler, S., Mechmer, U. K., Tiegs, G., and Brune, B., Modulation of glyceraldehydes-3-phosphate dehydrogenase in Salmonella abortus equi lipopolysaccaride-treated mice. *Eur. J. Pharmacol.*, 267, 105-112 (1994).

Geng, Y.J., Pertersson, A. S., Wennmalm, A., and Hansson, G. K., Cytokine-induced expression of nitric oxide synthase results in nitrosylation of heme and nonheme iron proteins in vascular smooth muscle cells. *Exp. Cell Res.*, 214, 418-428 (1994).

Han, Y. H., Chung, S. J., and Shim, C. K., Canalicular membrane transport is primarily responsible for the difference in hepatobiliary excretion of trirthylmethylammonium and tributylmethylammonium in rats. *Drug Metab. Dispos.*, 27, 872-879 (1999).

Hess, D. T., Lin, L. H., Freeman, J. A., and Norden, J. J., Modification of cystein residues within G0 and other neuronal proteins by exposure to nitric oxide. *Neuropharmacology*, 33, 1283-1292 (1994).

Hooiveld, G. J. E. J., Van Montfort, J. E., Meijer, D. K. F., and Muller, M., Function and regulation of ATP-binding cassette transport proteins involved in hepatobiliary transport. *Eur. J. Pharm. Sci.*, 12, 525-543 (2001).

Kim, J. W., Kang, K. W., Oh, G. T., Song, J., Kim, N. D., and Pak, Y. K., Induction of hepatic inducible nitric oxide synthase by cholesterol *in vivo* and *in vitro*. *Exp. Mol. Med.*, 34, 137-144 (2002).

Kuhn, D. M. and Arthur, R. E. Jr., Inactivation of brain tryptophan hydroxylase by nitric oxide. *J. Neurochem.*, 67, 1072-1077 (1996).

Liang, M. and Knox, F. G., Nitric oxide reduces the molecular activity of Na^+/K^+-ATPase in opossum kidney cells. *Kidney Int.*, 56, 627-634 (1999).

McDonald, L. J. and Moss, J., Nitric oxide and NAD-dependent protein modification. *Mol. Cell Biochem.*, 138, 201-206 (1994).

Pogun, S., Baumann, M. H., and Kuhar, M. J., Nitric oxide inhibits [^{3}H]dopamine uptake. *Brain Res.*, 641, 83-91 (1994).

Qayyum, I., Zubrow, A. B., Ashraf, Q. M., Kubin, J., Delivoria-Papadopoulos, M., and Mishra, O. P., Nitration as a mechanism of Na^+/K^+-ATPase modification during hypoxia in the cerebral cortex of the guinea pig fetus. *Neurochem. Res.*, 26, 1163-1169 (2001).

Smith, S. B., Huang, W., Chancy, C., and Ganapathy, V., Regulation of the reduced folate transporter by nitric oxide in cultured human retinal pigment epithelial cells. *Biochem. Biophys. Res. Commun.*, 257, 279-283 (1999).

Song, I. S., Chung, S. J., and Shim, C. K., Different activity of ATP dependent transport across the canalicular membrane for tributylmethylammonium and triethylmethylammonium as a potential mechanism of the preferential biliary excretion for the tributylmethylammonium in the rat. *Pharm. Res.*, 16, 540-544 (1999).

Trauner, M., Meier, P. J., and Boyer, J. L., Molecular regulation of hepatocellular transport systems in cholestasis. *J. Hepatol.*, 31, 165-178 (1999).

Trotti, D., Rossi, D., Gjesdal, O., Levy, L. M., Racagni, G., Fanbolt, N. C., and Volterra, A., Peroxynitrite inhibits glutamate transporter subtypes. *J. Biol. Chem.*, 271, 5976-5979 (1996).

Vos, T. A., Gouw, A. S., Klok, P. A., Havingga, R., van Goor, H., Huitema, S., Roelofsen, H., Kuipers, F., Jansen, P. L., and Moshage, H., Differential effects of nitric oxide synthase inhibitors on endotoxin-induced liver damage in rats. *Gastroentrology*, 113, 1323-1333 (1997).

Wei, C., Khoo, H., Lee, K., and Hon, W., Dfferential expression and localization of nitric oxide synthases in cirrhotic livers of bile duct-ligated rats. *Nitric Oxide*, 7, 91-102 (2002).