Enhancement of Cytotoxicity of Doxorubicin by Verapamil in the Hepatic Artery Infusion for Liver Tumors in Rats

Masaru Miyazaki, M.D., Tsukasa Shimoda, M.D., Hiroshi Itoh, M.D., Takashi Kaiho, M.D., Katsuhiro Iinuma, M.D., Takashi Koyama, M.D., Kohji Nakagawa, M.D., Katsuhiko Andoh, M.D., Satoru Anbiru, M.D., Satoshi Ohtawa, M.D., Akira Ogata, M.D., Norio Yasuda, M.D., Shinichi Hayashi, M.D., and Nobuyuki Nakajima, M.D.

Background. **The calcium channel blocker has been demonstrated to be effective in the accumulation and retention of chemotherapeutic agents in tumor cells.**

Methods. **The effect of verapamil on cytotoxicity of doxorubicin was investigated in a hepatic artery infusion (HAI) for liver tumors of Walker 256 carcinosarcoma in rats. Doxorubicin was infused by way of a hepatic artery by a bolus injection intra-arterially (IA) (1 mg/kg) and a continuous infusion intra-arterially (CIA) (6 mg/kg/day for 6 days).**

Results. **Doxorubicin increased 90% and 66% in tumor tissue following HAI of verapamil by a bolus and continuous infusion ($P < 0.05$), respectively. However, no enhancement of the accumulation of doxorubicin in the tumor tissue was found in an intravenous administration of verapamil. The CIA infusion of verapamil with doxorubicin inhibited the tumor growth by 73% in comparison with doxorubicin only ($P < 0.05$). Verapamil administered intravenously (IV) could not induce this inhibitory effect. The CIA administration of verapamil reduced the serum concentration by 45% ($P < 0.001$) in comparison with the CIV route. Furthermore, the administration of verapamil did not increase the accumulation of doxorubicin in the normal liver and heart tissues. No enhancement of bone marrow suppression and hepatic biochemical influence by doxorubicin was revealed by the concomitant use of verapamil.**

Conclusions. **The continuous HAI of verapamil remarkably enhanced the cytotoxicity of HAI with doxorubicin for the treatment of hepatic tumor without aggravating the side effects induced by doxorubicin.**

From The First Department of Surgery, School of Medicine, Chiba University, Chiba, Japan.

Address for reprints: Masaru Miyazaki, M.D., The First Department of Surgery, School of Medicine, Chiba University, 1-8-1 Inohana, Chuoh, Chiba, 260, Japan.

Accepted for publication March 1, 1993.

Key words: verapamil, hepatic artery infusion, doxorubicin, calcium blocker. *Cancer* 1993; 72:349–54.

The effect of the calcium channel blocker on the accumulation and retention of chemotherapeutic agents in tumor cells has been demonstrated by Tsuruo et al.[1,2] This effect could achieve the enhancement of cytotoxicity and overcome the multiple drug resistance in cancer chemotherapy. However, clinical application carries the risk of influencing the cardiovascular organ when the calcium channel blocker is administered systemically in doses sufficient to enhance antitumor effects. Intra-arterial (IA) infusion of cancer chemotherapeutic agents through a feeding artery overcomes the systemic administration of these agents in the antitumor effect for localized tumors, especially in hepatic tumors. The hepatic artery infusion (HAI) of the calcium channel blocker may not significantly influence the cardiovascular organ so much, despite the high drug level in the liver.

This study assesses whether the concomitant administration of the calcium channel blocker in hepatic artery infusion of chemotherapeutic agents for liver tumor treatment can enhance antitumor effects.

Materials and Methods

Animals and Chemicals

Sixty male Wistar rats (Shizuoka Experimental Animal Laboratory, Shizuoka, Japan) weighing 200–250 g were obtained and fed ad libitum. Verapamil, the calcium channel blocker, was provided by Tokyo Eisai Kogyo

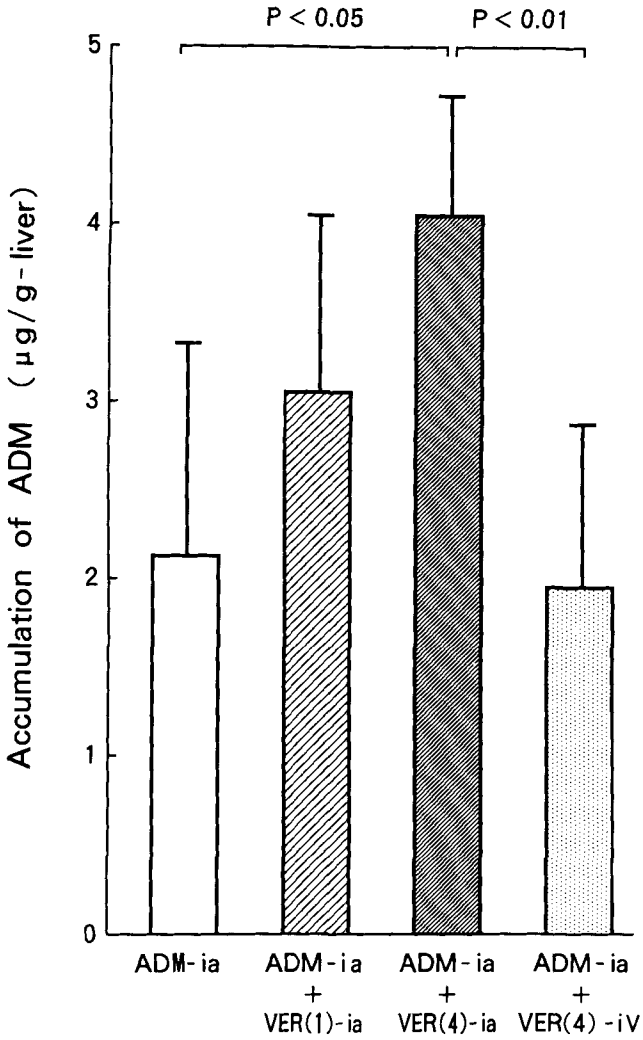

Figure 1. Effect of verapamil administration on the accumulation of doxorubicin through the hepatic artery. The hepatic arterial bolus injection of verapamil significantly enhances the accumulation of doxorubicin in the tumor tissue. Data are shown as the mean ± standard deviation (n = 5).

Co Ltd, Tokyo, and doxorubicin was provided by Kyowa Kogyo Co Ltd, Tokyo.

Liver Tumor Model

Walker 256 carcinosarcoma maintained in rats was sampled and dispersed to single cells by 0.2% trypsin solutions (Sigma, St. Louis, MO). Under ether anesthesia, 1 × 106 tumor cells were inoculated into the left lateral lobe of the liver. The rats were subjected to therapeutic experiments 9 days after tumor cell inoculation, when liver tumor grew to 0.49 mm in a mean diameter and 760 mg in a mean tumor weight.

Bolus HAI Studies

In 20 rats, polyethylene catheters (IDO, 28 mm; OD, 0.61 mm; Intramedic, PE10) were canulated into the hepatic artery in relaparotomy 15 days after tumor cell inoculation under pentobarbital anesthesia as previously reported.[3] Four experimental groups differed in the infusates of hepatic artery infusion. Doxorubicin was administered IA (1 mg/kg) in all experimental groups with simultaneous verapamil infusion IA (1 mg/kg, 4 mg/kg) or IV (4 mg/kg). After the bolus infusion, the IA catheter was withdrawn and the hepatic artery was ligated, then the abdomen was closed. The tumor, liver, and heart tissues were sacrificed 2 hours after HAI for assessment of doxorubicin tissue levels by a high-performance liquid chromatography (HPLC) method using reversed phase column, which was reported by Matsushita et al.[4]

Continuous HAI Studies

Nine days after tumor cell inoculation, the abdomen was reopened under the pentobarbital anesthesia in 40 rats. The catheter was inserted into the hepatic artery as in the bolus infusion study and was connected to an osmotic pump (Alza, CA) for a continuous IA infusion. The osmotic pump was placed subcutaneously and the abdomen was closed. Doxorubicin was administered continuously intra-arterially (CIA) (0.6 mg/kg/day), and the concomitant infusion of verapamil CIA (1.5 mg/kg/day) or continuously intravenously (CIV) (1.5 mg/kg/day) for 6 days. Two osmotic pumps were implanted subcutaneously in the group of CIA doxorubicin plus CIV verapamil. The hepatic artery was ligated in the control group without HAI. Rats were sacrificed 15 days after the tumor cell inoculation, and tumor weight and doxorubicin levels of the tumor, liver, and heart tissues were measured. Serum concentrations of verapamil were measured in the groups of CIA doxorubicin plus CIA verapamil and CIA doxorubicin plus CIV verapamil by a HPLC method reported by Kuwada et al.[5] For assessing the influence of a concomitant use of verapamil on the normal liver and bone marrow function, peripheral venous blood was sampled after 6 days of continuous HAI in the groups of control, CIA doxorubicin, and CIA doxorubicin plus CIA verapamil. White and red blood cells were counted, and the activity of serum glutamic-pyruvic transaminase and total bilirubin, albumin, and hepaplastin levels was measured. All rats could survive during the experimental period following the treatment modality.

Statistical Analysis

The data were analyzed by ANOVA using Fisher's least squares difference method,[6] except for a comparison of the serum verapamil concentrations, which were evaluated by Student *t* test. Significance was attributed to $P <$

Table 1. Doxorubicin Tissue Levels in the Normal Liver and the Heart 2 Hours After the Hepatic Artery Infusion of Doxorubicin

	Doxorubicin tissue levels			
Organ	*IA doxorubicin*	*IA doxorubicin + IA verapamil* (1)	*IA doxorubicin + IA verapamil* (4)	*IA doxorubicin + IV verapamil* (4)
Normal liver (n = 5)	1.77 ± 0.40	2.32 ± 0.52	1.94 ± 0.92	1.49 ± 0.61
Heart (n = 5)	3.18 ± 0.95	2.54 ± 0.57	2.83 ± 0.57	2.55 ± 0.21

Values are given as μg/g (mean ± SD). No significant differences among the groups. IA: intraarterially; IV: intravenously.

0.05. All data are expressed as the mean ± standard deviation of the mean.

Results

Doxorubicin Tissue Levels in the Bolus HAI

The tumor tissue levels of doxorubicin were significantly higher in the group of IA doxorubicin plus IA verapamil(4) than in the groups of IA doxorubicin and IA doxorubicin plus IV verapamil(4) (4.07 ± 0.66 μg/g versus 2.14 ± 1.23 μg/g; $P < 0.05$ and versus 1.96 ± 0.91 μg/g; $P < 0.01$) as shown in Figure 1. No differences were found between the levels in the group of IA doxorubicin plus IA verapamil(1) (3.04 ± 1.00 μg/g) and those in other groups. The concomitant administration of verapamil in the bolus HAI with doxorubicin could not increase the accumulation of doxorubicin in the normal liver and heart tissues when infused intra-arterially or even intravenously (Table 1).

Doxorubicin Tissue Levels in the Continuous HAI

As in the bolus HAI studies, the tumor tissue levels of doxorubicin were significantly higher in the group of CIA doxorubicin plus CIA verapamil than those in the group of CIA doxorubicin (0.48 ± 0.08 μg/g versus 0.29 ± 0.07 μg/g; $P < 0.05$). But there was no difference between the groups of CIA doxorubicin plus CIA verapamil and CIA doxorubicin plus CIA verapamil (0.27 ± 0.15 μg/g) (Fig. 2). However, the concomitant administration of verapamil in the continuous HAI of doxorubicin did not influence doxorubicin accumulation in the normal liver and heart tissues (Table 2).

Effects of Verapamil on Tumor Growth in the Continuous HAI

Tumor cell inoculation of Walker 256 carcinosarcoma made a solitary hepatic tumor with a mean diameter of 2.0 cm and a mean weight of 3.66 g 15 days after inoculation. In this study, the tumor weights in the control group were slightly less than those in the rats only inoculated with tumor cells without hepatic artery ligation. The tumor weights in the group of CIA doxorubicin were less than those in the control group (0.98 ± 0.41 g versus 1.35 ± 0.82 g) but not significantly. However, the tumor weights were significantly lower in the group of CIA doxorubicin plus CIA verapamil (0.24 ± 0.14 g)

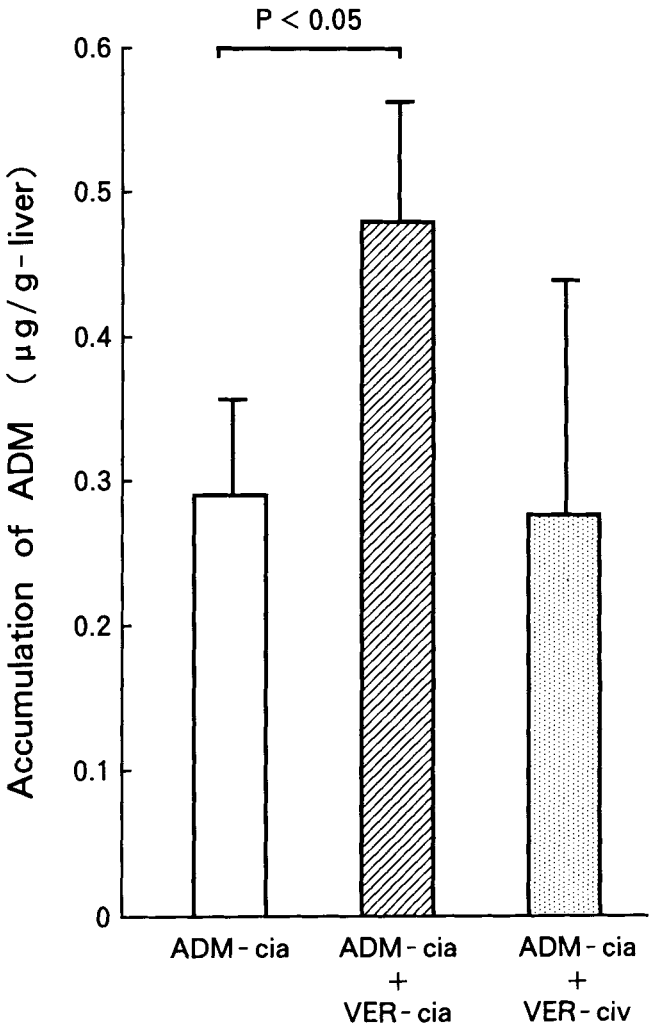

Figure 2. Effect of verapamil on the accumulation of doxorubicin in the tumor tissue following the continuous hepatic arterial infusion of doxorubicin. The enhancement of accumulation of doxorubicin in the tumor tissue is achieved by the intra-arterial administration of verapamil but not by the intravenous one. Data are shown as the mean ± standard deviation (n = 5).

Table 2. Doxorubicin Tissue Levels in Normal Liver and Heart After the Continuous Hepatic Artery Infusion of Doxorubicin

	Doxorubicin tissue levels		
Organ	*CIA doxorubicin*	*CIA doxorubicin + CIA verapamil*	*CIA doxorubicin + IV verapamil*
Normal liver (n = 4)	0.36 ± 0.11	0.46 ± 0.10	0.41 ± 0.27
Heart (n = 4)	0.34 ± 0.10	0.23 ± 0.13	0.50 ± 0.27

Values are given as μg/g (mean ± SD). No significant differences among the groups. Tissues were sampled 6 days after the induction of the continuous hepatic artery infusion. IV: intravenously; CIA: continuous intraarterially.

than those in the control group ($P < 0.05$), the CIA verapamil group (1.08 ± 0.17 g; $P < 0.001$), and the CIA doxorubicin group ($P < 0.05$). The systemic administration of verapamil with CIA doxorubicin (the group of CIA doxorubicin plus CIV verapamil) could not suppress tumor growth as did the intra-arterial administration of verapamil (0.94 ± 0.44 g; $P < 0.05$) (Fig. 3). No obvious differences of changes in body weight were found among experimental groups.

Comparison between the Serum Levels of Verapamil Administered IA and IV

The serum levels of verapamil were remarkably lower in the IA infusion than those in the IV one (31.3 ± 1.6 ng/ml versus 56.7 ± 4.0 ng/ml; $P < 0.001$) as shown in Figure 4.

Blood Counts and Hepatic Biochemistries Following HAI with Doxorubicin and Verapamil

The IA administration of doxorubicin significantly decreased white blood cell counts compared with the control group. However, no obvious differences were found among the three groups in other blood cell counts and hepatic biochemistries (Table 3).

Discussion

The extent of penetration into and accumulation and retention within tumor cells of some antitumor agents is the most important determinant of the cytotoxicity of these drugs.[7] Tsuruo et al.[1] have reported that verapamil, a calcium influx blocker, greatly enhances the cellular level of vincristine and vinblastine in P388 leukemia cells. This is especially apparent in P388/vincristine, where there is an inhibition of the vincristine efflux function of the cells. Verapamil has also been demonstrated to enhance doxorubicin cytotoxicity in P388 cells resistant to vincristine and doxorubicin.[2] The enhanced accumulation of vincristine and doxorubicin is directly related to a marked enhancement of the cytotoxicity of these drugs. Verapamil seems to interfere with the drug efflux function of tumor cells common to vincristine and doxorubicin.[2] The mechanism of the inhibitory effect on the drug efflux function by verapamil modulates the function of P-glycoprotein[8,9] of a mem-

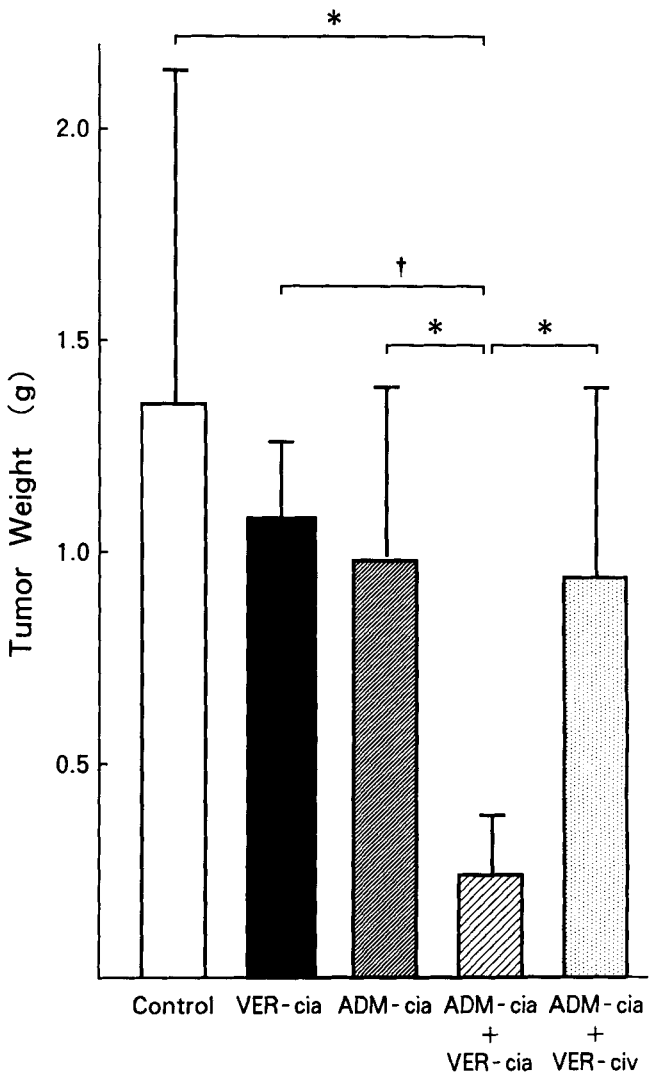

Figure 3. Tumor weights following continuous hepatic arterial infusion of doxorubicin with or without the concomitant administration of verapamil. Significant inhibition of tumor growth is achieved by the intra-arterial infusion of verapamil but not by the systemic one. Data are shown as the mean ± standard deviation (n = 5). *: $P < 0.05$; †: $P < 0.001$.

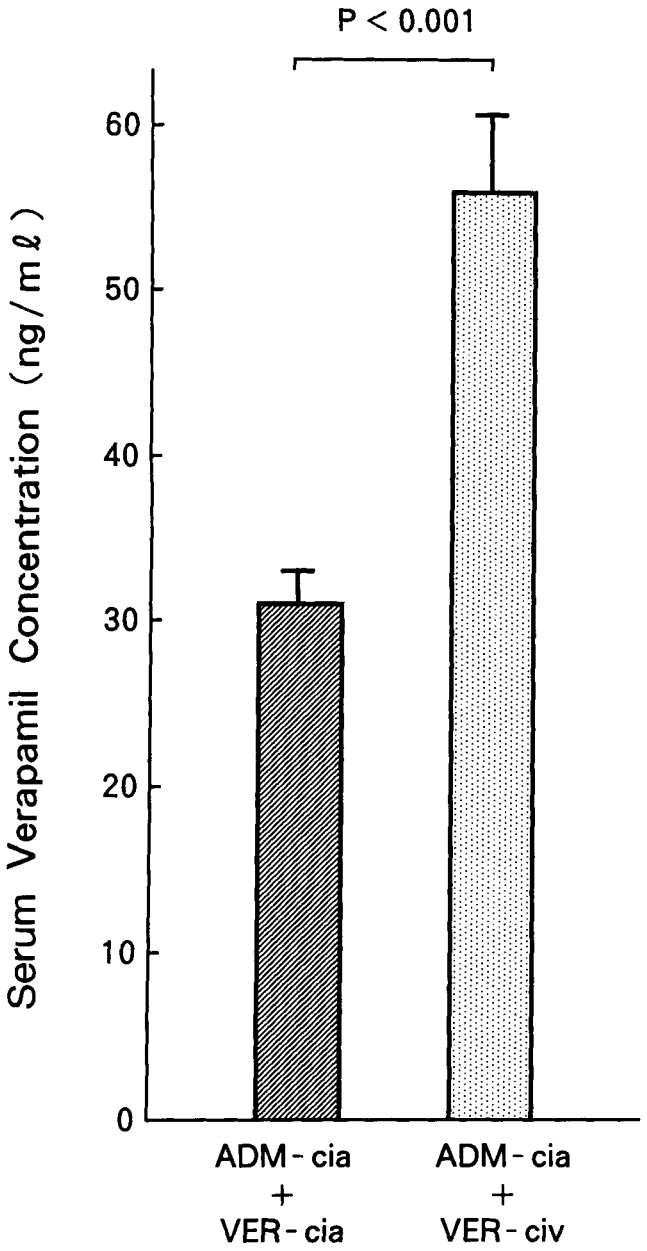

Figure 4. The serum levels of verapamil when administered continuously with the hepatic arterial infusion and the systemic intravenous one. Data are shown as the mean ± standard deviation (n = 5).

brane glycoprotein, a pump molecule that transports hydrophobic anticancer agents outside the cells.[7,10]

The concentration of verapamil of more than 500–1000 ng/ml is necessary to enhance accumulation of anticancer agents in tumor cells, as reported in in vitro experimental studies.[11,12] Clinical doses of verapamil of 0.1–0.2 mg/kg exhibiting an antiarrhythmic effect have been reported to induce a serum verapamil level of 100–200 ng/ml when administered IV.[13] To achieve a serum verapamil level of more than 500–1000 ng/ml in clinical cases, a large dose of verapamil inducing an influence on the cardiovascular organ has to be administered IV. This is a major problem for the clinical application of the calcium blockers to enhance the cytotoxicity of anticancer agents. To overcome this problem, we performed hepatic artery infusion of verapamil with anticancer agents for the treatment of hepatic tumors. When administered through the hepatic artery, the dose of some drugs reaching the liver is 10-fold compared to systemic administration.[14] This study shows that in a bolus infusion of verapamil through the hepatic artery, a dose of 4 mg/kg was necessary to obtain the enhancing effect on doxorubicin accumulation in the hepatic tumor tissues. Thom et al.[15] revealed that tumor doxorubicin levels were not increased with hepatic artery infusion of verapamil and doxorubicin in rabbits with hepatic VX-2 tumors. In their study, two doses of verapamil, 1 and 2 mg/kg, were evaluated, the doses of which could not increase tumor doxorubicin levels by a bolus infusion in our study. Although this effective dose of 4 mg/kg of verapamil is 20- to 40-fold larger than the dose used clinically, it is difficult to apply the bolus HAI of verapamil for clinical cases. However, the dose used in the continuous HAI of verapamil in this study was 0.06 mg/kg/hr, which was effective in suppressing tumor growth and increasing the drug accumulation in the tumor tissues. The continuous HAI of 0.06 mg/kg/hr of verapamil induced a serum level of 31 ng/ml, which is safe enough for a clinical application. Todd et al.[16] reported that in rats, the continuous administration of verapamil IV produced a serum drug level of 290 ng/ml with a reduction of mean arterial blood pressure of only 10%. Also in clinical cases, the CIV administration of verapamil produced a minor influence on the cardiovascular organ in doses of 0.125 mg/kg/hr and 0.15 mg/kg/hr by Reiter et al.[13] and Dalton et al., respectively.[17] It seems that if verapamil is administered continuously through the hepatic artery with cancer chemotherapeutic agents, the enhancement of cytocidal effect could be induced without a deteriorative influence upon the cardiovascular organ.

As previously reported, satisfactory results could not be obtained by clinical application of the calcium blockers such as verapamil in cancer chemotherapy, especially in solid tumors.[18,19] However, solid malignant tumors have been revealed to express P-glycoprotein.[20] In these investigations, the intravenously administered doses of verapamil was less than 0.30 mg/kg/hr, which is assumed to be too low to achieve an effective level of verapamil in tumor tissues. P-glycoprotein has been revealed to be expressed not only in tumor cells, but also in normal human cells, such as those of the adrenal, kidney, intestine, and liver.[20,21] In the concomitant administration of verapamil with anticancer agents, the enhanced toxicity of anticancer agents acts

Table 3. Blood Counts and Hepatic Biochemistries After the Hepatic Artery Infusion of Doxorubicin and Verapamil

Parameter	Control	CIA doxorubicin	CIA doxorubicin + CIA verapamil
		$P < 0.001$	
	$P < 0.05$		
WBC (10^3/mm^3)	10.1 ± 0.6	6.6 ± 2.7	5.2 ± 1.4
RBC (10^4/mm^3)	866.0 ± 53.0	813.0 ± 72.0	765.0 ± 36.0
GPT (kU/ml)	3.0 ± 11.0	40.0 ± 17.0	26.0 ± 5.0
Total bilirubin (mg/dl)	1.2 ± 0.2	1.2 ± 0.5	0.9 ± 0.2
Albumin (g/dl)	4.2 ± 0.2	4.2 ± 0.2	4.1 ± 0.3
Hepaplastin (%)	57.0 ± 3.0	57.0 ± 5.0	60.0 ± 2.0

Values are given as mean ± SD (n = 5). Blood was sampled 6 days after the induction of the continuous hepatic artery infusion. CIA: continuous intraarterially; WBC: white blood cells; RBC: red blood cells; GPT: glutamic-pyruvic transaminase.

against normal cells. However, Smith et al.[22] demonstrated that verapamil at concentrations within the clinically acceptable levels does not enhance the sensitivity of human bone marrow cells to anticancer agents. In this study, doxorubicin accumulation in the liver and heart tissues was not enhanced by verapamil administration, and neither bone marrow suppression nor hepatic dysfunction was found.

For the treatment of liver cancer, the continuous HAI of calcium blockers with anticancer agents seems to be a useful therapeutic modality to enhance the antitumor cytotoxicity.

References

1. Tsuruo T, Iida H, Tsukagoshi S, Sakurai Y. Overcoming of vincristine resistance in P388 leukemia in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Res* 1981; 41:1967–72.
2. Tsuruo T, Iida H, Yamashiro M, Tsukagoshi S, Sakurai Y. Enhancement of vincristine and adriamycin-induced cytotoxicity by verapamil in P388 leukemia and its resistant sublines to vincristine and adriamycin. *Biochem Pharmacol* 1982; 31:3138–40.
3. Miyazaki M, Makowka L, Falk RE, Falk J, Inaba T. Comparison of in-vivo thermo chemotherapy of isolated rat liver through portal vein and hepatic artery. *Can J Surg* 1983; 26:224–8.
4. Matsushita Y, Iguchi H, Kiyosaki T, Tone H, Ishikura T, Takeuchi T, et al. A high performance liquid chromatographic method of analysis of 4'-0-tetrahydropyra-nyladriamycin and their metabolites in biological samples. *J Antibiot (Tokyo)* 1983; 36:880–6.
5. Kuwada M, Tateyama T, Tsutsumi J. Simultaneous determination of verapamil and its seven metabolites by high performance liquid chromatography. *J Chromatogr* 1981; 222:507–11.
6. Miller JG. Simultaneous statistical inference. 2nd ed. New York: Springer-Verlag, 1981: 90–4.
7. Sirotnak FM, Chello PL, Brockman RW. Potential for exploitation of trans port system in anticancer drug design. *Methods Cancer Res* 1979; 16:381–447.
8. Gottesman MM, Pastan I. The multidrug transporter, a double-edged sword. *J Biol Chem* 1988; 263:12,163–6.
9. Bradley G, Juranka RF, Ling V. Mechanism of multidrug resistance. *Biochem Biophys Acta* 1988; 948:87–128.
10. Cornwell HM, Pasran I, Gottesman MM. Certain calcium channel blockers bind specifically to multi-drug resistance human KB carcinoma membrane vesicles and inhibit drug binding to P-glycoprotein. *J Biol Chem* 1987; 262:2166–70.
11. Tsuruo T, Iida H, Nojiri M, Tsukagoshi S, Sakurai Y. Circumvention of vincristine and adriamycin resistance in vitro and in vivo by calcium influx blockers. *Cancer Res* 1983; 43:2905–10.
12. Chang BK, Brenner DE, Gutman R. Dissociation of the verapamil induced enhancement of doxorubicins cytotoxicity from changes in cellular accumulation or retention of doxorubicin in pancreatic cancer cell lines. *Anticancer Res* 1989; 9:347–52.
13. Reiter JM, Skand GD, Annonsen ML, Wagoner R, McCarthy E, Pritchett CLE. Pharmaco kinetics of verapamil: experience with a sustained intravenous infusion regimen. *Am J Cardiol* 1982; 50:710–21.
14. Miyazaki M, Fujimoto S, Kitsukawa Y, Endoh F, Okui K, Hashiba N, et al. Clinicopharmacological effects of intra-arterial infusion of anticancer agents in gastrointestinal cancers. *Clinics of Cancer* 1981; 11:1339–43 (in japanese).
15. Thom KA, Zhang S, Deveney C, Daly MJ. Effects of verapamil and degradable starch microspheres during hepatic artery infusion of doxorubicin. *Surgery* 1990; 107:552–9.
16. Todd E, Abernethy DR. Physiological pharmacokinetics and pharmacodynamics of (+)-verapamil in female rats. *Biopharm Drug Dispos* 1987; 8:285–97.
17. Dalton WS, Grogan JM, Meltzer PS, Scheper RJ, Durie BGM, Taylor CW, et al. Drug resistance in multiple myeloma and non-Hodgkins lymphoma: detection of P-gly-coprotein and potential circumvention by addition of verapamil to chemotherapy. *J Clin Oncol* 1989; 7:415–25.
18. Cairo MS, Siegel S, Anas N, Sender L. Clinical trials of continuous infusion verapamil, bolus vinblastine and continuous infusion VP-16 in drug-resistant pediatric tumors. *Cancer Res* 1989; 49:1063–6.
19. Benson AB, Tramp DL, Koeller JM. Phase I study of vinblastin and verapamil given by concurrent iv infusion. *Cancer Treat Rep* 1985;69:795–9.
20. Fojo AT, Ueda K, Slamen DJ, Doplack DG, Gottesman MM, Pastan I. Expression of a multidrug-resistance gene in human tumors and tissues. *Proc Natl Acad Sci U S A* 1987; 84:265–9.
21. Thiebaut F, Tsuruo T, Hamada H, Gottesman HM, Pastan I. Cellular localization of the multidrug resistance gene product P-glycoprotein in normal human tissues. *Proc Natl Acad Sci U S A* 1987; 84:7735–8.
22. Smith MA, Merry S, Smith JG, Kaye SB. Clinically relevant concentrations of verapamil do not enhance the sensitivity of human bone marrow CFU-GM to adriamycin and VP. *Br J Cancer* 1988; 57:576–8.