

Research Article

Using Rhodamine 123 Accumulation in CD8⁺ Cells as a Surrogate Indicator to Study the P-Glycoprotein Modulating Effect of Cepharanthine Hydrochloride In Vivo

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The purpose of this study was the use of rhodamine 123 (Rho123) accumulation in peripheral blood CD8⁺ cells as a surrogate indicator to evaluate the modulating effect of P-glycoprotein (P-gp) inhibitors in the multidrug resistance (MDR) tumor-bearing mouse model. Rho123 was administered to mice, and the fluorescence level in CD8⁺ cells was measured. Cepharanthine hydrochloride (CH) and verapamil (VER), two P-gp inhibitors, were administered to mice 1 hour prior to Rho123 administration *in vivo* or added to peripheral blood 1 hour prior to Rho123 addition *ex vivo*. The tumor inhibition effect of 5-fluorouracil/adriamycin/cisplatin (FAP) protocol plus CH was also investigated. A concentration- or dose-response relationship was shown between the concentration and dose of CH and Rho123 accumulation or the antitumor activity. In conclusion, the measurement of Rho123 accumulation in CD8⁺ cells provides a surrogate assay for the screening of candidate P-gp inhibitors in preclinical trials, and CH is effective in modulating P-gp-mediated MDR *in vivo*.

1. Introduction

Multidrug resistance (MDR) remains the major obstacle to success in a wide variety of advanced malignancies, particularly in solid cancers. The overexpression of P-gp is one of the important mechanisms involved in MDR [1–3]. P-gp-mediated cellular uptake and the efflux of anticancer drugs from cancer cells are responsible for the cell elimination of the drugs which in turn decreases their therapeutic effect. Therefore, one of the best methods of reversing MDR and increasing the efficacy of anticancer drugs is to use the potent P-gp inhibitors modulating P-gp function and/or expression.

It is an important step to use assays to evaluate the effect of candidate drugs as inhibitors of P-gp function *in vivo* after the drugs were found to have an MDR-reversing effect *in vitro*. For nonsurgical solid cancer patients, there are currently no direct assays measuring intratumoral drug

concentrations or the alteration of P-gp function and/or expression. Thus, alternative approaches to evaluate the degree of functional inhibition of P-gp *in vivo* will be a useful aid in the development of MDR modulators. P-gp is also expressed in normal tissues in which they seem to have an important role in the protection against xenobiotics [4]. Several authors reported that P-gp was expressed in lymphocytes including CD3⁺, CD4⁺, and CD8⁺ T cells, as well as the CD56⁺ natural killer (NK) cells, with the highest expression levels and activities observed in CD56⁺ cells followed by CD8⁺ cells [5, 6]. However, the physiological roles that P-gp plays in these cells are unclear. The P-gp in the lymphocytes appears functionally identical to that observed in multidrug resistant cells; they have the same substrate and antagonist specificities [5, 7, 8]. Since the expression and function of P-gp in CD56⁺ cells are the highest, is Rho123 accumulation widely used in CD56⁺ populations as

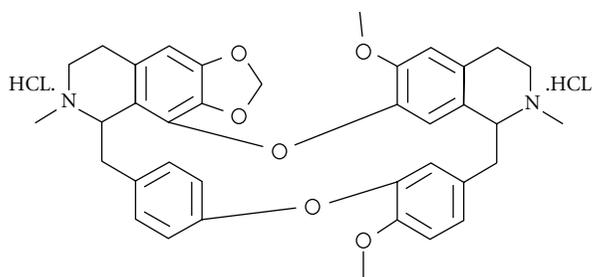


FIGURE 1: The chemical structure of CH used in the present study.

a surrogate indicator to evaluate the degree of functional inhibition of P-gp in clinical trials of P-gp inhibitors, for example, the work of Tariquidar [9] and Zosuquidar [10]. As mouse NK cells do not express CD56⁺ [11], we used Rho123 accumulation in CD8⁺ cells as a surrogate indicator to evaluate the reversal activity by P-gp reversors in the mouse MDR tumor-bearing model.

The search for MDR modulators has extended to the natural products and their derivatives; natural source compounds have become the most widely used of fourth-generation P-gp inhibitors because they are less toxic and more potent than the disappointing first- and second-generation MDR modulators [12–14]. CH, manufactured by salification from cepharanthine, which is a biscochlorine alkaloid, extracted from *Stephania cepharantha* Hayata has a variety of biological activities (Figure 1) [15–17]. Recently, it has been reported that CH has an MDR-reversal effect, and P-gp inhibition is one of the reversal mechanisms of MDR *in vitro* [18, 19]; however, the effect was not evaluated *in vivo*.

We present here a method to detect the effect of the inhibitor of the P-gp function on Rho123 accumulations of the CD8⁺ cells in whole blood from the MDR tumor-bearing mouse. This method was first used to develop and validate the efficacy of CH against P-gp functions in the mouse model for potential use in subsequent clinical evaluations.

2. Materials and Methods

2.1. Animals. The experiments were carried out on 2–4-month-old BALB/c male mice, obtained from the Henan Experimental Animal Center (Zhengzhou, China). The animals had been raised from birth in a specific pathogen-free environment and were handled according to the institutional guidelines complying with Chinese legislation.

2.2. Cell Lines. Hca/FAP variants were developed from the parental Hca hepatocarcinoma cells, which were obtained from KeyGen Biotechnology Co. Ltd. (Nanjing, China). By step-by-step, continuous exposure to increasing doses of FAP, the overexpression of P-gp in the variant was confirmed by the Western blot analysis and the cells that have been previously described [20].

2.3. Drugs. CH was supplied by the Henan Academy of Medical and Pharmaceutical Sciences (Zhengzhou, China).

VER was purchased from Harvest Pharmaceutical Co., Ltd (Shanghai, China). Adriamycin (ADR) was purchased from Hisun Pharmaceutical Co., Ltd (Zhejiang, China). Cisplatin (CDDP) was purchased from Qilu Pharmaceutical Co., Ltd (Shandong, China). 5-Fluorouracil (5-Fu) was purchased from Xudong Haipu Pharmaceutical Co., Ltd (Shanghai, China). Rho123 was purchased from Sigma-Aldrich (St. Louis, Mo, USA) and dissolved in PBS at a concentration of 1 mg/ml. The Ficoll-Histopaque mouse lymphocyte separation medium was obtained from Solarbio Science & Technology Co., Ltd (Beijing, China). The PE antimouse CD8 antibody (clone 53-6.7) and PE Rat IgG2 α , κ Isotype Ctrl were obtained from BioLegend (BioLegend Corp., USA). All drugs were freshly prepared.

2.4. Animal Treatment. Hca/FAP cells were collected from the ascitic fluid of BALB/c mice harboring 5–7 day-old ascitic tumor. The 1×10^7 Hca/FAP cells were injected intramuscularly in the right axilla of BALB/c male mice selected for the experiment on Day 0. The next day, the animals were randomized and divided into different groups; each group comprised 10 mice.

To study the effects of Rho123 administration on peripheral blood CD8⁺ cells, the retention of fluorescence in these cells was investigated as described previously [21] with some modifications and a dose-response curve established. On the 8th day after the Hca/FAP injection, the mice received a single intravenous (i.v.) injection of Rho123. The doses used were 0.5, 1.0, 2.5, 5.0, and 7.5 mg/kg, and the volume of administration was 10 μ l/g. The control group was injected with the vehicle alone. One hour after administration, peripheral blood was collected and cell suspension was prepared as described below.

For the investigation of the effects of P-gp inhibitors *ex vivo*, blood samples were collected as described below on the 8th day after Hca/FAP injection. CH was then added at final concentrations of 10.0, 5.0, and 2.5 μ M; VER was added at a final concentration of 5.0 μ M, an equal volume of the vehicle was added as control. The samples were incubated at 37°C for one hour before Rho123 was added at a final concentration of 50 ng/mL, and the tubes were incubated further at 37°C for one hour. Cell suspension was prepared as described below.

To study P-gp function *in vivo*, Rho123 was injected with or without CH or VER as described before [6] with small modifications on the 8th day after Hca/FAP injection. Briefly, mice, respectively, received a single intravenous (i.v.) injection of the vehicle as control; 2.5, 5.0, and 10.0 mg/kg of CH or 2.5 mg/kg of VER followed one hour later by a single i.v. injection of 2.5 mg/kg of Rho123. The volume of administration was 10 μ l/g. After one hour, blood samples were collected and cell suspension was prepared as described below.

2.5. Cell Preparation and Rho123 Accumulation by Flow Cytometry. Peripheral blood was collected from the orbital sinus of each mouse in a tube containing K₂EDTA as an anticoagulant. Mononuclear cells were obtained by the separation of anticoagulated blood by a Ficoll-Histopaque density-gradient centrifugation and transferred to 1.5 mL

Eppendorf tubes and kept on ice. Cells were washed twice with ice-cold phosphate buffered saline (PBS, pH 7.4). After that, the pellet was gently resuspended in ice-cold PBS and adjusted to a concentration of 1×10^6 cells/ml. Single-cell suspensions were incubated with either the PE-conjugated antimouse CD8⁺ antibody or PE-conjugated Rat IgG2 α , κ Isotype Ctrl as a negative control. After staining for 30 minutes in the darkness at 4°C, the cells were washed twice with ice-cold PBS and then resuspended in PBS and kept on ice in the dark until analyzed as previously described [22]. A life-gate based on forward scatter (FSC) and side scatter (SSC) parameters were made to analyze only viable cells; other gates were made to determine the subpopulations. Amplifier settings for FSC and side SSC were used in linear mode and those for fluorescence channels were used in a logarithmic mode. Fluorescence compensation was manually set for FL1 channel (Rho123) and FL2 channel (PE) with single Rho123-stained cells and PE-stained cells separately. At least 30,000 events were acquired per sample. Multicolor flow cytometry analyses were used to evaluate the proportions of the CD8⁺ population and the mean fluorescence intensity (MFI) of Rho123 in the population. All analyses were performed in duplicate in at least four separate experiments. Cells were analyzed on Epics-XL MCL, and data were analyzed with Expo32 ADC software (Beckman Coulter, Fullerton, Calif, USA).

2.6. Tumor Inhibition of FAP Chemotherapy Protocol plus CH. To evaluate the antitumor effect of FAP chemotherapy protocol plus various concentrations of CH or VER *in vivo* as previously described [23]. Twenty four-hours after the Hca/FAP injection, the mice were randomly divided into different groups and treated as follows:

- Group I: control (normal saline i.v. injected consecutively from Day 1 to 9),
- Group II: 10 mg/kg of CH alone i.v. injected from Day 1 to 8,
- Group III: FAP chemotherapy: 4.2 mg/kg of ADR and 85.0 mg/kg of 5-FU i.v. injected on Day 1, 2.8 mg/kg of CDDP i.v. injected from Day 1 to 8,
- Group IV: 2.5 mg/kg of CH i.v. injected from Day 1 to 8 with FAP chemotherapy,
- Group V: 5.0 mg/kg of CH i.v. injected from Day 1 to 8 with FAP chemotherapy,
- Group VI: 10.0 mg/kg of CH i.v. injected from Day 1 to 8 with FAP chemotherapy,
- Group VII: 2.5 mg/kg of VER i.v. injected from Day 1 to 8 with FAP chemotherapy.

The volume of administration was 10 μ l/g. Tumors were resected on the second day following the last injection. The tumor length (*L*) and width (*W*) were measured, and the tumor weight (*WR*) was calculated as follows:

$$WR = \frac{1}{2} \times L \times W^2. \quad (1)$$

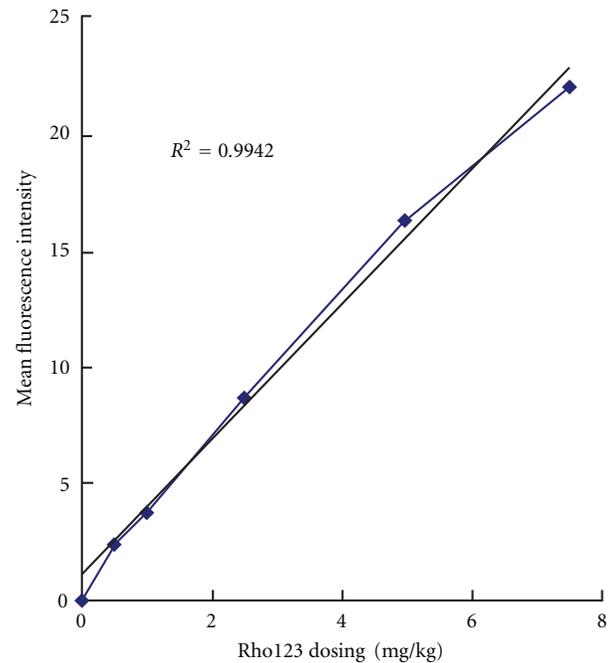


FIGURE 2: Mean fluorescence intensities of CD8⁺ cells were linearly correlated to the dose of Rho123 administered ($r^2 = 0.9942$). The treatment protocol and experimental procedures are described in the text. Responses to treatments were significantly different from each other ($P < .05$).

The percent of tumor growth inhibition was calculated on Day 8 by comparing the average values of the treated groups with those of the tumor-bearing control group. Tumor growth in saline treated control animals was taken to be at 100%.

2.7. Statistical Analysis. For all the experiments, data are presented as the mean \pm SD. Statistical comparison between the groups with different doses or concentrations was carried out using two-sided Student's *t*-tests. Tests for significant differences among the groups were done using one-way ANOVA with multiple comparisons (Fisher's pairwise comparisons) using SPSS 13.0 (IBM software, Somers, NY, USA). A minimum *P* value of .05 was estimated as the significance level for all tests.

3. Results

The i.v. administration of Rho123 (0.5–7.5 mg/kg) in mice produced a dose-dependent fluorescence fashion in peripheral blood CD8⁺ cells (Figure 2). All doses were well tolerated without significant toxicity.

The lower dose of Rho123, 2.5 mg/kg, was selected to investigate the effects of CH on the accumulation of Rho123 in peripheral blood CD8⁺ cells. Using this dose, Rho123 accumulations were increased after administration of either VER (positive control) or CH and presented a clear dose-dependent relationship with CH (2.5–10 mg/kg) compared with vehicle control, as shown in Figure 3. The efficacy of

TABLE 1: The Tumor inhibition of the Hca/FAP solid tumor after treatment.

Treatment groups	Avg. tumor weights (mg)	%Tumor growth inhibition	Mortality
FAP+CH 10.0 mg/kg	248.0 ± 88.0*	71.4%	0/10
FAP+CH 5.0 mg/kg	364.0 ± 82.2*	57.9%	0/10
FAP+CH 2.5 mg/kg	479.0 ± 96.8*□	44.7%	0/10
FAP+VER 2.5 mg/kg	482.8 ± 59.4*□	44.2%	2/10
FAP	479.0 ± 187.5*	44.7%	0/10
CH 10.0 mg/kg	689.0 ± 104.8*	20.4%	0/10
Control	866.0 ± 153.3	—	0/10

* $P < .05$, different from the control, □ no significant difference from each other ($P > .05$).

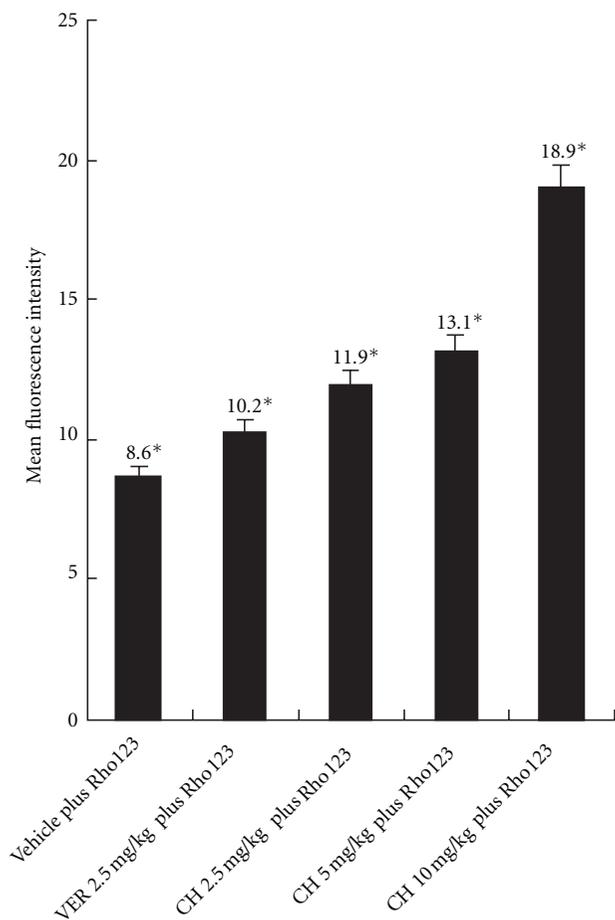


FIGURE 3: Accumulation of Rho123 in CD8⁺ cells by different doses of reversors with or without Rho123 at a dose of 2.5 mg/kg *in vivo*. The increase of the mean fluorescence intensity that reflects the efflux is inhibited. * $P < 0.05$, different from the control. The results are the mean ± SD of at least 4 independent experiments.

the P-gp reversors was not equal; VER was less efficient and potent than CH. There has similar result in *ex vivo* blood samples; the MFIs of CD8⁺ cells in the samples treated with the vehicle, 5.0 μM of VER and 2.5, 5.0 and 10.0 μM of CH were 14.4 ± 0.3, 20.0 ± 0.3, 22.9 ± 0.5, 25.4 ± 1.0 and 29.8 ± 0.9, respectively. The MFI was related to the presence of CH in a concentration pattern in *ex vivo* blood samples, as

shown in Figure 4. This demonstrated that P-gp is active in CD8⁺ cells and can be modulated by CH *in vivo* and *ex vivo*.

The antitumor effects of FAP combination chemotherapy plus i.v. 2.5 mg/kg of VER or 2.5, 5.0, and 10.0 mg/kg of CH were evaluated in the Hca/FAP tumor-bearing mouse. The FAP combination chemotherapy plus 10.0 mg/kg of CH provided the maximum antitumor activity of the treatments studied. Compared with the FAP chemotherapy group, the antitumor effect was increased in a dose-dependent manner when the FAP combined chemotherapy plus various concentrations of CH was used, as shown in Table 1. This suggested that the MDR resistance was reversed by CH *in vivo*.

4. Discussion

Hepatocellular carcinoma (HCC) represents the third leading cause of cancer-related death [24]. Chemotherapy consisting of FAP was commonly used to treat HCC in clinical situations [25]; however, HCC is often resistant to these drugs. Therefore, one of the key approaches is to overcome drug resistance in the success of chemotherapy against HCC. Previous studies of P-gp inhibitors such as VER, cyclosporine A, and its analog PSC833 [26–28] have been limited because of its serious toxicity at the time of dosage for reversing drug resistance.

An important step to develop the MDR reversors targeting P-gp is to determine the inhibition level of P-gp in the preclinical animal model. An ideal approach is to detect the real concentrations of the investigated drug directly *in vivo*, but it is almost impossible, even in the animal model. If there is no related data on the inhibition level of P-gp, it is difficult to interpret the efficacy of P-gp inhibitors in pre- or clinical trials. Recently, the functional study of P-gp was of great concern. Among lymphocytes in the mouse, CD8⁺ cells expressed the highest levels of P-gp followed by CD4⁺ > CD3⁺ [6, 21, 29–32]. Since blood samples are easy to be collected, we selected peripheral blood CD8⁺ cells as a surrogate indicator to investigate the alteration of P-gp activities before and after the administration of P-gp inhibitors and then evaluate the reversal effect of the drug in a solid-tumor-bearing mouse. Rho123 is a fluorescent substrate and has been shown to be a sensitive reagent for the detection of P-gp function by flow cytometry [14, 33]. Some authors reported that Rho123 was a substrate for MRP and

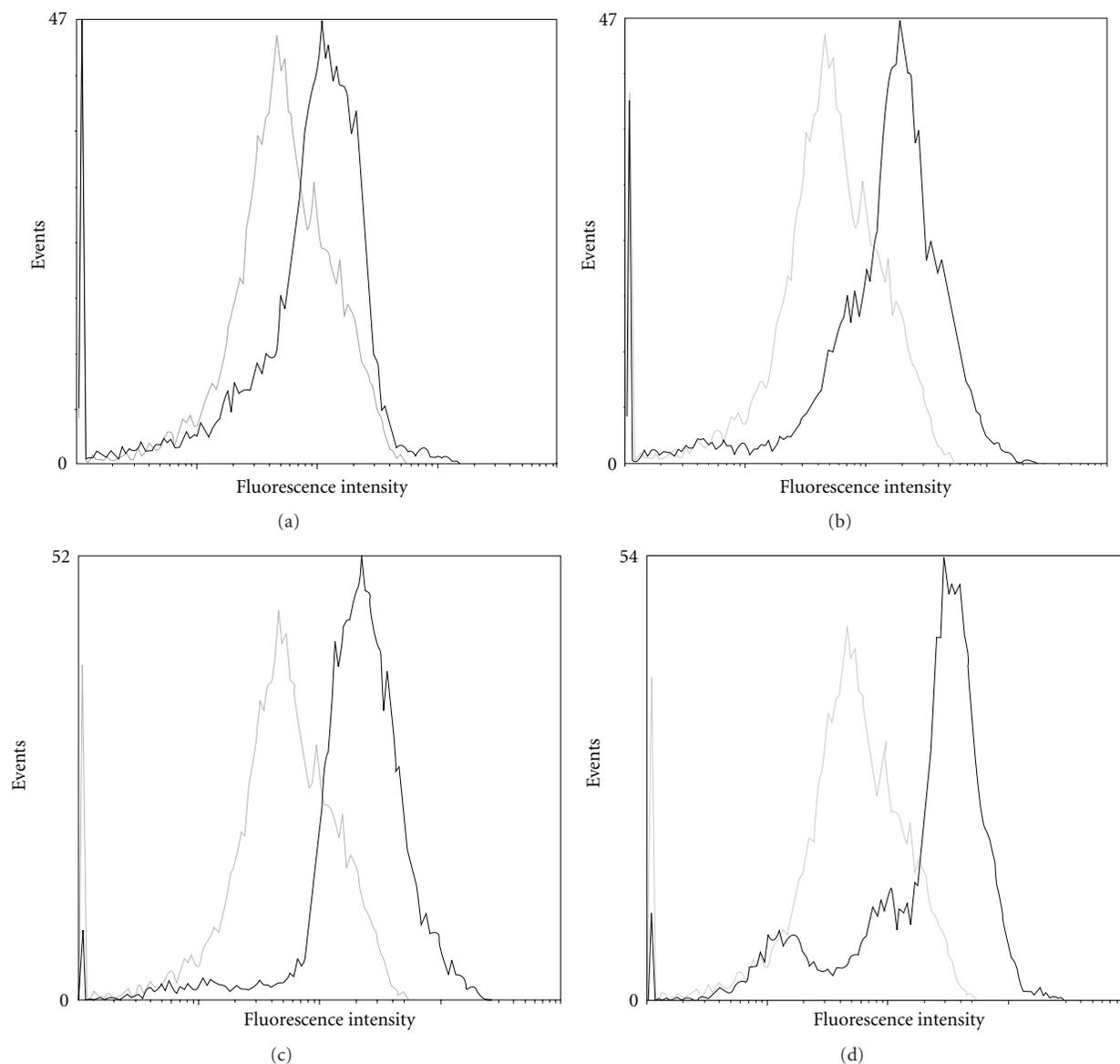


FIGURE 4: Rho123 accumulations in CD8⁺ cells *ex vivo*, where the grey lines stand for cells treated with Rho123 plus the vehicle and it was set as the control in the four sub-figures and the black lines represent cells treated with Rho123 in the presence of various concentrations reversors: A. VER 5.0 μM , B. CH 2.5 μM , C. CH 5.0 μM , D. CH 10.0 μM . This figure is representative of at least four independent experiments.

P-gp [34], but Leite et al. [6] reported that classical MRP1 reversors were unable to enhance Rho123 accumulation in lymphocytes suggesting that MRP1 might not be active or Rho123 is not a good substrate for MRP1 expressed in these cells. Therefore, the alteration of Rho123 fluorescence intensities in these cells responded to the alteration of the P-gp function. In this study, we demonstrated that the mean fluorescence intensities of CD8⁺ cells were related to the dose of Rho123 in a dose-dependent manner suggesting that it is a good choice to use the accumulation of Rho123 in CD8⁺ cells as a surrogate indicator for the study of P-gp inhibitors.

We further studied the modulation effect of CH *in vivo* and *ex vivo*; the results showed that the mean fluorescence intensities of CD8⁺ cells were related to CH in

a dose- or concentration-dependent manner either *in vivo* or *ex vivo*. This suggested that the activities of P-gp were inhibited by CH. In our previous study, it was shown that the enhancement of cytotoxicity of FAP against cultured Hca/FAP cells by CH occurs *in vitro*; the efflux rate of Rho123 in Hca/FAP cells was 2 times less than that of Hca cells after 1 hour exposure to CH plus FAP. We have determined the LD₅₀ for i.v. mice is 0.47 times of clinical dosing of FAP protocol, that is, 85.0 mg/kg of 5-FU, 4.2 mg/kg of ADR, and 2.8 mg/kg of CDDP [20]. In the present study, we established an Hca/FAP solid tumor mouse model and assessed the antitumor effect of FAP combination protocol plus CH. The i.v. dosing for a mouse is 42.50 mg/kg of 5-Fu, 2.10 mg/kg of ADR, 1.40 mg/kg of CDDP. The results showed

TABLE 2: Body weights of the animal after treatment.

Treatment groups	Body weights (g)		
	Day 1	Day 4	Day 8
FAP+CH 10.0 mg/kg	25.9 ± 1.4	25.6 ± 1.3	25.1 ± 1.2
FAP+CH 5.0 mg/kg	25.2 ± 1.6	25.0 ± 1.6	24.7 ± 1.8
FAP+CH 2.5 mg/kg	25.0 ± 1.9	24.5 ± 1.9	23.8 ± 2.1
FAP+VER 2.5 mg/kg	25.1 ± 1.2	25.0 ± 1.1	24.3 ± 1.3
FAP	25.5 ± 1.8	24.6 ± 1.7	24.6 ± 1.5
CH 10.0 mg/kg	24.9 ± 1.3	25.0 ± 1.0	24.9 ± 0.9
Control	25.3 ± 1.2	25.8 ± 1.4	26.9 ± 1.9

that a single i.v. dose of 10.0 mg/kg CH partly inhibited the tumor growth, but the effect was weak; this is consistent with conclusions drawn from other authors' reports [35]: a single i.v. dose of 5.0 mg/kg CH has no antitumor effect (data not shown). When FAP combined chemotherapy plus various concentrations of CH was used, the antitumor effect was increased in a dose-dependent manner. Therefore, with this correlation with the results of the inhibition of P-gp function by CH in CD8⁺ cells, we think that the antitumor activity recovered by CH in a dose-dependent manner was related to the inhibition of the P-gp function. All of the efficacious combination protocols with CH appeared to be well tolerated, as indicated by the minimal changes in body weights of the various treatment groups (as shown in Table 2). Since an LD₅₀ of VER for the i.v. administration in mouse was reported to be 7.6 mg/kg [36], but a single i.v. dose of 5.0 mg/kg VER caused the death of 60% of animal in the treated BALB/c mice (data not shown), we selected the dose of 2.5 mg/kg as a positive control. There is no mortality in the FAP treated group; we think the 20% mortality in the FAP plus VER treated group was caused by the increasing toxicity when VER combined with ADR [37].

Although one can argue that what occurs at the level of peripheral blood CD8⁺ cells may not accurately reflect the real inhibition level of P-gp in the solid tumor in mouse, ^{99m}Tc-sestamibi scans performed in some studies have shown effective P-gp inhibition in both normal tissues and solid tumors [9, 38], suggesting the inhibition level of P-gp was modulated simultaneously. We have to note that no model is perfect and there are obvious differences between humans and mice; the BALB/c mouse is unique for its features, making even more difficult extrapolations to human conditions.

With regard to the results obtained, we conclude that CH is a potent P-gp inhibitor for clinical application. Although limitations may exist in the surrogate assay in CD8⁺ cells in the mouse model, we are convinced that this assay can provide us with much useful information in screening potential P-gp inhibitors *in vivo*.

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