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# Amelioration of methotrexate-induced malabsorption by vitamin A

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**Abstract** Methotrexate (MTX) induces damage to the small intestine, resulting in malabsorption and diarrhea. We found that the coadministration of vitamin A (VA) with MTX protected the small intestine from MTX-induced damage. In this study, the permeability of D-glucose, D-xylose and L-leucine through the small intestine of rats treated with MTX and/or VA was studied using everted segments of small intestine. MTX treatment decreased permeability and VA coadministration prevented the decrease. The transport of D-glucose in the small intestine of MTX plus VA- and VA-treated rats and of control rats followed Michaelis-Menten kinetics, in contrast to the transport kinetics in MTX-treated rats. The pharmacokinetics of orally administered [$^{14}$C]-D-glucose in control rats and MTX- and/or VA-treated rats was also studied. The bioavailability of D-glucose in MTX-treated rats was lower than in the other three groups. VA coadministration improved the bioavailability of D-glucose. Thus, it seems likely that VA ameliorates MTX-induced malabsorption.

**Key words** Methotrexate · Vitamin A · Malabsorption

## Introduction

Methotrexate (MTX) has been extensively used for patients with osteosarcoma. MTX chemotherapy is effective against the cancer, but is often accompanied by side effects such as nausea, vomiting, diarrhea, stomatitis, gastrointestinal ulceration and mucositis [5, 17]. In recent years, various regimens for MTX chemotherapy have been tried to reduce the side effects. A high-dose MTX regimen with leucovorin rescue has been recommended as a useful regimen [8, 18]. MTX treatment is known to induce malabsorption syndrome characterized by serious malabsorption of nutrients and diarrhea [1, 16]. MTX depresses the absorption of certain drugs in the small intestine [2, 4, 21]. It also inhibits the metabolic activity and active transport capacity of the intestinal mucosa [3, 16]. Damaged and shortened microvilli and a decreased surface area of the small intestine are observed in this syndrome [1]. In addition to the morphological changes in the intestine, the present authors have reported that the amounts of small intestinal membrane components (lipids, proteins) of MTX-treated rats are decreased and the physical structures of the brush border membrane are changed [19, 20].

We have found that vitamin A (VA) can protect the small intestine of rats from this morphological, biochemical and physical damage induced by MTX [7, 20] by protecting the crypt cells [9]. Further, treatment with VA has been shown not to disturb the antitumor activity of MTX both in vitro and in vivo [14, 22]. In this study, with the aim of clarifying the effect of VA on the malabsorption induced by MTX treatment, we investigated the in vitro and in vivo absorption of glucose and amino acids in the small intestine of MTX- and/or VA-treated rats.

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## Materials and methods

### Drugs and chemicals

MTX, fluorescein isothiocyanate-dextran (FITC-dextran), average molecular weight 70 000 Da), D-(+)-glucose, D-(+)-xylose, L-leucine, and glucose C-Test and GOT-UV Test kits were purchased from Wako Pure Chemical Industry (Osaka, Japan). VA was from Sigma Chemical Co. (St. Louis, Mo.). [$^{14}$C(U)]-D-glucose and AQUASOL-2 were purchased from New England Nuclear (Boston, Mass.). All other reagents were of analytical grade.

### Animals

Male Wistar rats (8–10 weeks of age and 180–250 g body weight (Japan SLC, Shizuoka, Japan) were used and acclimatized for at least 1 week before the experiments. Rats were treated in four groups as follows: (A) saline solution alone as a control, (B) MTX (15 mg/kg body weight), (C) MTX (15 mg/kg) plus VA (5000 IU/kg), and (D) VA (5000 IU/kg) alone. Each drug solution (about 0.5 ml) was administered orally to the rats once daily for 4 days.

### Experimental method of in vitro intestinal absorption

The in vitro intestinal absorption study was carried out using an everted sac of small intestine as described previously [20]. Treated rats were fasted overnight before experimental use. The rats were anesthetized with ethylether and the intestines were perfused through the portal vein at a rate of 30 ml/min with saline solution at 37 °C and excised. Segments (7 cm) of the jejunum were cut off at a distance of 3 cm from the end of the duodenum. The segments were everted in saline solution. An L-shaped glass cannula was inserted into each end of the everted segments and a 10-ml syringe was attached to the exposed end of each cannula. The segments were then immersed in 40 ml Krebs-Henseleit bicarbonate buffer (KHBB), pH 7.4, containing various concentrations of the substrates D-glucose, D-xylose and L-leucine. 5 ml of KHBB containing FITC-dextran (0.05 mg/ml) was applied to the serosal side of the segments via the syringe. The two syringes were gently moved up and down and the absorption experiments were started after the intestinal segments had been incubated for 7 min at 37 °C. Gas (95% $O_2$ –5% $CO_2$) was gently bubbled through the mucosal side solution during the absorption experiments. At designated times after the start of the experiments, the solution (0.1 ml for D-glucose and L-leucine and 1 ml for D-xylose) was taken from the serosal side for determination of absorbed substrate and at the same time, the same volume of KHBB solution (0.1 ml for D-glucose and L-leucine and 1 ml for D-xylose) was added to maintain the volume. The substrate permeability through the segments was determined from the amount of substrate in the serosal side solution. The volume change on the serosal side was corrected by determining the FITC-dextran concentration.

### In vivo absorption of D-glucose

D-Glucose solution (1 ml, 10 m*M*) containing [$^{14}$C]-D-glucose (0.1 μCi/ml) was administered orally to the rats treated with MTX and/or VA or with saline solution alone as described above. Blood (0.3 ml) was withdrawn from the jugular vein at designated times to determine the plasma concentration of [$^{14}$C]-D-glucose. The blood samples were centrifuged for 3 min at 14000 rpm in a Beckmann Microfuge B and [$^{14}$C]-D-glucose in the separated plasma was determined.

### Determination of D-glucose

D-Glucose in the sample solutions was determined using the Wako glucose C Test based on the method of Miwa et al. [11].

### Determination of D-xylose

Orcinol reagent (2 ml) containing 1% orcinol in 0.1% $FeCl_3$/HCl solution was added to 200 μl sample solution containing D-xylose and the reaction mixture was boiled for 1 h. The optical density of the sample was determined at 630 nm.

### Determination of L-leucine

Sulfosalicylic acid solution (80 μl, 0.5%) was added to 20 μl sample solution containing L-leucine and the mixture was filtered through a 0.45- μm membrane filter (chromatodisk 13A). The L-leucine in the samples was determined using a Hitachi model L-8500 high-speed amino acid analyzer.

### Determination of FITC-dextran

KHBB (2.5 ml) was added to 20 μl sample solution on the serosal side. The fluorescence intensity of FITC-dextran in the sample solution was determined at an excitation wavelength of 495 nm and an emission wavelength of 515 nm using a Hitachi fluorescence spectrophotometer 650–60.

### Determination of [$^{14}$C]-D-glucose

Scintillation fluid (10 ml AQUASOL-2) was added to 100 μl plasma in scintillation vials. The radioactivities in the plasma were counted in an Aroca LSC-903 liquid scintillation counter.

### Kinetic analysis of in vitro D-glucose permeability

The D-glucose permeability in the small intestine as a function of D-glucose concentration was analyzed by fitting the data to the Michaelis-Menten equation (Eq. 1) using a nonlinear least squares computer program (MULTI program) [23].

$$V = \frac{V_{max} C}{K_m + C} \qquad \text{(Eq. 1)}$$

where V is the velocity of D-glucose transport at a certain concentration (C) of D-glucose, $K_m$ is the apparent Michaelis-Menten constant and $V_{max}$ is the apparent maximum velocity.

### Pharmacokinetics of D-glucose administered orally to rats

The time course of the appearance of [$^{14}$C]-D-glucose in the plasma after oral administration to rats was measured. The plasma concentration [C(t)] vs time (t) curves were analyzed using the following two-exponential equation describing a one-compartment open model with first order absorption [6], using a nonlinear least squares computer program (MULTI program) [23].

$$C(t) = \frac{\text{Dose F } k_a}{V(k_a - k_{el})}(e^{-k_{el}t} - e^{-k_a t}) \qquad \text{(Eq. 2)}$$

where $k_a$ and $k_{el}$ are the absorption and elimination rate constants of D-glucose, respectively, V is the apparent volume of distribution of D-glucose and F is the absorbed fraction of the administered dose. $C_{max}$, the maximum concentration of D-glucose in the plasma, was calculated as $C_{max} = (\text{dose F/V})\ R^{1/(1-R)}$, where $R = k_a/k_{el}$. $T_{max}$, the time at which the maximum concentration of D-glucose in the plasma occurred was calculated as $T_{max} = \ln R/(k_a - k_{el})$. MRT, the mean residence time, was obtained by analyzing the data based on the equation, $MRT = \int_0^\infty tC(t)dt / \int_0^\infty C(t)dt$.

### Statistical analysis

Statistical analysis was performed using Student's *t*-test. Results were considered significant at $P < 0.05$.

## Results

### Periods of treatment of rats and D-glucose permeability in the small intestine

The effects of the period of MTX and/or VA treatment on the in vitro permeability of D-glucose through the everted segments of small intestine were studied (Fig. 1). Intestine from rats treated orally daily for 1 or 2 days with MTX or MTX plus VA showed a similar D-glucose permeability to

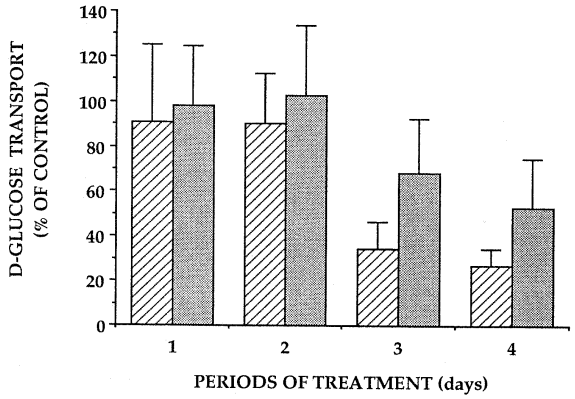



**Fig. 1** Effect of MTX and VA treatment on D-glucose permeability of the small intestine. Three groups of rats were treated as follows: saline solution alone, MTX 15 mg/kg (*hatched column*), MTX 15 mg/kg plus VA 5000 IU/kg (*dotted column*). The treatments were carried out once daily for 1 to 4 days. The D-glucose permeability was examined in vitro in everted segments of the small intestine. The amounts of D-glucose transported from the mucosal side to the serosal side during a 20-min period were determined. The D-glucose permeability in the drug-treated rats is expressed as the percentage of that in the control rats receiving saline solution alone for the corresponding periods of treatment. The values are the means ± SD for three to five rats

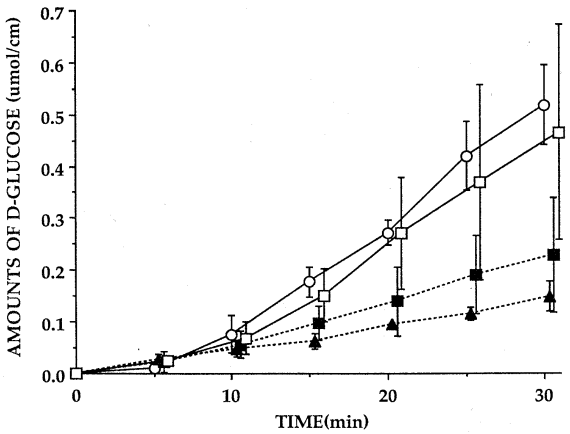



**Fig. 2** Time course of D-glucose permeability of the small intestine. Four groups of rats were treated once daily for 4 days as follows: (A) saline solution alone, (B) MTX 15 mg/kg, (C) MTX 15 mg/kg plus VA 5000 IU/kg, and (D) VA 5000 IU/kg. The D-glucose permeability was examined in vitro in everted segments of small intestine using a D-glucose concentration of 10 m*M* and is expressed as the amount of D-glucose transported per 1 cm length of intestine. The values are the means ± SD for three rats in the control groups (○), seven rats in the MTX group (▲), four rats in the MTX plus VA group (■) and five rats in the VA group (□)

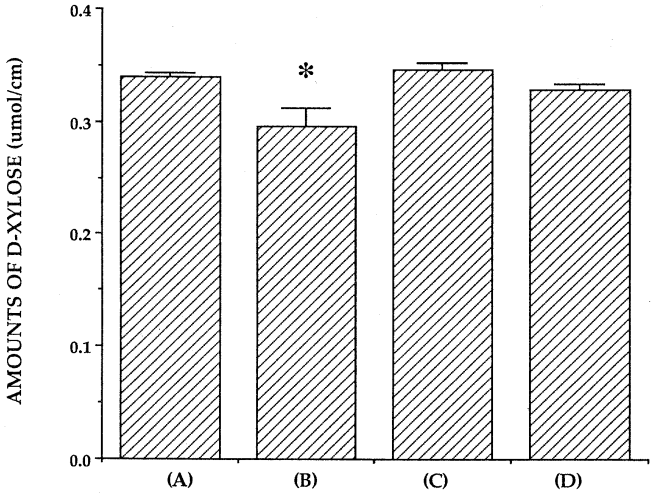
intestine from rats treated with saline alone. Intestine from rats treated with MTX once daily for 3 days showed a decrease in D-glucose permeability to less than 40% of that observed in intestine from control rats, and that from rats treated with MTX plus VA showed a decrease to about 70% of that in intestine from control rats. The intestine from rats treated with MTX or MTX plus VA once daily for 4 days showed a decrease in D-glucose permeability to less than 30% and about 50% of that observed in intestine from control rats, respectively.

## In vitro permeability of D-glucose in the small intestine of rats treated with MTX and/or VA

The time course of D-glucose permeability in the everted segments of small intestine from rats treated once daily for 4 days with MTX, MTX plus VA, VA or saline solution alone was examined using a concentration of 10 m*M* D-glucose (Fig. 2). The D-glucose permeability increased linearly in the intestine from both the treated and control rats after 5 min from the start of the experiments. The D-glucose permeabilities in the intestine from MTX plus VA- and VA-treated rats were slightly less than that of control rats for 30 min and in the intestine from MTX-treated rats decreased compared to that from control rats.

The permeabilities of D-glucose in the small intestine increased linearly from 5 min to 25 min as shown in Fig. 2. The permeabilities of D-glucose were examined at various concentrations of D-glucose (5−30 m*M*) and the permeation rates of D-glucose were evaluated by determining the amounts of D-glucose transported during periods of 20 min. The permeation rates in the intestine from control, MTX plus VA- and VA-treated rats were concentration-dependent, following Michaelis-Menten kinetics. The Michaelis-Menten kinetic parameters were obtained by fitting the data into Eq. 1. The apparent $K_m$ and $V_{max}$ were as follows: 21.0 m*M* and 0.0102 mg/cm per min for the control rats, 28.0 m*M* and 0.0091 mg/cm per min for the MTX plus VA-treated rats, and 8.6 m*M* and 0.0055 mg/cm per min for the VA-treated rats, respectively. In contrast, the

**Fig. 3** D-Xylose permeability in the small intestine. Rats were treated once daily for 4 days with (A) saline solution alone, (B) MTX 15 mg/kg, (C) MTX 15 mg/kg plus VA 5000 IU/kg and (D) VA 5000 IU/kg: The D-xylose permeability was examined in vitro in everted segments of small intestine using a D-xylose concentration of 10 m*M*. The amounts of D-xylose transported during a 20-min period between 5 and 25 min after the start of the transport experiment, were determined. The results are expressed as the amounts of D-xylose transported per 1 cm length of intestine. The values are the means ± SD for three rats (* $P < 0.01$ vs control rats)



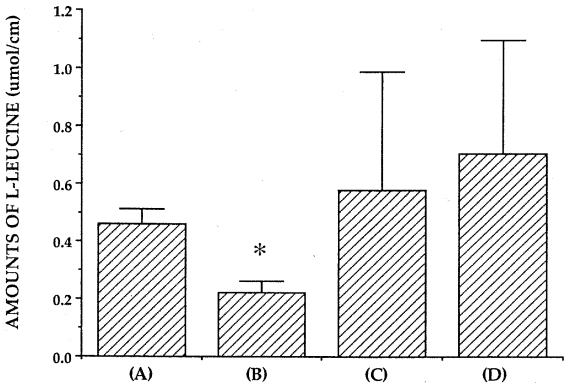



**Fig. 4** L-Leucine permeability in the small intestine. Four groups of rats were treated once daily for 4 days as follows: (A) saline solution alone, (B) MTX 15 mg/kg, (C) MTX 15 mg/kg plus VA 5000 IU/kg and (D) VA 5000 IU/kg. The L-leucine permeability was examined in vitro in everted segments of small intestine using an L-leucine concentration of 10 m*M*. The amounts of L-leucine transported during a 20-min period between 5 and 25 min after the start of the transport experiment were determined. The results are expressed as the amounts of L-leucine transported per 1 cm length of intestine. The values are the means ± SD for four rats in the control group, three rats in the MTX group, five rats in the MTX plus VA group, and four rats in the VA group. (* $P < 0.01$ vs control rats)

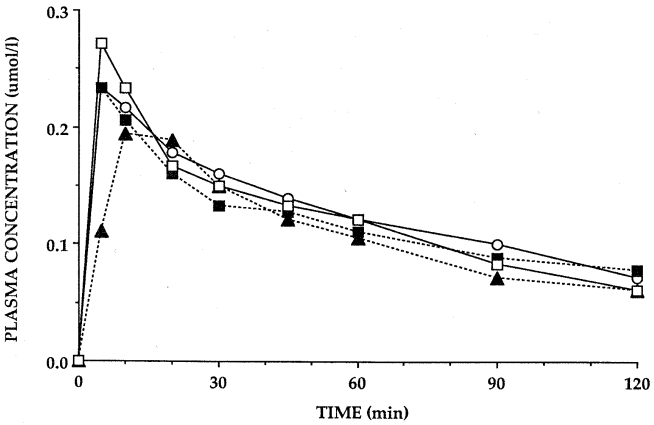



**Fig. 5** Plasma concentration-time curves after oral administration of [$^{14}$C]-D-glucose to rats treated once daily for 4 days with saline solution alone (○), MTX 15 mg/kg (▲), MTX 15 mg/kg plus VA 5000 IU/kg (■) or VA 5000 IU/kg (□)

permeation rates in the MTX-treated rats increased with increasing D-glucose concentration and did not follow Michaelis-Menten kinetics.

### In vitro permeability of D-xylose in the small intestine of rats treated with MTX and/or VA

The D-xylose permeabilities in the everted segments of small intestine from rats treated once daily for 4 days with MTX, MTX plus VA, VA or saline solution alone were examined using a concentration of 10 m*M* D-xylose (Fig. 3). The D-xylose permeability in the intestine of MTX-treated rats decreased significantly ($P < 0.01$) compared with that observed in intestine from control rats. In contrast, in intestine from MTX plus VA- or VA-treated rats, D-xylose was transported to the same extent as in control rats.

### In vitro permeability of L-leucine in the small intestine of rats treated with MTX and/or VA

The L-leucine permeabilities in the everted segments of small intestine from rats treated once daily for 4 days with MTX. MTX plus VA, VA or saline solution alone were examined using a concentration of 10 mM L-leucine (Fig. 4). The L-leucine permeability in the intestine of MTX-treated rats decreased significantly ($P < 0.01$) compared with that observed in the intestine from control rats. In contrast, the L-leucine permeabilities in the intestine from MTX plus VA- or VA-treated rats were comparable to that in control rats.

**Table 1** Pharmacokinetic parameters of D-glucose in the rats treated with MTX and/or VA

| Treatment | $C_{max}$ (μmol/l) | $T_{max}$ (min) | $AUC_{0-120\ min}$ (μmol/l×min) | $MRT_{0-120\ min}$ (min) |
|---|---|---|---|---|
| Control | 0.250±0.020 | 8±3 | 14.701±1.254 | 47.506±2.349 |
| MTX | 0.198±0.007 | 10±0 | 14.018±1.174 | 51.697±3.355 |
| MTX+VA | 0.235±0.058 | 8±3 | 15.628±1.852 | 50.469±1.504 |
| VA | 0.309±0.036 | 7±3 | 16.174±1.239 | 46.385±1.377 |

### D-glucose in plasma after oral administration

The time course of [$^{14}$C]-D-glucose appearance in plasma after its oral administration to control rats or MTX- and/or VA-treated rats was examined (Fig. 5). The plasma concentration-time curves of D-glucose were fitted to the one-compartment open model with first-order absorption according to Eq. 2. The pharmacokinetic parameters of D-glucose are shown in Table 1. The following results were obtained, although the differences were not statistically significant. The $C_{max}$ value of the MTX-treated rats was lower than that of the control rats, while that of the MTX plus VA-treated rats was comparable with that of the control rats. The $T_{max}$ value of the MTX-treated rats was larger than that of the control rats, while that of the MTX plus VA-treated rats was comparable with that of the control rats. The area under the plasma concentration curve (AUC) of the MTX-treated rats was lower than that of the control rats, and that of the MTX plus VA-treated rats was larger than that of the MTX-treated rats. The MRT value of the MTX-treated rats was larger than those of the other three groups.

## Discussion

Some antitumor drugs have been reported to decrease intestinal absorption. 5-Fluorouracil and mitomycin C administered to rats or hamsters decreases the absorption of D-glucose and other nutrients in the small intestine [3, 12, 15].

MTX treatment of rats depresses not only the active transport capacity of the intestinal mucosa [3, 16], but also decreases the passive transport of some acidic, basic and neutral drugs and mannitol in the small intestine [2, 4, 21].

The small intestinal permeability of D-glucose has been reported to decrease as a result of MTX-induced intestinal toxicity [13, 16]. We investigated D-glucose permeability using everted segments of intestine isolated from rats treated with MTX and/or VA. MTX treatment of rats for 3 or 4 days decreased the small intestinal permeability to D-glucose, although treatment for 1 or 2 days had no effect on permeability (Fig. 1). This time course of the change in intestinal permeability to D-glucose is coincident with that of the change in the constituents of the small intestine (e. g. proteins, lipids, phospholipids, cholesterol) following MTX treatment [20]. It is noteworthy that VA coadministration improved the intestinal permeability to D-glucose, compared with that of rats treated with MTX alone. This effect of VA is also coincident with the effects of VA on the morphological, biochemical and physicochemical damage to the intestine [20]. The transport kinetics of D-glucose in the small intestine were further investigated to clarify this effect of VA (Fig. 2). The transport kinetics of D-glucose in the control, MTX plus VA- and VA-treated rats followed Michaelis-Menten kinetics. In contrast, transport in the MTX-treated rats did not follow Michaelis-Menten kinetics, but was simply passive transport. These results indicate that the function of the glucose transporter in the small intestine is abolished by MTX treatment, but that the function of the transporter is preserved by VA coadministration.

This enhancement of intestinal permeability by VA was further evaluated by studying D-xylose permeability as a passive transport marker (Fig. 3). D-Xylose permeability in MTX-treated rats decreased compared with that in the control rats, but in the MTX plus VA-treated rats D-xylose permeability was almost equivalent to that in the control rats. This indicates that VA protected the nonspecific transport system of the small intestine from MTX-induced damage.

With regard to the amino acid transport system in the small intestine, the permeabilities of L-tyrosine, L-phenylalanine and L-glycine have been reported to decrease following MTX treatment [3, 13]. As shown in Fig. 4, L-leucine transport in the small intestine decreased in MTX-treated rats and VA coadministration ameliorated this effect of MTX, suggesting that VA protected the L-leucine transport system from MTX-induced damage.

We investigated the pharmacokinetics of D-glucose in control, and in MTX-, MTX plus VA- and VA-treated rats in order to evaluate the in vivo effect of VA on intestinal absorption. [$^{14}$C]-D-Glucose was administered orally to the rats and the D-glucose concentration in the plasma was monitored (Fig. 5). The pharmacokinetic parameters were obtained (Table 1). The values of $C_{max}$, $T_{max}$, AUC and MRT from the MTX-treated rats among the three actively treated groups were most different from the values from the control rats, although the differences were not statistically significant. This indicates that MTX treatment decreased the bioavailability of D-glucose. In contrast, the $C_{max}$, $T_{max}$, AUC and MRT values for the MTX plus VA-treated rats were comparable with the values for the control rats, respectively. It should be noted that the coadministration of VA and MTX improved the bioavailability of D-glucose. This suggests that VA ameliorates the malabsorption resulting from MTX-induced damage to the small intestine, although the oral MTX doses required to decrease the intestinal absorption of D-glucose and L-leucine were much higher than those given clinically except for the high-dose MTX regimen.

MTX has been reported to induce chronic liver injury [10]. The coadministration of VA with MTX under the present experimental conditions did not cause liver injury. In addition, VA does not affect the antitumor activity of MTX and rather profoundly kills the cells due to its cell-killing activity [22]. Further, the coadministration of VA does not inhibit the in vivo antitumor activity of MTX [14]. Thus, VA may be a useful biochemical modulator capable of preventing MTX-induced damage to the small intestine.

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