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# Interactions of vinblastine and vincristine with methotrexate transport in isolated rat hepatocytes*

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**Abstract.** The accumulation of methotrexate (MTX) in the presence of vinblastine (VBL) and vincristine (VCR) was studied in isolated rat hepatocytes. In accordance with our recent study on vindesine (VDS), we found VBL and VCR to reduce net MTX accumulation significantly at 15 min after MTX addition. Drug concentrations of 100 μ*M* VBL and 500 μ*M* VCR led to 67% and 82% reductions in intracellular MTX, respectively. Since there was only a slight inhibition of MTX efflux by 100 μ*M* VBL, the accumulation data demonstrate that the major effect of VBL is on MTX influx. Dixon-plot analyses are suggestive of competitive inhibition of the MTX influx, yielding inhibition constants ($K_i$ values) of 55 μ*M* for VBL and 110 μ*M* for VCR. Since the $K_i$ values correspond grossly to plasma levels obtained in humans shortly after the infusion of therapeutic doses of the vinca alkaloids studied herein, the interaction with MTX uptake could serve to diminish the toxicity of MTX to nonmalignant cells.

## Introduction

Vinca alkaloids have been employed as investigative and therapeutic tools in vivo and in vitro in studies aimed at increasing the tumor response rate and reducing the non-tumor-cell toxicity of methotrexate (MTX) [9, 39, 42, 51, 54]. Vincristine (VCR), in particular, has been observed to enhance the intracellular accumulation of MTX in tumor cells in vitro by several mechanisms [4, 10, 11, 15, 19, 57, 58], including decreased efflux [3, 16]. Similar effects were found in tumor cells in vivo, but higher concentrations of VCR were needed [4, 10]. In nonmalignant mouse intestinal epithelial cells, Chello et al. [10] could not show any effect of VCR on MTX uptake in vitro or in vivo.

We have demonstrated that the vinca alkaloid vindesine (VDS) inhibits the influx and accumulation of MTX in freshly isolated rat hepatocytes [7]. On the basis of these observations, we questioned whether the vinca alkaloids VCR and vinblastine (VBL) share these properties of VDS. In this report we present data in support of a probable competitive inhibition of MTX transport into isolated rat hepatocytes by VCR and VBL, suggestive of a common perturbation of MTX transport by vinca alkaloids.

## Materials and methods

*Drugs and chemicals.* L-Glutamyl-3,4-[$^3$H]-MTX (specific activity, 41.0 Ci/mmol; purity, 99.3% as determined by high-performance liquid chromatography, HPLC) was purchased from New England Nuclear (Boston, Mass., USA). Formulated MTX (purity, 99% as determined by HPLC) was a gift from Nycomed A/S (Oslo, Norway). Formulated VCR was kindly provided by Eli Lilly & Co. (Indianapolis, Ind., USA). VBL (Velbe) was obtained from Eli Lilly S. A. (Oslo, Norway). Collagenase (type I, 300 U/mg), bovine albumin (fraction V, defatted), and HEPES buffer were obtained from Sigma Chemical Company (St. Louis, Mo., USA). Insta-Gel II scintillation liquid was supplied by Packard Instruments Co. (Groningen, The Netherlands). All other reagents were of analytical grade. All samples containing MTX were stored protected from light.

*Preparation of hepatocyte cell suspensions.* Nonfasted male Wistar rats weighing 210–250 g (Charles River, WIGA GmbH, Sulzseld, Germany) were used for the experiments. Following ether anesthesia and laparotomy, the liver cells were prepared essentially by the methods of Berry and Friend [5] and Seglen [47], with our previously reported modifications [7]. Cell viability was 95.5% ± 1.9% (mean ± SD) at the start and 91.5% ± 4.1% at the end of the incubation maximum 3 h later. There was no difference in viability between VBL- and VCR-treated hepatocytes.

The incubation medium contained 5.5 m*M* glucose, 137 m*M* NaCl, 5.37 m*M* KCl, 0.81 m*M* $MgSO_4$, 4 m*M* $CaCl_2$, 0.34 m*M* $KH_2PO_4$, 20 m*M* HEPES buffer, and 2% bovine serum albumin and was adjusted to pH 7.4 at 37° C with NaOH.

*Transport experiments with VBL and VCR.* For influx studies, hepatocyte cell suspensions of 0.5 ml for Dixon-plot analysis and 1.5 ml for dose-response studies (mean, $0.87 \times 10^6$ cells/ml) were incubated under agita-

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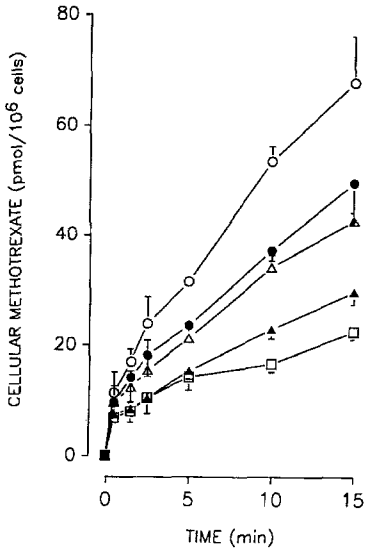


**Fig. 1.** Effect of VBL on hepatocellular uptake of MTX. Freshly isolated rat hepatocytes were exposed to 1 μ*M* [$^3$H]-MTX after 20 min preincubation with increasing concentrations of VBL. Data represent mean values ± SD ($n$ = 3). *Open circles*, no VBL; *filled circles*, 12.5 μ*M* VBL; *open triangles*, 25 μ*M* VBL; *filled triangles*, 50 μ*M* VBL; *open squares*, 100 μ*M* VBL

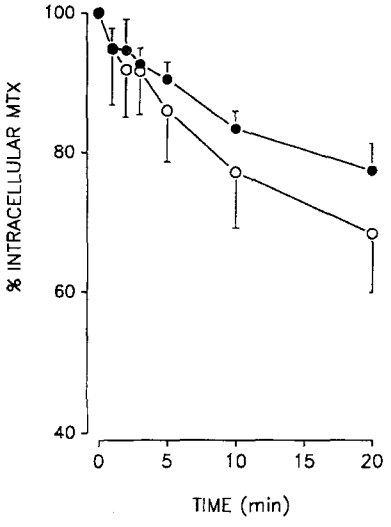


**Fig. 2.** Efflux of MTX in the presence of 100 μ*M* VBL. Hepatocytes were preloaded with 1 μ*M* [$^3$H]-MTX for 60 min. After the addition of 100 μ*M* VBL or isotonic saline, the hepatocytes were incubated for 20 min prior to a washing procedure. The cells were then reincubated in 100 μ*M* VBL (*filled circles*) or isotonic saline (*open circles*). Data represent mean values ± SD ($n$ = 5)

tion for 30 min at 37° C prior to preincubation with 0–100 μ*M* VBL and 0–500 μ*M* VCR for 20 min. [$^3$H]-MTX was then added to final concentrations of 1–10 μ*M*. Both MTX and the vinca alkaloids were added at levels 100 times higher than the final concentrations in the hepatocyte suspensions. Samples (100 μl) of the incubation medium were removed at 0.5, 1.5, 2.5, 5, 10, and 15 min after MTX addition.

For the efflux study, hepatocytes were preloaded for 60 min with 1 μ*M* MTX, which was followed by the addition of 100 μ*M* VBL or isotonic saline and a further incubation for 20 min. Efflux was initiated by washing the hepatocyte suspensions once in ice-cold saline. Cell pellets were immediately resuspended in MTX-free incubation medium

**Table 1.** Decreased net [$^3$H]-MTX accumulation in isolated rat hepatocytes pretreated with VBL

| VBL (μ*M*) | MTX (pmol/10$^6$ cells) | % Decrease in cellular MTX |
|---|---|---|
| 0 | 67.8 ± 8.5 | – |
| 12.5 | 49.5 ± 7.3* | 27.0 |
| 25 | 42.6 ± 1.5 | 37.2 |
| 50 | 29.6 ± 2.3 | 56.3 |
| 100 | 22.4 ± 1.4 | 67.0 |

Hepatocytes were preincubated with VBL for 20 min prior to incubation in 1 μ*M* [$^3$H]-MTX for 15 min. Data represent mean values ± SD ($n$ = 3)
* $P$ <0.05 between 0 and 12.5 μ*M* VBL

(37° C) containing 100 μ*M* VBL or isotonic saline. Aliquots of 100 μl were removed at 0, 1, 2, 3, 5, 10, and 20 min following the completion of washing procedures.

The obtained samples were immediately pipetted into 1.5-ml polyethylene microcentrifuge tubes containing a mixture of dinonyl phtalate and dibutyl phtalate (1 : 3, v/v; 250 μl), overlying 1 ml ice-cold isotonic saline (0° C). The cells were separated from the medium by centrifugation within less than 10 s [1]. Further handling prior to the determination of cell radioactivity has been described in detail elsewhere [1].

*Calculations and statistics.* MTX efflux was analyzed according to a one-compartment model. The half-life parameter was calculated as described elsewhere [6]. Statistical analyses were performed using the nonparametric Mann-Whitney $U$-test (Microstat; Ecosoft Inc., Indianapolis, Ind.). Statistical significance was defined as $P$ <0.05. All results are expressed as mean values ± SD.

## Results

### *Effects of VBL on MTX transport*

Hepatocellular influx of 1 μ*M* [$^3$H]-MTX over 15 min at increasing concentrations of VBL is illustrated in Fig. 1. VBL at 100 μ*M* significantly inhibited MTX uptake at as early as 30 s after MTX addition.

Table 1 shows the effects of escalating VBL concentrations on the accumulation of [$^3$H]-MTX in hepatocytes. Concentrations as low as 12.5 μ*M* VBL inhibited MTX accumulation significantly, whereas 100 μ*M* VBL reduced the accumulation of MTX by 67.0%.

To examine whether the vinca alkaloids would also interfere with MTX efflux (Fig. 2), hepatocytes were preloaded with 1 μ*M* [$^3$H]-MTX for 60 min to approach the steady state for intracellular MTX. At 20 min after a washing procedure, intracellular MTX was significantly less reduced in VBL-treated hepatocytes (22.6% ± 3.9%) as compared with controls (31.6% ± 8.4%). The half-life ($t_{1/2}$) values obtained for MTX efflux in VBL-treated cells and controls were 60.3 ± 16.0 and 39.7 ± 10.4 min, respectively, demonstrating a slight inhibitory effect of VBL on MTX efflux. Thus, the decline in net MTX accumulation by VBL is caused by a gross reduction in MTX that is not counterbalanced by a moderate effect on MTX efflux.

We therefore determined the kinetics of inhibition of MTX influx by VBL using Dixon-plot analysis at various concentrations of [$^3$H]-MTX (1–10 μ*M*) and VBL

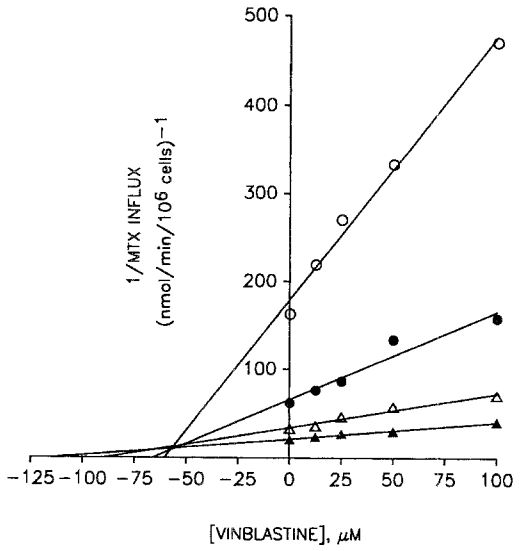


**Fig. 3.** Dixon-plot analysis of MTX influx in isolated rat hepatocytes exposed to VBL. Hepatocytes were preincubated with 0–100 μ*M* VBL before the addition of 1–10 μ*M* [$^3$H]-MTX. At 10 min after [$^3$H]-MTX addition, hepatocyte suspensions were collected for examination of the rate of MTX influx. *Open circles*, 1 μ*M* [$^3$H]-MTX; *filled circles*, 2.5 μ*M* [$^3$H]-MTX; *open triangles*, 5 μ*M* [$^3$H]-MTX; *filled triangles*, 10 μ*M* [$^3$H]-MTX

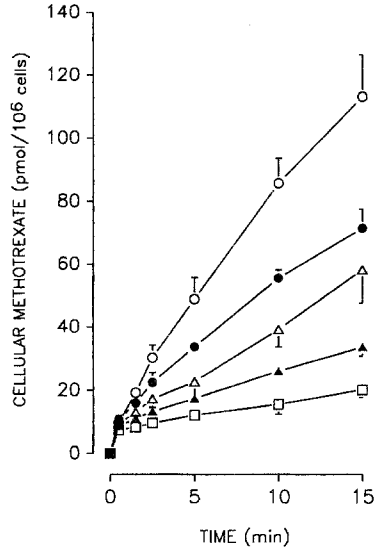


**Fig. 4.** Effect of VCR on hepatocellular uptake of MTX. Freshly isolated rat hepatocytes were exposed to 1 μ*M* [$^3$H]-MTX after 20 min preincubation with increasing concentrations of VCR. Data represent mean values ± SD ($n$ = 3). *Open circles*, 0 μ*M* VCR; *filled circles*, 50 μ*M* VCR; *open triangles*, 100 μ*M* VCR; *filled triangles*, 250 μ*M* VCR; *open squares*, 500 μ*M* VCR

(0–100 μ*M*). Figure 3 displays an apparent $K_i$ value of 55 μ*M* for inhibition of MTX influx.

### *Effects of VCR on MTX transport*

Figure 4 illustrates the influence of 0–500 μ*M* VCR on the uptake of 1 μ*M* [$^3$H]-MTX over a 15-min exposure period.

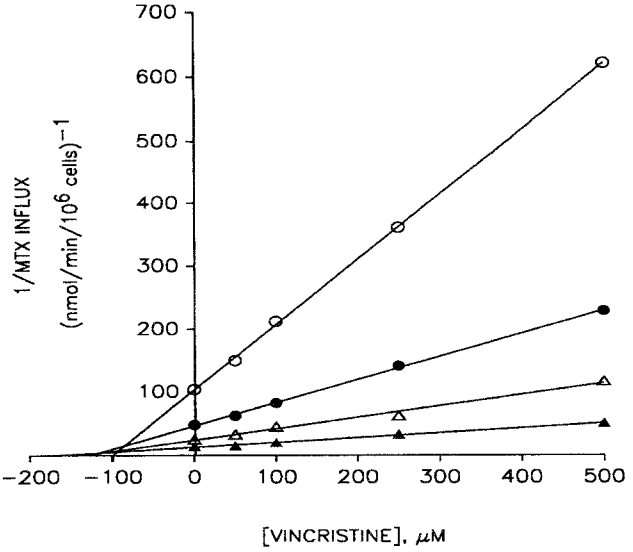


**Fig. 5.** Dixon-plot analysis of MTX influx in isolated rat hepatocytes exposed to VCR. Hepatocytes were preincubated with 0–500 μ*M* VCR before the addition of 1–10 μ*M* [$^3$H]-MTX. At 10 min after [$^3$H]-MTX addition, hepatocyte suspensions were collected for examination of the rate of MTX influx. *Open circles*, 1 μ*M* [$^3$H]-MTX; *filled circles*, 2.5 μ*M* [$^3$H]-MTX; *open triangles*, 5 μ*M* [$^3$H]-MTX; *filled triangles*, 10 μ*M* [$^3$H]-MTX

Statistically significant inhibition occurred at 50 μ*M* VCR, whereas 500 μ*M* VCR decreased hepatocellular MTX by as much as 82.2% (Table 2). A Dixon-plot analysis for determination of the kinetics of inhibition of MTX transport by VCR is presented in Fig. 5. The apparent $K_i$ value obtained for inhibition of MTX influx was 110 μ*M*.

## Discussion

The effects of vinca alkaloids on MTX transport have been described in several reports. However, the results remain contradictory [3, 4, 7, 10, 11, 23, 53]. We recently reported the observation of decreased MTX uptake in isolated rat hepatocytes induced by the vinca alkaloid VDS [7]. Herein, we present data in support of a similar effect of VBL and VCR on the hepatocellular accumulation of

**Table 2.** Inhibition of net [$^3$H]-MTX accumulation in isolated rat hepatocytes pretreated with various concentrations of VCR

| VCR (μ*M*) | MTX (pmol/10$^6$ cells) | % Decrease in cellular MTX |
|---|---|---|
| 0 | 113.0 ± 13.3 | – |
| 50 | 71.4 ± 6.1* | 36.8 |
| 100 | 58.1 ± 10.7 | 48.6 |
| 250 | 33.5 ± 2.9 | 70.4 |
| 500 | 20.1 ± 2.4 | 82.2 |

Hepatocytes were preincubated with VCR for 20 min prior to incubation in 1 μ*M* [$^3$H]-MTX for 15 min. Data represent mean values ± SD ($n$ = 3)

* $P$ <0.05 between 0 and 50 μ*M* VCR

MTX. We also report a moderate inhibition of the efflux of MTX by VBL.

Net accumulation of MTX was reduced 67% and 82% by VBL and VCR, respectively. Hepatocytes were preincubated for 20 min with the vinca alkaloids, since it has been reported that a 10- to 20-min incubation interval is essential for VCR to influence cell transport [25]. Although MTX efflux was slightly inhibited by VBL, the net reduction in cellular MTX accumulation demonstrates that inhibition of influx is by far the dominating effect of VBL.

Our data corroborate the report of Strum et al. [53], who found VCR inhibition of amethopterin uptake by the isolated perfused rat liver. In contrast, experiments using VCR in different tumor cells have demonstrated augmented MTX uptake [3, 10, 19, 57]. In one of the few studies examining MTX transport interactions in nonmalignant cells, Chello et al. [10] showed no effect of VCR on the cellular transport of MTX in normal mouse intestinal epithelium. Whether there is a difference between tumor cells and nonmalignant cells in general in their handling of the combination of MTX and VCR remains ambiguous.

Dixon-plot analysis revealed $K_i$ values for VBL (55 $\mu M$) and VCR (110 $\mu M$) that were, respectively, similar to and slightly higher than that determined for VDS (57 $\mu M$) [7]. As is the case for VDS [7], the inhibition of MTX uptake by both VBL and VCR appears to be competitive, as the extrapolated lines intersect above the abscissa. However, since the intersection of the lines is fairly close to the x-axis, it is difficult to exclude the possibility of a noncompetitive interaction. The concentrations of vinca alkaloids applied in the hepatocyte suspensions correspond grossly to the human plasma levels obtained shortly after the infusion of therapeutic doses [40, 41].

Most mammalian cell types have similar systems for the uptake of reduced folates and MTX [27, 30, 49, 53]. In isolated rat hepatocytes, however, the carrier-mediated uptake of 5-methyltetrahydrofolate has been found to differ from that responsible for MTX transport [17, 20, 29, 31, 32]. In this system, Gewirtz et al. [20] described a high- and a low-affinity route for MTX influx. Both routes have been suggested to be energy- and sodium-dependent [21, 49]. In an attempt to characterize further the uptake routes, those authors could not detect a significant difference between the two routes in the presence of inhibitory substances [21] and under the various conditions [20] investigated. The rapid and substantial decrease in cellular MTX accumulation noted in the present study is compatible with an inhibitory effect on both influx routes by vinca alkaloids.

With regard to efflux, mechanisms in tumor cells have been extensively studied [24–26, 45, 50]. Part of the efflux proceeds via the bidirectional reduced-folate influx system [24], but whether a single [45, 50] or multiple [24, 25] unidirectional, energy-dependent route(s) handle the main MTX efflux remains elusive. In isolated hepatocytes, Gewirtz et al. [22] have induced MTX release by $\alpha$-adrenergic agents in the presence of calcium and metabolic energy. It is presently unknown whether this is the main or only the functional efflux route for MTX in hepatocytes. Further studies are in progress in our laboratory to explain the effect of the vinca alkaloids on MTX efflux.

The observation that VBL, VCR, and VDS interact with MTX by generating a detrimental effect on the cellular drug uptake may be suggestive of a mutual target of action for all three vinca alkaloids. But whether all vinca alkaloids act uniformly [14, 28, 44, 48] or whether each substance undergoes a specific interaction with cellular tubulin [55] has not been resolved. A possible interaction between vinca alkaloids and MTX could occur at the level of the cytoskeleton/plasma membrane connection. By interrupting the assembly of microtubules and hence possibly altering the cytoskeleton/plasma membrane [36, 43, 46], vinca alkaloids could disturb the MTX transport system.

Our previous study on VDS [7] was initiated as part of a series of experiments aimed at outlining strategies for reducing MTX toxicity and improving the rates of response to this established anticancer agent. By decreasing the hepatocellular influx of MTX, VDS reduced the formation of the metabolite 7-hydroxymethotrexate (7-OH-MTX) [7]. Since 7-OH-MTX may limit the toxicity of MTX to malignant cells [18, 37] by reducing cellular MTX entry and synthesis of the potent MTX polyglutamates [12, 13, 35], an interaction leading to reduced 7-OH-MTX synthesis may be advantageous. Furthermore, 7-OH-MTX has been suggested to be a possible mediator of both hepato- and nephrotoxicity [2, 8, 33, 34, 38, 56]. We assume that by limiting the hepatocellular influx of MTX, VBL and VCR inhibit 7-OH-MTX formation as well. Such an interaction may contribute to a decrease in unwanted MTX-associated toxicity in chemotherapy regimens combining vinca alkaloids and MTX.

We conclude that VBL and VCR as does VDS, inhibit the hepatocellular uptake of MTX in a probably competitive manner. This interaction should be further exploited to clarify the mechanisms involved.

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