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Research paper

Effect of etoposide on the pharmacokinetics of methotrexate *in vivo*

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The effect of etoposide on the pharmakokinetics of methotrexate (MTX) was examined *in vivo*. High-dose (5 g/m^2/24 h) MTX therapy was combined with two etoposide (100 mg/m^2/1 h) infusions as a part of the medulloblastoma protocol developed in our department. Vepesid therapy was administered in two different schedules. The first group of patients received etoposide immediately before and at the end (24 h) of MTX treatment. The second group was treated with etoposide at 24 and at 48 h after starting MTX infusion. In this latter group both treatment-related grade III and grade IV toxicity developed more frequently than in the first group (58.6 versus 29.2%, for grade 3 toxicity p=0.019, for grade 4 toxic signs p=0.040, respectively). We observed that after the second dose of etoposide given at 48 h (second group) both total and unbound serum MTX levels (determined by high-performance liquid chromatography) were elevated by 53–109 and 26–65%, respectively, by the third hour after completion of Vepesid infusion. This effect was detectable for 6 h. All the liver and kidney functions of the patients were within the normal range. These results suggest the possibility of partial recirculation of extra/intracellular MTX into the blood after etoposide administration. Based on these results, the therapeutic protocol has been modified, and Vepesid is given prior to and at the end (24 h) of high-dose MTX treatment. Under these conditions only a slight decrease of MTX elimination has been detected between 25 and 28 h. These results emphasize the role of possible schedule-dependent interactions of cytostatic drugs. [© 1998 Lippincott Williams & Wilkins.]

Key words: Brain tumor, children, etoposide, methotrexate, pharmacokinetics.

Introduction

The incidence of central nervous system tumors in childhood is approximately 30%. Nowadays, besides surgery, chemotherapy combined with radiotherapy is being applied more frequently. Those drugs could only be incorporated into the chemotherapeutical protocols for patients with brain tumors which have a good serum/cerebrospinal fluid concentration ratio or in which the drugs could be accumulated in the tumor tissue with minimal toxicity to the host organism.[1] Taking pharmacokinetic and pharmacodynamic considerations into account, we have been using the medulloblastoma protocol (Table 1) since 1991.[2–5] The protocol prescribes the high-dose methotrexate (MTX, Methotrexat) and etoposide (VP-16, Vepesid) combination block once in the pre-irradiation phase. In spite of using the same dose and duration of MTX administration as is prescribed in the ALL BFM-90 protocol for leukemic patients, slight toxic signs typical for MTX were more frequent in patients treated with VP-16 at 24 and 48 h after starting MTX, compared to leukemic patients, even when the serum MTX levels were in the normal range. Knowing the high serum protein binding properties of MTX, and of VP-16 in particular, the possibility has arisen of etoposide administered at 43–48 h causing an increase in unbound, effective MTX.

The aim of the present *in vivo* study was to determine how VP-16 influences the pharmacokinetics and pharmacodynamics of MTX in serum.

Materials and methods

Medulloblastoma treatment protocol

Patients were treated according to the medulloblastoma protocol developed at our department.[2–5]

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Table 1. Medulloblastoma protocol

Drug	Dose	Route of administration	Time of the treatment (weeks)
Vincristine	1.5 mg/m^2, maximum 2 mg	i.v.	on weeks 1–2, 19–20, 29–30, 38–39 (on the first day of the week)
Elobromol (dibromodulcitol)	500 mg/m^2	orally	on weeks 1–3, 19–21, 29–31, 38–40 (on the first day of the week)
Natulan (procarbazine)	100 mg/m^2/day	orally	on weeks 1–3, 19–21, 29–31, 38–40
MTX with leucovorin rescue	5 g/m^2/24 h according to the serum MTX levels	in a 24 h infusion i.v. or orally	on week 4
MTX	according to the age	i.t.	at the end of the 24 h infusion
Vepesid (etoposide)	100 mg/m^2	i.v. 1 h infusion	on week 4, on the day of methotrexate administration and the day after
Radiotherapy	tumor dose 55 Gy cranio-spinal dose 30 Gy		from week 7 to 13
Elobromol (dibromodulcitol)	50 mg/m^2/day	orally	from week 7 to 13 during the radiotherapy
Vepesid (etoposide)	50 mg/m^2	i.v. bolus	on weeks 15, 24, 34
Platidiam (cisplatin)	90 mg/m^2/5 h	5 h infusion	on weeks 15, 24, 34

Cytotoxic drugs prescribed in the postoperative period of the protocol are listed in Table 1. Therapy and sample collection were undertaken in compliance with the Geneva nomenclature and with the written consent of the parents/relatives. To circumvent technical reasons, in cases where the time between VP-16–MTX block and radiation therapy would be more than 3 weeks, an additional VP-16–MTX block was administered.

All of the patients were prehydrated with 3000 ml/m^2/24 h 0.45% NaCl + 5% glucose infusion, containing 90 mmol/m^2/24 h KCl and 180 mmol/m^2/24 h $NaHCO_3$ before high-dose MTX treatment. Extra i.v. or oral alkalization was given when urine pH decreased below 7. One-tenth of the 5 g/m^2/24 h MTX was administered in 30 min, the remaining nine-tenths being completed in 23.5 h. Following treatment, posthydration was given until 72 h with the above-mentioned infusion solution. Ca-leucovorin rescue dose and rate of administration was determined according to the serum MTX levels.

Dose of VP-16 was 100 mg/m^2 in a 60 min infusion twice: one group of patients [22 patients, 29 treatments, including the first (15 years old) and the second (11 years old) patients] received VP-16 at the end of MTX (24 h) treatment and at 43–48 h; the second group [20 patients, 24 treatments, including the third (7 years old) and fourth (13 years old) patients] received VP-16 immediately before and at the end (24 h) of MTX infusion.

Classification of toxic effects

Classification of toxic effects developed after VP-16–MTX combination chemotherapy was based on 'common toxicity criteria' (NCI) published by SIOP[6] in *General methodology for phase II therapeutic trials in paediatric oncology* 1992.

Statistical analysis

For statistical evaluation data were processed by Student's *t*- and *F*-tests.

Collection of samples

Following high-dose MTX therapy, we determined serum MTX levels routinely at the end of MTX infusion (*24 h*) and at 48 h. In the case of the presented patients additional measurements were taken before the administration of VP-16 and after it every hour for 4 h. For adequate rescue administration serum MTX levels were monitored until 0.4 μM (4×10^{-7} M).

Materials

We used Methotrexat (Ebewe, Unterach, Austria) and Vepesid (Bristol, New York, NY) for the rescue, and Calciumfolinat (Ebewe) for preparation of infusion

solutions and standards. HPLC grade chemicals were obtained from Merck (Whitehouse Station, NJ).

Chromatography system

HPLC was performed using a LaborMIm Model 2010 instrument (Liquopump 312/1, 308 UV Decetor, Radelkis OH-850 recorder), with a 10u 250 × 4 mm Hypersil ODS column connected with a 30 mm guard column. Composition of the eluent was 0.08 M sodium acetate buffer (pH 5.0) containing 11.5% acetoitrile at a flow rate of 1 ml/min. Injected volume was 500 μl. Quantitative MTX analysis was performed at 313 nm by measuring peak height. Detection limit was 0.05 μM.

Determination of the total and free MTX concentrations from the frozen samples of the first and second patients was performed using a Beckman System Gold HPLC instrument (equipped with a Beckman System Gold 126 pump and a 166 detector), with the above-mentioned column and eluent. The injected volume was 20 μl resulting in the same detection limit. Data were processed automatically by a computer connected to the HPLC system according to the area under the peak of the chromatography curve. All the samples were analyzed after calibration with plasma from healthy volunteers.

Sample preparation

The native blood after clotting was centrifuged (3200 r.p.m. for 10 min). To 2500 μl serum, 500 μl 50% trichloroacetic acid was added and, after thorough mixing, centrifuged again. The supernatant was injected directly. The variation coefficient of the method was (V) 3.12%. To determine the total MTX concentrations we used solid-phase extraction (Varian Bond Elut 200 mg C18) according to Belz *et al.*[7] The variation coefficient of the method was (V) 1.65%.

The free (unbound) MTX fraction was determined by filtration (Ultrafree-MC 10 000 NMWL 400 μl) after centrifugation (3000 r.p.m. for 30 min). Filtrate was injected directly. The variation coefficient of the method was (V) 2.35%.

Results

Evaluation of toxicity

We examined the two groups of patients according to the different time schedules of VP-16 administration.

VP-16 administered at 24 and 48 h. We detected grade III-IV toxicity in 17/29 cases (58.6%) after VP-16-MTX treatment of 22 patients by day 2-16. In one case (patient ID 18/2) the grade III Hb on the first day (the day of MTX administration) could not be attributed to the treatment. The most frequent toxic sign was the decrease of Hb by day 8-16 (6/29, 20.7%) with or without other hematologic toxicity. Neutropenia (5/29, 17.2%) and granulocytopenia (4/29, 13.8%) were independent of the occurrence of sepsis (2/29, 6.9%). Thrombocytopenia (4/29, 13.8%) appeared between day 10 and 14. Liver function tests (bilirubin 1/29, 3.4%; GPT 2/29, 6.9%) showed a low frequency of severe toxicity. In one case severe vomiting was detected (3.4%), while deep stomatitis, mucositis developed in 2/29 (6.9%) cases.

The serum MTX levels measured at 48 h were not significantly different for cases with (4.1 ± 12.2 μM, 0.1-51.3 μM) and without (0.9 ± 0.8 μM, 0.3-2.3 μM) grade III-IV toxicity (p=0.4). Elevated (higher than 1 μM) serum MTX levels were detected at 48 h in 6/17 cases (35.3%) with grade III-IV toxicity. In 11/17 (64.7%) cases the toxic signs developed after normal MTX levels (0.5 ± 10.2 μM, 0.1-0.8 μM).

There was no grade III-IV toxicity in 11/29 (37.93%) cases with 0.9 ± 0.8 μM, 0.3-2.3 μM MTX levels including three cases higher than 1 μM MTX.

The details are shown in Table 2.

VP-16 administered before and after MTX (0 and 24 h). Grade III-IV toxicity occurred in 8/24 cases (33.3%) after VP-16-MTX treatment of 20 patients by day 2-11. The most frequent toxic sign was the decrease of Hb by day 4-8 (4/24, 16.6%) and of white blood cells (5/24, 20.8%). Granulocytopenia (1/24, 4.2%) was related to the occurrence of sepsis (2/24, 8.3%). Thrombocytopenia (2/24, 8.3%) appeared between day 7 and 9. Similar to the above-mentioned group, liver function tests (bilirubin 2/24, 8.3%; GPT 2/24, 8.3%) showed a low frequency of toxicity. In one case severe vomiting was detected (4.2%). There was no grade III-IV stomatisis.

Serum MTX levels measured at 48 h were significantly different for cases with (3.9 ± 5.7 μM, 0.56-5.93 μM) and without (0.89 ± 1.03 μM, 0.2-4.2 μM) grade III-IV toxicity (p=0.018). Elevated (higher than 1 μM) serum MTX levels were detected at 48 h in 4/8 cases (50.0%) with grade III-IV toxicity. In 4/8 (50.0%) cases the toxic signs developed after normal MTX levels.

There was no grade III-IV toxicity in 16/24 (66.7%) cases with 0.9 ± 1.03 μM, 0.2-4.2 μM MTX levels

Table 2. List of toxic signs of patients treated with VP-16 infusions at 24 and 48 h following high-dose MTX

Patient ID and treatment code	Toxic signs (day, organ, grade)	Serum MTX levels at 48 h (μM)
1		2.300
2		0.830
3	10.ANC.III	0.588
4/1	2.SBi.III	1.500
4/2		1.900
5	8.WBC.III	0.816
6/1		2.120
7/2	8.Hb.III	51.300
6/3		0.337
8/2		0.476
9/1		0.732
10/1	15.Hb.III, 15.WBC.IV, 15.ANC.IV, 15.PLT.III	1.330
9/2		–[a]
11	7.WBC.IV, 7.SGPT.III, 10.SGPT.III, 11.SGPT.IV	–[b]
10/2		0.347
12	2.Vo.III	3.600
13		0.309
14/1	16.Hb.III, 16.WBC.III, 16.PLT.III	0.714
15	7.ANC.III, 7.WBC.III	0.603
16	14–24.sepsis, 14–24.PLT.III–IV	0.359
17	7.Hb.III	0.278
14/2	12.Hb.IV	0.621
18/1	9.PLT.III, 10.PLT.III	0.322
19	11.Sto.III	0.679
18/2	1.Hb.III	0.522
20		0.360
21	7.SGPT.IV	0.123
22/1	7.Sto.III, 7–15.sepsis	0.726
22/2	2.ANC.IV	1.240

[a]Patient ID 9/2 is identical to the second patient, whose results are presented in Figures 3 and 4.
[b]Patient ID 11 is identical to the first patient, whose results are shown in Figures 1 and 2.
Abbreviations: Hb, Hemoglobin (g/l); WBC, white blood cell (g/l); ANC, absolute neutrophil count (g/l); PLT, platelets (g/l); SGPT, glutamate-piruvate transaminase (U/l); Sto, stomatitis; Sbi, serum bilirubine (μmol l); Vo, vomiting.

including three cases higher than 1 μM MTX.

The details are shown in Table 3.

Pharmacokinetic studies

In four patients, who were chosen randomly, serum MTX levels were closely monitored as described above.

Results of the first patient. The second VP-16 infusion was administered at 45.5 h after the starting of high-dose MTX. The serum MTX level determined at 43.5 h was 2.7 μM; at 46.5 h (at the end of VP-16 infusion) it was 2.9 μM; and 2.8, 5.0, 3.4 and 0.99 μM at 47.5, 48.5 , 49.5 and 63.5 h, respectively (Figure 1). By 3 h the MTX level measured immediately after the end of VP-16 was increased by 74.6%.

The determination of free (unbound) and total MTX levels from the frozen samples (Figure 2) showed a 64.9 and 53.1% increase also by 3 h, respectively. The ratio of free MTX to total did not change significantly during the monitored period (47.5 $\pm$ 5.4%, 43.7–77.3%).

Results of the second patient. VP-16 infusion was started at 48 h. The serum MTX level determined at 45 h was 0.57 μM, at 49 h (at the end of VP-16 infusion) 0.5 μM; and 0.6, 0.7, 0.6 and 0.14 μM at the 50, 51, 52 and 65 h, respectively (Figure 3). By the 3 h, the MTX level measured immediately after the end of VP-16 was increased by 36.8%.

Total MTX levels determined by solid phase extraction from the frozen serum samples showed a 109.2% increase, while the filtrated unbound fraction increased by 25.9% also by 3 h (Figure 4). There was no significant change in the calculated free/bound MTX ratio (48.2 $\pm$ 2.2%, 30.5–62.1%).

Monitoring of serum MTX levels of *patient 3* (Figure 5) and *patient 4* (Figure 6) treated according to the modified protocol showed a temporary and slight

Table 3. List of toxic signs of patients treated with VP-16 before and after (at 24 h) high-dose MTX infusion

Patient ID and treatment code	Toxic signs (day, organ, grade)	Serum MTX levels at 48 h (μM)
23		1.080
24	2.Vo.III, 3.WBC.III, 6.WBC.III	1.620
7/1	6.Hb.III	4.130
25		0.900
26		0.848
27	7.Hb.III, 7.WBC.III, 7.SGPT.III	5.930
8/1	3.WBC.III, 4.WBC.IV, 4.Hb.III, 7.Hb.III, 7.WBC.IV, 7.PLT.III, 3–12.sepsis, 9.WBC.III, 9.PLT.III	17.100
6/2	1.SBi.III, 8.WBC.III, 8–15.sepsis	0.558
28		0.640
29	9.PLT.III, 9.SGPT.III	0.749
30[a]		0.299
31[b]		0.535
32/1	2.SBi.III	0.510
32/2		4.200
33/1		0.294
34/1		0.229
33/2		0.413
34/2		0.736
34/3		0.641
35	8.Hb.III, 11.WBC.III, 11.ANC.III	0.572
36		0.512
37		0.250
38		0.220
39		2.430

[a]Patient ID 30 is identical to the third patient, whose results are shown in Figure 5.
[b]Patient ID 31 is identical to the fourth patient, whose results are shown in Figure 6.
Abbreviations: Hb, Hemoglobin (g/l); WBC, white blood cell (g/l); ANC, absolute neutrophil count (g/l); PLT, platelets (g/l); SGPT, glutamate-piruvate transaminase (U/l); Sto, stomatitis; Sbi, serum bilirubine (μmol/l); Vo, vomiting.

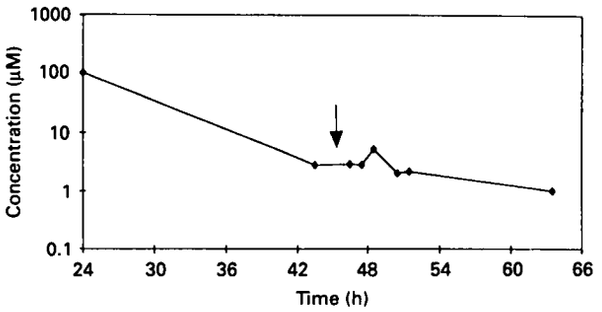

Figure 1. Serum MTX levels of the first patient (patient ID is 11) determined after deproteinization by trichloroacetic acid. The start of the etoposide infusion is indicated by the arrow.

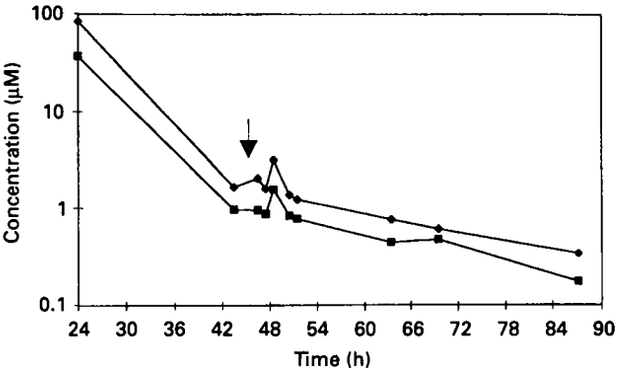

Figure 2. Total (◆) and unbound (■) serum MTX levels of the first patient (patient ID is 11). The start of the etoposide infusion is indicated by the arrow.

decrease in the rate of elimination between 25 and 28 h. After this period elimination returned to that of leukemic average. Serum protein values of all patients were in the normal range (65–75 g/l) during the monitored period.

We compared the results of the different deproteinization methods used in this study. Drug levels determined by trichloroacetic acid protein precipitation showed a good correlation with the free drug levels measured by filtration (R^2=0.99, p=0.095, data not shown). Total MTX (levels calculated from the results of trichloroacetic acid protein precipitation and measured after solid-phase extraction also showed good correlation (R^2=0.99, p=0.04, data not shown).

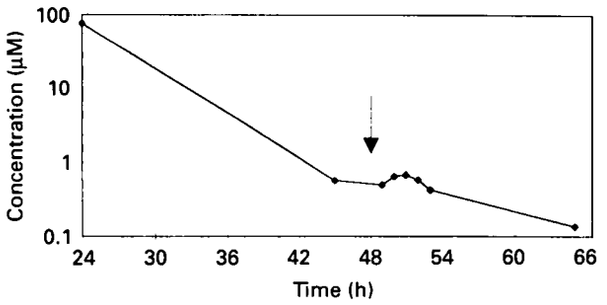

Figure 3. Serum MTX levels of the second patient (patient ID is 9/2) determined after deproteinization by trichloroacetic acid. The start of the etoposide infusion is indicated by the arrow.

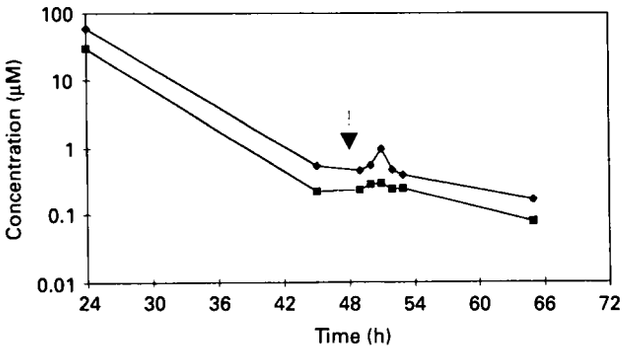

Figure 4. Total (◆) and unbound (■) serum MTX levels of the second patient (patient ID is 9/2). The start of the etoposide infusion is indicated by the arrow.

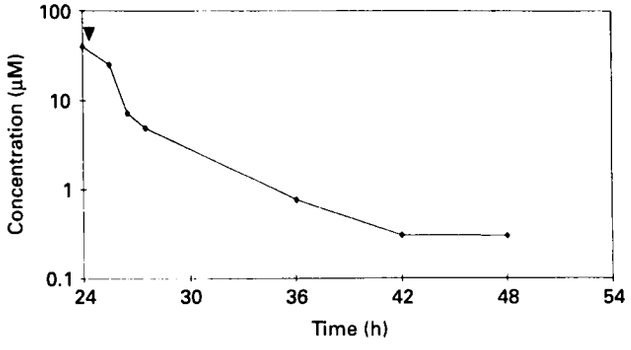

Figure 5. Serum MTX levels of the third patient (patient ID is 30) determined after deproteinization by trichloroacetic acid. The start of the etoposide infusion is indicated by the arrow.

Discussion

Drugs absorbed or administered directly are present in the blood in a certain ratio of free (unbound)-bound

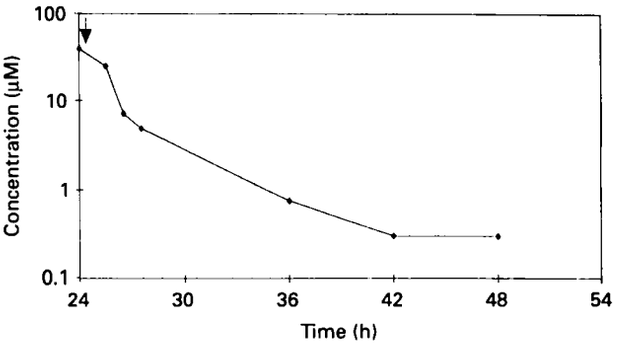

Figure 6. Serum MTX levels of the fourth patient (patient ID is 31) determined after deproteinization by trichloroacetic acid. The start of the etoposide infusion is indicated by the arrow.

forms according to their properties in relation to serum albumin, α-1-glycoprotein and lipoprotein fractions. This ratio, the binding site(s) and the strength of binding depend on the structural features of the pharmacon.

To have effect the drug has to reach and enter into the target cell, but only the free drug is able to leave the vessels. The most plausible reason for the unusually intensive pharmacologic effect or toxicity is the increased intracellular drug concentration caused by either the inhibition of efflux or by the higher rate of influx due to the increased free drug ratio in the blood. Both pathways can be affected by endogenous (hypoproteinemia, impaired liver and kidney function or an increase in the concentration of endogenous substrates, such as bilirubine) and exogenous factors. To the latter group belong the chemotherapeutic drug combinations. Despite the protein binding of MTX and VP-16 being well described individually, we have found no published data on the pharmacokinetic or pharmacodynamic consequences of their simultaneous administration, nor any studies *in vitro* or *in vivo* relating the changes in their protein binding.

MTX has been reported to bind serum proteins in the range of $47.2 \pm 4.4\%$[2] predominantly to serum albumin. The percentage of MTX bound to albumin is 87.3%.[3] Two classes of MTX binding sites have been observed, a high-affinity, low-capacity group and a low-affinity group with higher capacity.[8-10] No significant linear correlations were observed between the extent of MTX binding and serum concentrations of total proteins, albumin, total bilirubin or triglyceride.[11] Salicylate in therapeutic concentrations produced a 20-60% decrease in the binding of MTX to albumin.[12] The organic acid probenicid has been shown to interfere with the active extrusion of MTX from

L1210 tumor cells leading to enhanced MTX accumulation. In Ehrlich ascites tumor cells probenicid may act by increasing the level of free (unbound) MTX. Probenicid (0.1 mM) reduced the albumin-bound MTX by 50%, while at 1 mM probenicid only 6% of the MTX was bound to serum proteins. Probenicid may also act by the direct inhibition of MTX efflux. Maximal cellular accumulation of MTX in the presence of albumin occurred with 3 mM probenicid. In these conditions MTX influx was stimulated by approximately 50%, while the efflux was inhibited by approximately 75%. At lower probenicid concentrations the stimulation of influx would, presumably, play a greater role.[13] Paxton[10] demonstrated that probenicid displaces MTX from human plasma proteins. At concentrations higher than or equal to 5×10^{-4} M, the reduction of plasma protein binding of MTX was more than 30%. Drugs used in cancer chemotherapy, such as adriamycin, vincristine sulfate and cyclophosphamide, may be administered in combination with MTX. They compete with MTX at the serum protein binding level. The competition involves only one of the MTX binding sites. The value of liberated MTX can reach 15–20% at high MTX concentrations, which is not negligible in pharmacological terms.[14] An effect of vincristine (VCR) increasing the intracellular pool of MTX has also been reported.[15]

Plasma protein binding of VP-16 is extensive (approximately 94%) but showed large interindividual variance. Binding is significantly related to serum albumin concentration. Binding parameters in human plasma were characterized by a single class of binding sites of moderate affinity and high capacity.[16] The fraction of etoposide unbound in plasma was significantly higher in patients with increased bilirubin[17] due to competition for the binding.[16] Steward *et al.*[18] used a mathematical model based on serum albumin and total bilirubin in order to calculate the unbound fraction of VP-16. The measured and predicted values were highly correlated. Impaired renal function or hypoproteinemia both in adults[19] and in children[20] causes more frequent toxicity due to the reduced clearance. The intracellular accumulation of VM-26 was associated with various cell organelles. However, only the nuclear fraction demonstrated high-affinity. Intracellular fractionation of bound drug indicated that a substantial proportion of VM-26 can be removed by exhaustive dialysis at 4°C, whereas the tightly bound drug remains after a variety of denaturing treatments. VCR was able to alter the proportion of free or loosely bound drug to tightly bound without affecting the steady-state accumulation of the drug.[21] Allen *et al.*[22] have devised a therapeutic regimen whereby VM-26 is given 2 h prior to VP-16 in order that VM-26 should become widely distributed in the body, and bound to albumin in plasma and in interstitial space. The systemic bioavailability of VP-16 should be enhanced in accordance with the extent to which its protein binding is diminished. They demonstrated that VM-26 displacement of VP-16 protein binding was greater than VP-16 displacement of VM-26 binding. The consequence of protein binding displacement at the cellular level is greater cellular uptake and steady-state accumulation of drug. This increases intracellular irreversible binding of VM-26 or VP-16. Patients who received the drug i.v. regimen experienced greater toxicity and became more leukopenic than those who received i.p.

In our present work the possibility that competition for protein binding sites may occur during VP-16–MTX treatment as well as elevation of circulating drug concentrations has been investigated by detailed pharmacokinetic examination of patients. In the case of the first and second patients, treated according to the schedule for the second group, both total and unbound serum MTX level elevation could be detected (53.1–109.2 and 25.9–64.9%, respectively) without significant change in the unbound fractions. This increase was not accompanied by any detectable impairment/change in liver or kidney function. Thus we suppose that the higher rate of development of grade III–IV toxic signs in the second group may be attributed to the withdrawal of rescue therapy after the determination of normal serum MTX levels before VP-16 administration. Following etoposide administered at the end of MTX infusion, we could detect a slight and temporary decrease in the rate of MTX elimination. This may be indirect proof of the recirculation of the extra/intracellular MTX into the blood.

The effect of etoposide on the protein binding of MTX is currently being investigated *in vitro*.

A good correlation between the different deproteinization methods used in this study was found.

Although, the frequency of elevated MTX levels at 48 h was similar in each group (7/24, 29.2% in the first and 9/29, 31.0% in the second group), the frequency of grade III–IV toxic signs was significantly higher in patients treated with VP-16 at 24 and 48 h (p=0.019). Whereas in the group treated with etoposide at 0 and 24 h only one patient had grade IV toxicity, in the other group grade IV toxicity developed in six cases. The difference was also significant (p=0.04). Liver function tests showed a similar frequency of toxicity in both groups. There was only one patient in each group with several vomiting episodes on day 2, but both of them were hypoproteinemic (total serum protein was 62 and 57 g/l) on that day. All the other

patients had normal (higher than 70 g/l) serum protein levels.

Based on the significant differences in the toxicity profile between the treatment groups the therapeutic protocol has been modified without collecting the appropriate number of cases for a more precise statistical analysis. Presently Vepesid is given prior to and at the end of high-dose MTX infusion.

These results emphasize the role of possible schedule-dependent interactions of cytostatic drugs.

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