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UPTAKE AND BINDING OF TENIPOSIDE (VM 26) IN KREBS II ASCITES CELLS

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Abstract—With [^{3}H] VM26 as marker, the uptake and binding of teniposide have been made in cells of Krebs II ascitic tumors. The intracellular accumulation of drug displayed a passive diffusion and a saturation kinetics with an apparent Michaelis–Menten constant of 37.54 10^{-6} M and a flux of 13.4 nM/min/mg of protein.

VM26 was rapidly taken and an equilibrium was established with the extracellular drug in about 30 min. The steady-state accumulation was diminished by Na^+ and Ca^{2+} absence and VP16-213, whereas, K^+ and Mg^{2+} have no effect.

Energy dependence of the system was characterized by a Q_{10} of 1.75 ± 0.2 and the uptake was reduced by ouabain and iodoacetamide, when 2–4-dinitrophenol and glucose absence were without appreciable change.

The study of the efflux showed that about 87% of the uptaken drug was removed, the residual amount being probably irreversibly bound.

The intracellular accumulation of the drug was associated with various cell organelles, however, only the nuclear fraction demonstrated a high affinity binding.

VM26, a semi synthetic derivative of podophyllotoxin had gained importance because of its effectiveness against a variety of experimental and human tumors [1–3]. Few papers have studied the transport mechanisms and uptake of this drug in biological systems. Only Allen [4] using L 1210 cells as biological model has shown that the rate of uptake, as well as the steady-state cellular concentration of VM26, were temperature and concentration dependent, and a fraction of the cellular drug was irreversibly retained in the nucleus. Lee and Roberts [5] have demonstrated that resistance of L 1210 cells to VM26 is associated with changes in the flux of the drug across the cell membrane.

Recently, we have shown the efficacy of VM26 in the mice bearing Krebs ascitic tumors and high intracellular levels of this drug following i.p. administration [6]. The present work was undertaken to investigate the uptake and binding of VM26 in Krebs II ascites cells, with the further aim to elucidate the resistance mechanisms of VM26 sublines of Krebs II cells and to compare these phenomenoms to those described with L 1210 cells [5].

MATERIALS AND METHODS

[^{3}H] VM26, [4-^{3}H]demethylepipodophyllotoxin-9-(4,6-0-2-thenylidene-D-glucopyranoside) with a specific activity of 58.6 μCi/mg was kindly supplied by Sandoz Laboratories (Switzerland). Teniposide and Etoposide (VP16-213) were also purchased from Sandoz Laboratories.

Fatty acid free bovin albumin (FFA) was from Sigma Chemical Co. (U.S.A.). Other reagents of analytical grade were obtained from Prolabo, Paris (France). Antibiotics (10^6 units penicillin plus 10^6 μg streptomycin per 5 ml) from Diamant (Puteaux, France).

Radioactivity determination of samples solubilized in 10 ml of picofluor was performed using a Packard tri-carb 460 CD liquid scintillation system counter (Downers Grove, IL).

Cellular suspension

The Krebs II ascitic tumors were maintained in female N.M.R.I. swiss mice, 9–12 weeks old by i.p. transfer of the ascitic fluid, between the eighth and tenth day after their inoculation [7].

All manipulations except incubations were performed at 1–4°. Harvested cells were added with the solution of penicillin and streptomycin (1% v/v), separated from the exsudate by centrifugation at 700 *g* (10 min) and washed twice in 3 vol. of hepes buffer (*N*-2-hydroxyethyl-piperazine *N*-2 ethane sulfonic acid 40 mM, Na_2HPO_4 2 mM, $CaCl_2$ 1 mM, $MgCl_2$ 10 mM, KCl 5 mM, NaCl 93 mM; glucose 11 mM, antibiotics, pH 7.34, 300 mosmol/kg). Contaminating erythrocytes were removed by hypotonic lysis.

Incubations and post treatment of cells

Influx experiments. The cell pellet (1 ml corresponding to about 2.10^8 cells) was suspended in Hepes buffer (1/60 v/v) containing albumin FFA 0.1 mM, antibiotics (1% v/v), VM26, in the presence or absence of various effectors and [^{3}H] VM26 as

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marker. The total volume of the media was 5 ml and osmolarity was adjusted to 300 mosmol/kg by varying the NaCl concentration.

In the experiment, when NaCl concentration was lowered, LiCl was used instead.

At the end of the incubation (37° with gentle shaking), cells were separated at 700 *g* (10 min) from the medium which was used for lactate deshydrogenase determination according to Wroblewski and La Due [8] using a commercial kit (Merck). Then, cell pellets were washed thrice with 20 ml of ice-cold Hepes buffer.

For kinetic studies, the incubation time was experimentally determined to be the midpoint of the linear phase of influx.

Efflux experiments. For efflux experiments, cells were treated as in influx experiments, washed thrice and suspended in free drug fresh 40 mM Hepes buffer. Incubation was performed at 37°, controls received unlabelled VM26 such as in influx determination. When incubation was stopped, extracellular medium was isolated as above.

Extraction and analysis

For uptake measurements, loaded cells were lysed with 1 ml of distilled water and placed in 10 ml of scintillator. Non-specific uptake (diffusion and adsorption) was determined by a control system in which 5 mM unlabelled VM26 were simultaneously added to [^{3}H] VM26 [9, 10]. This experiment is based on the rapid equilibrium of the diffusion and the strong reduction of [^{3}H] VM26 active transport induced by a straight competitition between labelled and unlabelled drug.

Non-specific uptake was always subtracted and only the specific uptake was considered.

Protein measurements were made by the method of Lowry *et al.* [11].

Cellular fractionation

After washing, preloaded cells were homogenized (2.5% v/v in PBS) with a Teflon pestle apparatus, and the nuclear fraction was collected by centrifugation at 1000 *g* (10 min) [7]. The post nuclear supernatant was used to isolate mitochondria, microsomes and cellular sap [4].

Statistical significances are computed according to the Student's *t*-test.

RESULTS

After a 3 hr incubation, the cell morphology remained normal under phase microscopy control, pH was above 7.3 and the lactate dehydrogenase extruded into the medium was less than 4.7% of the total cell enzymatic activity (Fig. 1).

For washing, less than 0.1% of the added radioactivity remained in the third wash of the cell pellet.

VM26 uptake by Krebs II ascites cells

Figure 1 shows the time course of accumulation of radiolabelled VM26 into the Krebs II ascites cells. The total uptake remained linear with time for about 10 min with an important rate of 11.04 ± 0.1 nM/min/2.10^8 cells (N = 4). After 10 min, the accumulation declined and an equilibrium was established in about 30 min.

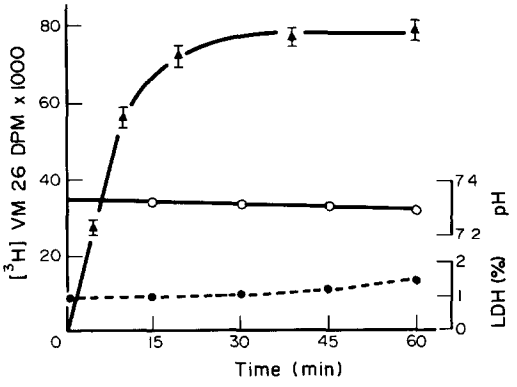

Fig. 1. Accumulation of radioactivity by Krebs II ascites cells as a function of incubation time. Krebs II cells, equivalent of 3.3 · 10^6 cells were incubated in 40 mM Hepes buffer in presence of 0.5 μCi [^{3}H] VM26 and 100 μM VM26. At the end of incubation time, cells were separated as described above and used for label determination (▼). The extracellular medium was used for pH (○) and LDH (●) determinations (N = 3, average values).

As shown in Fig. 2 [^{3}H] VM26 incorporation proceeded by a non-specific uptake (diffusion) governed only by the extracellular drug concentration, and a specific uptake. In experiments related below, only the latter was considered. Under conditions described above, the specific uptake increased proportionally with the increased of medium drug until 60 μM, declined beyond and attained steady-state at about 100 μM.

The curve indicates that specific uptake approximated Michaelis–Menten kinetics which were determined against extracellular drug concentration (Fig. 3a). When the data were plotted according to Lineweaver–Burk construction (Fig. 3b), a single straight line was obtained with a correlation coef-

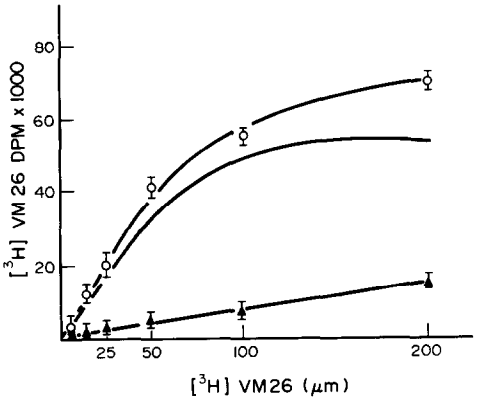

Fig. 2. Effect of [^{3}H] VM26 concentration on VM26 uptake. Effect of [^{3}H] VM26 concentration (abcissa) on VM26 uptake (ordinates) into Krebs ascites cells. 3.3 · 10^6 cells were incubated under standard conditions for 60 min at 37°. Non-specific uptake (▼) was measured by adding 5 mM unlabelled VM26. Specific uptake (■) was the difference between total uptake (○) and non-specific uptake (N = 3, average values).

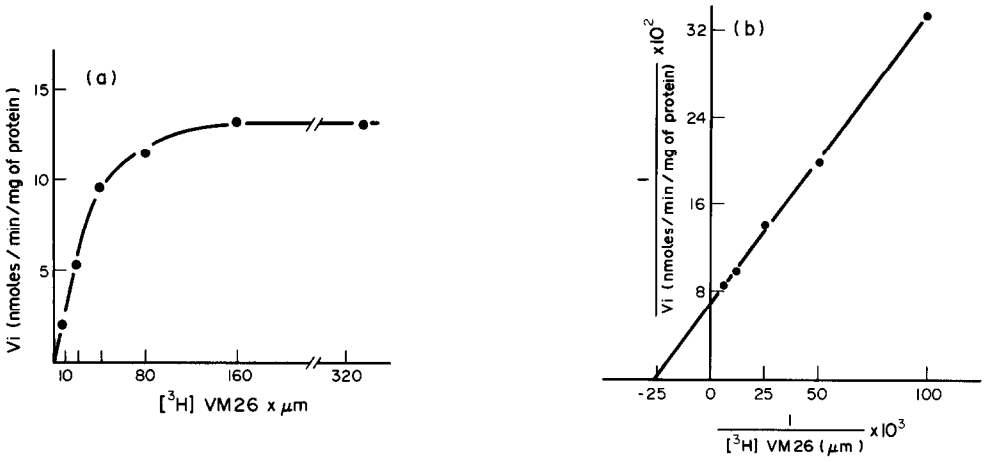

Fig. 3. (a) Plot of the initial velocities of [^{3}H] VM26. Accumulation into Krebs II ascites cells as a function of respective labelled concentrations. (b) Double reciprocal plot. Krebs cells were incubated for 5 min under standard conditions at varying concentrations of labelled substrate. Initial velocities of VM26 uptake are the means of triplicate determinations.

ficient of 0.9924 and an apparent Michaelis–Menten kinetics, with a K_m of 37.54×10^6 M and a V_m of 13.4 nM/min/mg of protein.

The owering of temperature affected the uptake of VM26, incubation at 4° for 10 min reduced the teniposide accumulation by about 95%. When the temperature incubation was lowered from 37° to 27°, uptake decreased with a Q_{10} of 1.75 ± 0.2 (N = 4).

Efflux of [^{3}H] *VM26*

Efflux of [^{3}H] VM26 is reported in Fig. 4. When released products were analysed by an HPLC method using an electrochemical detector [12], only one radioactive peak co-migrating with standard VM26 was identified.

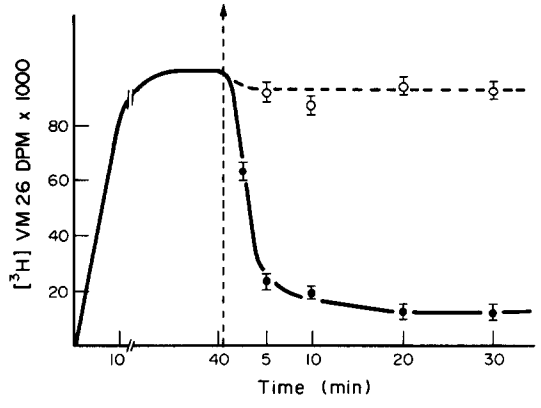

Fig. 4. Efflux of [^{3}H] VM26. Time course of efflux of [^{3}H] VM26 was determined after the step of Fig. 1. Equivalent of $3.3 \cdot 10^6$ cells were preloaded with 100 μM [^{3}H] VM26 for 40 mn at 37° under standard conditions according to Materials and Methods section. After separation and washings, cells were incubated again in free drug fresh medium. At the end of incubation, the label was determined as in influx experiments (●), controls (○) received 100 μM of unlabelled VM26 (N = 3, average values).

After the preloading by [^{3}H] VM26 100 μM and reincubation in fresh free drug medium (assay), or fresh medium with 100 μM of unlabelled VM26 (controls), the cell pellet radioactivity of control indicated a non-significant decrease, when assays decreased by about 87%. Most the drug loss by cells occurred in the first 10 min after transfer.

*Interaction of ions, metabolic inhibitors and VP*16-213 *on VM26 uptake*

In order to optimize the Hepes buffer composition and investigate the ionic requirement for Na^+, K^+, Ca^{2+}, Mg^{2+} and EDTA, cells were incubated in extracellular medium free or containing one of these ions. Results are summarized in Table 1. There is no effect of K^+ and Mg^{2+}, while VM26 uptake displayed a partial dependence on Na^+ and Ca^{2+}. Since in extracellular media free of these ions, the VM26 carrier mediated uptake was reduced by about 18% and 27%. When media was supplemented by EDTA 20 mM, inhibition is in the same order of magnitude than with Ca^{2+} absence.

Table 1 shows also the effects of some metabolic inhibitors. Ouabain (0.2 mM), an inhibitor of adenosine triphosphate (ATP phosphohydrolase (Na^+–K^+) ATPase, EC 3.6.13) lowered the uptake by about 42%. Whereas 2–4 dinitrophenol (0.2 mM), a well known uncoupler of oxydative phosphorylation was ineffective. Iodoacetamide (2 mM), which inhibits glycolysis and oxygen consumption and decreases ATP significantly, lowered the total VM26 uptake by about 35%.

Potassium ferricyanure and glucose absence were not effective. When [^{3}H] VM26 100 μM was added after a 15 min incubation of cells in medium containing VP 16–213 (100 μM), a structural analogue of VM26, the rate of the specific uptake was strongly diminished (37.5% of control (N = 6) indicating a possible use of the same carrier.

The intra-cellular distribution of [^{3}H] VM26 (Table 2) indicates that most of the radioactivity of the homogenate was found in the nucleus pellet.

Table 1. Effect of ions, metabolic inhibitors and VP16-213 on [^{3}H] VM26 accumulation into Krebs II ascites cells

	Concentration		[^{3}H] VM26 uptake
	Assay (A) (mM)	Control (C) (mM)	$\frac{A}{C} \times 100$
Sodium	3	96	82.8 ± 2.9 *
Potassium	—	5	105.0 ± 1.5 NS
Calcium	—	1	78.7 ± 2.7 *
Magnesium	—	10	100.8 ± 3.2 NS
E.D.T.A.	—	20	76.5 ± 4.2 †
Glucose omission	—	11	95.2 ± 3.5 *
Potassium ferricyanide	2	—	97.1 ± 3.5 NS
2-4 Dinitrophenol	0.2	—	99.2 ± 3.2 NS
Iodo acetamide	2	—	61.1 ± 5.7 †
Ouabain	0.2	—	61.7 ± 2.9 †
VP16-213	0.100	—	36.7 ± 3.4 †

The accumulation of [^{3}H] VM26 was examined as described under Influx experiments. After incubation for 15 min in the presence of EDTA, VP16-213, inhibitors, or the absence of ions and glucose, [^{3}H] VM26 was added and the reaction was stopped 20 min later. Values are the means of four determinations (NS, not significant).
* $P < 0.01$.
† $P < 0.001$.

Little or none of the drug was found in mitochondria or microsomes fractions. The high specific activity of the cellular sap was very likely due to the free cytosolic drug which is computed as protein bound.

DISCUSSION

In their broadest sense, the results reported above establish that VM26 enters the Krebs II ascites cells via two mechanisms. First, a simple diffusion described in earlier studies [4, 5] and characterized by a steady-state concentration not greater, nor less, than the extracellular drug levels. In such a process there exists no specific interaction between the drug and any membrane constituent that could require energy process or ionic dependence. Passive diffusion is not influenced by inhibitors such as structural analogues and cannot be involved in cell resistance to the drug, the consequence of an altered flux without any changes in membrane structure [13].

The second translocation process is a carrier-mediated mechanism since it fulfils a number of well-established criteria [14]. The uptake is saturable ($K_m = 37.54\ 10^6$ M, $V_m = 13.4$ nM/min/mg protein) has characteristic temperature coefficient ($Q_{10} = 17.5 \pm 0.2$) and is inhibited by a structural analogue: VP 16-213.

According to earlier kinetics [15–17] and because of its high affinity constant and sodium dependence, the VM26 carrier of our model is a low affinity uptake.

Energy dependence of the system is difficult to establish, because the inhibitions observed with ouabain or iodoacetamide could be subsequent to the inhibition of (Na^+–K^+) dependent ATPase; so, extracellular Na^+ can be completely substituted by Li^+, and K^+ omitted without suppressing the VM26 uptake. The energy required for the carrier should not be provided by glycolysis, because dinitrophenol has no significant effect, which corroborates the results obtained in the glucose-free experiments. Moreover, an active carrier system can act in the absence of metabolic energy like a facilated transport system.

Analysis of the VM26 efflux from preloaded cells shows a releasing of more than 80% of the intracellular drug and a steady-state obtained in less than 10 min. Our results were partly in agreement with those reported concerning L 1210 cells [3]. The fast efflux and the small remaining cellular drug which is

Table 2. Intracellular distribution of [^{3}H] VM26 in Krebs ascites cells

Cell fraction	Centrifugation	Protein (mg)	SA = dpm/mg protein	Ratio: $\frac{\text{fraction dpm}}{\text{homogenate dpm}} \times 100$
Homogenate		62.82 ± 8.3	6112 ± 264	
Nuclear pellet	1000 g, 15 min	25.71 ± 6.72	11,320 ± 423	74.79 ± 2.8%
Mitochondria	10,000 g, 20 min	9.26 ± 1.2	317 ± 97	0.76 ± 0.22%
Microsomes	100,000 g, 120 min	1.61 ± 0.78	1696 ± 58	0.71 ± 0.02%
Cellular SAP		5.26 ± 1.7	12,128 ± 219	23.85 ± 3.0%

Krebs II ascites cells ($5 \cdot 10^7$, N = 4) were incubated with radiolabelled VM26 for 40 min and then were washed and resuspended in PBS. Cells were homogenized with a Teflon pestle apparatus [6] and fractionated as described in Materials and Methods.

probably irreversibly bound [3] suggest a passive diffusion.

Concerning VM26 intracellular distribution, this is expected since it has been shown that this drug can affect a number of different biochemical systems within tumor cells [18, 19]. It has been reported that VM26 reversibly prevents mitochondrial NADH reoxidation [19] whereas an irreversible blockade of the G2 phase of the cell cycle takes place after incubating cells with VM26 [20].

In addition, exhaustive dialysis indicates that only a substantial proportion of the drug can be removed after efflux experiments. These results are in good agreement with those related in our efflux experiments and cellular fractionation (Table 2), which indicated that about 13% of intracellular drug was not affected by dialysis and, only a little of cellular radioactivity is bound to mitochondria proteins.

The general conclusion of this study is that Krebs cells take up VM26 through two mechanisms. A passive diffusion governed by the drug lipophilicity and its extracellular concentration. The second mechanism is a carrier mediated transport not reported by Allen for his biological model. This discrepancy may be due to the difference between the cytoplasmic membranes of L 1210 cells and Krebs cells. However, the passive diffusion reported by Allen cannot explain that the intracellular rate of the drug is 20-fold greater than the incubating medium concentration, whereas, less than 1% of the drug (free from protein) [21] is osmotically active. In addition, the osmotic diffusion cannot lead to the alterated flux established with teniposide-resistant sublines of L 1210 cells [22].

In order to obtain more data on the VM26 transport and its interaction with cell drug resistance acquisition, we have undertaken an approach to the isolation of VM26 resistant sublines of Krebs II ascites cells.

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