Analysis of the Inhibitory Effects of VP-16-213 (Etoposide) and Podophyllotoxin on Thymidine Transport and Metabolism in Ehrlich Ascites Tumor Cells in Vitro¹

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ABSTRACT

These studies explore the effects of an epipodophyllotoxin on the membrane transport and metabolism of thymidine in Ehrlich ascites tumor cells. Uptake of \(^{3}H\) after exposure of cells to \(^{3}H\)thymidine is characterized by a rapid initial velocity that approximates membrane transport followed by a slower rate of uptake that parallels the accumulation of phosphorylated derivatives of thymidine, primarily thymidine triphosphate, within the cell. The high rate of thymidine transport relative to thymidine metabolism to the triphosphate within the cell decreases as the extracellular nucleoside concentration is reduced due to a much greater decrease in membrane transport than the subsequent metabolic step. Hence, as extracellular thymidine is decreased, transport becomes increasingly rate limiting to metabolism within the cell. VP-16-213 (etoposide) or podophyllotoxin inhibits the initial uptake rate for thymidine and, as a consequence, inhibits the intracellular formation of thymidine triphosphate. When extracellular thymidine is high, inhibitory effects on transport are transient, and the net rate of thymidine triphosphate accumulation within drug-treated cells rapidly approaches a velocity comparable to that of control cells, indicating no direct VP-16-213 or podophyllotoxin effect on nucleoside and nucleotide phosphorylation. When extracellular thymidine is reduced so that transport is rate limiting to metabolism, the duration of the inhibitory effects of VP-16-213 on thymidine triphosphate formation is prolonged. A secondary effect of VP-16-213 becomes manifest beyond 10 min of incubation with \(^{3}H\)thymidine with the virtual complete cessation of thymidine incorporation into the acid precipitate without any change in the thymidine triphosphate level. This late effect is not observed with podophyllotoxin and indicates a direct effect of VP-16-213 on DNA synthesis that is distinct from the earlier inhibitory effect on thymidine phosphorylation, which is secondary to membrane transport.

INTRODUCTION

VP-16\(^{\alpha}\) (3, 12, 16, 24, 26), a semisynthetic derivative of PDT, induces a premitotic blockade in either the late-S or early G\(_{2}\) phase of the cell cycle (7, 10, 18, 22) and has been found to cause chromosomal damage in human hematopoietic cells (15). Loike and Horwitz (21) demonstrated VP-16-induced DNA damage in Hela cells that was reversed after removal of the drug. Recently, Wozniak and Ross (33) demonstrated that this damage includes both single- and double-strand breaks as well as DNA-protein cross-links.

Although VP-16 causes premitotic alterations probably related to DNA damage, and although PDT acts as a classical spindle poison inducing a mitotic block (13, 20), several laboratories have demonstrated that, in addition, epipodophyllotoxins such as VP-16 and VM-26 as well as PDT share the ability to inhibit net cellular nucleoside uptake into a variety of mammalian cells (10, 11, 13, 20, 23, 30, 32). VP-16 and PDT inhibition of radio-labeled precursor uptake into cells has been attributed to inhibition of macromolecular biosynthesis (10, 11), perturbation of nucleoside and nucleotide metabolism (10), and inhibition of nucleoside transport (20, 23, 32). In this paper, experimental techniques were applied to distinguish between VP-16 and PDT effects on dThd transport, dThd nucleotide formation, and incorporation of dTTP into DNA within the cell in order to characterize more precisely the pharmacological effects of these agents.

MATERIALS AND METHODS

Chemicals. Nonlabeled dThd was purchased from Sigma Chemical Co. (St. Louis, MO). \([6-{ }^{3}H]dThd\) (16.2 Ci/mmol) was purchased from New England Nuclear (Boston, MA) and was purified by HPLC. VP-16, provided by Bristol Laboratories (Syracuse, NY), and podophyllotoxin, from Aldrich Chemical Co. (Milwaukee, WI), were brought into solution in 100% DMSO. Dipiridamole was provided by Boehringer-Ingleheim (Indianapolis, IN). Final DMSO concentrations did not exceed 0.8% and were present in control suspensions at equivalent concentrations.

Cells and Medium. Ehrlich ascites tumor cells were grown in male CF\(_{1}\) mice (Sprague-Dawley, Madison, WI) and passed weekly by i.p. inoculation of 0.2 ml of undiluted ascitic fluid. Cells were harvested 7 to 10 days postinoculation, washed, and centrifuged (500 \(\times\) g for 2 min) twice in 0.85% NaCl solution to remove erythrocytes. The cells were finally suspended in a buffer composed of 136 mM NaCl, 4.4 mM KCl, 16 mM NaHCO\(_{3}\), 1.1 mM KH\(_{2}\)PO\(_{4}\), 1 mM MgCl\(_{2}\), and 1.9 mM CaCl\(_{2}\). The pH was maintained at 7.4 by passing warm and humidified 95% O\(_{2}-5%\) CO\(_{2}\) over the cell suspension. Cells were stirred in specially designed flasks by revolving Teflon paddles in a 37° water bath as described previously (9).

Uptake Studies. For determination of cellular \(^{3}H\) uptake over very brief intervals, 0.5 to 20 sec (determined on a Cronus chronometer; Precision Products, Santa Clara, CA), 1 ml of the suspension was injected rapidly into a 15-ml glass conical centrifuge tube resting on an operating vortex and containing \([^{3}H]dThd\) with or without VP-16 or podophyllotoxin; appropriate DMSO controls were included. Uptake was stopped by the injection of 10 ml of 0.85% NaCl solution containing 50 \(\mu\)M dipiridamole at 0°.

Cell fractions were then separated by centrifugation (500 \(\times\) g for 2

1 Supported by Grant CA-16906 from the National Cancer Institute, NIH, and a grant from Bristol Laboratories.

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The abbreviations used are: VP-16, VP-16-213 [4'-demethylepipodophyllotoxin 9-(4,6-O-ethyldiene-\(\beta\)-D-glucopyranoside)]; dThd, thymidine; FdUrd, 5-fluorodeoxyuridine; TCA, trichloroacetic acid; PDT, podophyllotoxin; DMSO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; MTX, methotrexate; VM-26, 4'-demethylepipodophyllotoxin 9-(4,6-O-2-thienylidene-\(\beta\)-D-glucopyranoside).

Received July 25, 1983; accepted November 28, 1983.
were collected into mini-scintillation counting vials, and radioactivity was measured. Between each analysis, the column was equilibrated for 30 min with aqueous buffer containing the ion-pairing agent. Typical elution times were: thymine, 9 to 11 min; dThd, 24 to 27 min; dTMP, 34 to 36 min; dTDP, 41 to 43 min; and dTTP, 46 to 48 min.

Acid-soluble intracellular dThd and thymine were corrected for extracellular dThd and thymine carried with cells through the silicon oil layer on the basis of the inulin space, and the concentrations of extracellular dThd and thymine were measured by HPLC.

RESULTS

Characteristics of dThd Transport and Metabolism and Effects of VP-16 and PDT. Chart 1A illustrates the effects of 50 µM PDT or VP-16 on the uptake of ³H over 20 sec after exposure of cells to 10 µM [³H]dThd. Uptake of ³H in control cells is biphasic. There is an initial rapid uptake phase followed by a progressive decline in the net uptake rate. PDT and VP-16 inhibit the initial rate of dThd uptake by 53.6 ± 14.1 and 30.6 ± 12.3%, respectively. However, the inhibitory effects of both agents on the net uptake rate of ³H are transient. After 5 sec, the net rate

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**Table 1:**

<table>
<thead>
<tr>
<th>Condition</th>
<th>dThd Uptake Rate (µmol/liter cell water)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>VP-16 (50 µM)</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>PDT (50 µM)</td>
<td>0.8 ± 0.1</td>
</tr>
</tbody>
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**Graph 1:**

- **A:** Control
- **B:** VP-16 (50 µM)
- **C:** PDT (50 µM)

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**Figure 1:**

- **A:** Control
- **B:** VP-16 (50 µM)
- **C:** PDT (50 µM)

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**Figure 2:**

- **A:** Control
- **B:** VP-16 (50 µM)
- **C:** PDT (50 µM)

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**Figure 3:**

- **A:** Control
- **B:** VP-16 (50 µM)
- **C:** PDT (50 µM)
of $^3$H accumulation in the presence of VP-16 or PDT is comparable to that of control cells. The inhibitory effects of VP-16 and PDT on the initial phase of dThd uptake are consistent with inhibition of the transport process, while the failure of these drugs to inhibit the slower later phase of $^3$H uptake, a process which reflects primarily the rate of conversion to phosphorylated derivatives (see below), indicates that phosphorylation of dThd is not directly perturbed by VP-16 or PDT over this interval.

As extracellular dThd is decreased to 1 and 0.1 μM (Chart 1, B and C, respectively), the initial rate of dThd uptake slows relative to the uptake phase and inhibition of the net rate of $^3$H uptake by VP-16, and PDT becomes more sustained over the interval of observation, suggesting that transport is becoming increasingly rate limiting to metabolism under these conditions (Table 1). However, while the inhibitory effects of VP-16 and PDT are protracted as extracellular dThd is decreased from 1 to 0.1 μM, Table 1 indicates that the percentage of inhibition of the initial uptake rate by 50 μM VP-16 remains essentially the same over this extracellular dThd concentration range, suggesting noncompetitive inhibition.

HPLC Analysis of dThd Uptake and Metabolism: Discrimination between Transport and Metabolism. Previous studies with FdUrd (4) demonstrated that the biphase uptake of this analogue of dThd represents an initial rapid unidirectional flux of nucleoside into the cell, a process that reflects the rate and characteristics of the membrane transport system, followed by a decline in the uptake rate for $^3$H which represents the rate of accumulation of phosphorylated derivatives which are retained within the cell. As illustrated in Chart 2, uptake of $^3$H on exposure of cells to 1 μM $[^3$H]dThd reflects similarly the sequence of rapid initial transport of dThd followed by metabolism to phosphorylated derivatives. Indeed, influx of dThd is so rapid that initial rates cannot be measured with this technique, and intracellular dThd is at its peak level within 30 sec (Chart 1B). Within 30 sec, the predominant phosphorylated derivative is dTTP, and beyond 30 sec, dTTP build-up parallels and accounts for the total net accumulation of TCA-soluble $^3$H. There is a slower appearance of $^3$H in the TCA precipitate, and by 3 min, the rate of this process is comparable to the rate of the accumulation of $^3$H in the TCA-soluble compartment. In these experiments, rapid intracellular consumption of dThd results in rapid depletion of extracellular dThd. Eighty to 85% of the decline of extracellular dThd is accounted for by intracellular dTTP accumulation and its incorporation into the TCA precipitate, while 15 to 20% is accounted for by the formation of thymine which is distributed in the intracellular and extracellular compartments. Formation of dTMP and dTDP accounts for very little of the consumption of extracellular dThd observed over this interval (note the difference in ordinate scale between A and B of Chart 2). The ratio of intracellular to extracellular dThd remains near constant within a range of 0.40 to 0.45 over this interval. When extracellular dThd is 10 μM, however, this ratio is increased to 0.8 (data not shown).

HPLC analysis of the effects of VP-16 on intracellular dThd and its metabolites over 5 min is indicated in Chart 3. When extracellular dThd is 1 μM, 50 μM VP-16 reduces the accumulation of $^3$H in the TCA-soluble fraction (Chart 3A). This reduction is due entirely to decreased dTTP, and the effect is maximal at 30 sec, the first measured point; beyond this, accumulation of total TCA-soluble $^3$H and dTTP follows a course parallel to that of control cells. This confirms that VP-16 does not have a direct inhibitory effect on nucleoside and nucleotide phosphorylation under these conditions. Rather, the inhibitory effects of VP-16 on accumulation of dTTP are transient. This is consistent with the transient initial suppression of dThd influx and free intracellular levels indicated in Chart 3B. Here, the intracellular dThd...
VP-16 and PDT Inhibition of dThd Transport

Chart 3. Analysis of the effects of VP-16 on (b) intracellular dThd levels and (A) total cell tritium and dTTP. Cells were treated with or without VP-16 (50 μM) along with 1 μM [3H]dThd for the specified times and then centrifuged through silicon oil into 10% TCA. The TCA-soluble fractions were analyzed by HPLC to separate dThd from its metabolites (see ‘Materials and Methods’).

Chart 4. Time course of [3H]dThd incorporation into the TCA precipitate in the presence or absence of VP-16 or PDT. Cells were incubated with 1 μM [3H]dThd alone (○) or with simultaneously added VP-16 (50 μM) (△) or PDT (50 μM) (○). At the specified times, portions of the cell suspension (~10^7 cells) were centrifuged through silicone oil into 10% TCA. The TCA-precipitable fraction was analyzed for radioactivity as described in ‘Materials and Methods’.

level is suppressed by VP-16 over the first 60 to 90 sec following which the intracellular levels for control and VP-16-treated cells are comparable.

Effects of VP-16 on dThd Incorporation into the TCA Precipitate. As illustrated in Chart 2, incorporation of dThd into the TCA-insoluble pool increases rapidly over the first 5 min of exposure. While VP-16 and PDT effects on the rate of dTTP accumulation in the acid-soluble pool are no longer observed by 30 sec (Chart 3) under these conditions, the inhibitory effects on incorporation of dTTP into the TCA precipitate persist for at least 5 min of incubation with 1 μM [3H]dThd (Chart 4).

To evaluate the effects of these agents over a longer interval, VP-16 (50 μM) or PDT (50 μM) was added to cell suspensions containing 10 μM [3H]dThd to minimize depletion of extracellular dThd and transport-mediated inhibitory effects on phosphorylation of dThd. Incorporation of 3H into the TCA-insoluble fraction was measured over 10 to 50 min (Chart 5). At 10 min, the cellular 3H levels are comparable at this extracellular dThd level. However, beyond this, there is a marked inhibition of 3H incorporation into the TCA precipitate by VP-16, but no inhibition is observed with PDT. HPLC analysis reveals that the dTTP levels in the TCA-soluble fractions are the same in control and VP-16-treated cells (not shown). Hence, alterations in synthesis of dTTP cannot account for this late VP-16 inhibition of dTTP incorporation into DNA.

DISCUSSION

VP-16 probably damages cells at the level of DNA by inducing single- and double-strand breaks and DNA-protein cross-links (21, 33). Unlike the DNA damage caused by VP-16 and other epipodophyllotoxins such as VM-26 (28), the parent compound PDT does not induce DNA strand breaks (21). Rather, this agent inhibits mitosis by binding to tubulin, thus preventing subsequent polymerization into microtubules (5, 19, 21). Earlier studies by Loike and Horwitz (20) suggested that VP-16 and PDT inhibit nucleoside transport without a direct effect on phosphorylation, and work from this laboratory indicates that epipodophyllotoxins inhibit energy-dependent transport processes (34), not unlike the inhibitory effects observed with Vinca alkaloids (8, 35). In this paper, we explore the effects of these agents on the membrane transport of dThd using rapid sampling and HPLC techniques that distinguish membrane transport from subsequent intracellular events. Of particular interest are studies that clarify the ramifications of the inhibition of membrane transport on the subsequent metabolism of dThd to its phosphorylated derivatives and their subsequent polymerization into DNA.

Previous studies from this laboratory (4) characterized the rapid transport of FdUrd that is distinct from its much slower metabolism to 5-fluorodeoxyuridine monophosphate, a reaction that dominates the uptake of radiolabel within 10 sec after exposure of cells to this compound. The major limiting step in the accumulation of this agent in the Ehrlich ascites tumor cell is its rate of phosphorylation to 5-fluorodeoxyuridine monophosphate. The rapidity of membrane transport relative to subsequent intracellular metabolism to retentive forms is a sequence of events that has been recognized for a variety of substrates that are phosphorylated within cells (6, 25, 29). Similarly, the data
reported here indicate rapid transport of dThd followed by slower intracellular metabolism to phosphorylated derivatives which represent primarily dTTP. Of particular interest is the clarification in this study that this relationship between transport and metabolism is dependent upon the extracellular dThd level. When extracellular dThd is high, the rate of transport is far in excess of the rate of metabolism and is not limiting to that process; under these conditions, the intracellular dThd level is close to the extracellular level, i.e., near equilibrium for this "equilibrating" transport system can be achieved. However, when extracellular dThd is reduced, transport slows relative to metabolism and is no longer sufficiently fast to maintain intracellular dThd near equilibrium with extracellular dThd. Under these conditions, the free intracellular dThd level falls below that of the extracellular compartment, and transport limits the rate of phosphorylation within the cell. Hence, when cytotoxic nucleosides are administered in vivo, transport influences the intracellular disposition of drug only when the extracellular drug level is low, as with a drug infusion, or after the drug level declines when the agent is administered as a pulse.

Within this context, the effects of VP-16 and PDT on transport and metabolism of dThd can be deciphered. First, neither VP-16 nor PDT directly affects the rate of formation of dThd nucleotides; this confirms the earlier suggestion of Loike and Horwitz (20). Rather, the effects of these agents on this process are secondary to their inhibitory effects on the membrane transport of dThd. Hence, when extracellular dThd is high and where transport is fast relative to metabolism, the inhibitory effect of influx is transient. Intracellular dThd rapidly approaches the same steady state as in control cells, and the rate of dTTP formation rapidly becomes the same as that of control cells. On the other hand, as extracellular dThd is reduced and transport becomes limiting to metabolism, inhibition of influx now has a more sustained inhibitory effect on the intracellular dThd level and, in turn, results in a more prolonged suppression of the rate of dTTP formation. Hence, PDT or VP-16 effects on membrane transport of dThd become important only at low extracellular nucleoside levels, but this may be most relevant to the pyrimidine levels found in patients with cancers (14).

Of interest is the translation of the inhibitory effects of these agents on membrane transport to the rate of dTTP incorporation into DNA. Over 30 sec to 5 min, the rate of dTTP accumulation within the acid-soluble pool in the presence of VP-16 is similar to that of control cells at an extracellular dThd level of 1 μM (Chart 3). The difference is in the absolute intracellular dTTP levels that reflect the earlier transient inhibition of dThd transport. However, because of this sustained difference in the intracellular dTTP substrate levels, there is, in turn, a sustained difference in the rate of dTTP incorporation into DNA over 5 min. Again, this effect should become much more important when the extracellular dThd level is decreased and/or the rate of dThd metabolism is high, resulting in a sustained reduction in the rate of dTTP synthesis with a consequent greater suppression of dTTP incorporation into DNA. Finally, in addition to this transport-related inhibition of dThd incorporation into DNA, another later-appearing inhibition by VP-16 was detected (Chart 5) which probably relates to a direct inhibitory effect on DNA synthesis in that this is unaccompanied by any late effects on the cellular dTTP pool. Similar results in human leukemia cells have been reported recently (17). The fact that PDT is a more potent inhibitor of transport than is VP-16 but that the late inhibitory effect on dTTP incorporation into DNA is expressed only with VP-16 further supports the conclusion that this effect is distinct from the transport-related phenomenon and is compatible with the known direct effects of VP-16 on DNA in contrast to the action of PDT only at the level of cellular microtubules (23).

These data suggest that VP-16 and PDT alter utilization of dThd, and possibly other nucleosides, by mammalian cells, although the consequences of this effect on cellular function are unclear. In addition, these studies provide a basis for some potential interactions between epipodophyllotoxins and other agents which are currently utilized in clinical regimens. For instance, combined regimens with VP-16 and cytoxic nucleosides, such as FdUrd or 1-β-D-arabinofuranosylcytosine, could be antagonistic under some circumstances, particularly when the nucleoside concentration is low, to result in reduced transport and consequent reduced activation of these compounds to their phosphorylated derivatives. However, the potential interactions between the agents are complex; indeed, VP-16 and 1-β-D-arabinofuranosylcytosine have been observed to synergize in vivo (27). There are other potential drug interactions, for instance, between these agents and MTX. Earlier studies indicate that epipodophyllotoxins act like the Vinca alkaloids, as well as inhibitors of energy metabolism, to block the energy-dependent loss of MTX from cells (34). This augments the free intracellular MTX concentration which, in turn, results in the enhanced formation of active polyglutamylated MTX derivatives. Studies in this paper raise the possibility of another interaction with MTX in that addition of epipodophyllotoxins after MTX might enhance the pharmacological effect of MTX by depressing the membrane transport of dThd into cells at a time when the utilization of dThd by salvage pathways could be critical. This may, in part, be the basis for the observation that the antitumor effect of VP-16 or VM-26, when administered long after MTX to L1210 leukemia-bearing mice in vivo, is synergistic (31). However, these effects of epipodophyllotoxins require high drug concentrations, and while these are levels that are achieved clinically, the high degree of protein binding may result in free epipodophyllotoxin levels that are too low to perturb nucleoside transport significantly (1). Interestingly, only low levels of VP-16 (10⁻⁷ to 10⁻⁶) are sufficient to achieve DNA breakage, inhibition of DNA synthesis, and cytotoxicity in vitro even in the presence of serum (17), supporting the concept that these effects, rather than perturbation of transport processes, are the major basis for the pharmacological activity of the epipodophyllotoxins.

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