Relationship of VP-16 to the Classical Multidrug Resistance Phenotype

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ABSTRACT

The classical multidrug resistance (MDR) phenotype is characterized by cross-resistance between a number of chemically unrelated drugs due to an increased efflux across the plasma membrane via a P-glycoprotein-mediated mechanism. The epipodophyllotoxin derivatives etoposide (VP-16) and teniposide (VM-26) are usually included among the drugs recognized by this MDR phenotype, and the MDR EHR2/DNR cell line is >50-fold cross-resistant to VP-16. The steady-state accumulation of VP-16 in EHR2/DNR cells is only half that of wild-type EHR2 cells, and deprivation of energy by sodium azide surprisingly increased accumulation to a similar extent in both sublines. Efflux was rapid (half-life of 32-35 s) and similar in both sublines, while initial influx was markedly lower in the resistant cells. The temperature coefficients over 10°C for VP-16 in- and efflux indicated passive transport in both sublines. In agreement with this finding, up to 10-fold molar excess (50 μM) VM-26 had no effect on VP-16 accumulation in MDR cells. VP-16 at a 100-fold molar excess inhibited azidopine photoaffinity labeling of P-glycoprotein by only 36% and vincristine binding to plasma membrane vesicles from EHR/DNR cells by 45%. However, VP-16 itself did not differentially bind to plasma membrane vesicles from EHR2 and EHR2/DNR cells. Finally, neither VP-16 accumulation nor cytotoxicity in EHR2/DNR cells were increased to the same degree as for daunorubicin and vincristine by verapamil, and the modulation was similar in wild-type and resistant cells. Thus, although VP-16 may be a substrate for P-glycoprotein, its effect on VP-16 accumulation in MDR cells is different from what is usually observed with Vinca alkaloids and anthracyclines (7, 8). Because several clinical trials of the modulation of MDR are in planning or in progress, it is important to define the relationship between VP-16 and the MDR phenotype.

INTRODUCTION

The classical MDR phenotype is a well-characterized entity involving cross-resistance between unrelated drugs, decreased intracellular drug concentration, expression of Pgp in the plasma membrane, and modulation of drug activity by lipophilic agents such as Tween 80 and VER (reviewed in Ref. 1). The phenotype is explained by Pgp mediating a rapid drug efflux leading to decreased intracellular levels, a mechanism which can be competitively inhibited by other drugs in the MDR group and by modulators (1). Drugs included in the MDR phenotype are typically anthracyclines, Vinca alkaloids, actinomycin D, and colchicine (1). The epipodophyllotoxin derivatives VP-16 and VM-26 are usually included in the MDR phenotype as being cross-resistant (1–6), although their transport characteristics and relationship to Pgp are not as well characterized as the anthracyclines and Vinca alkaloids. Recent reports have, however, indicated that VP-16 transport in MDR cells is different from what is usually observed with Vinca alkaloids and anthracyclines (7, 8). Because several clinical trials of the modulation of MDR are in planning or in progress, it is important to define the relationship between VP-16 and the MDR phenotype.

MATERIALS AND METHODS

Cell Lines. The Ehrlich ascites wild-type EHR2 and MDR EHR2/DNR cells have previously been described and show all the characteristics of the classical MDR phenotype (9–15). Both sublines were maintained as ascitic tumors as described previously (15).

Chemicals. Methoxy-labeled [14C]VP-16 (11 mCi/mmol) and sugar-labeled [3H]VP-16 (16 mCi/mmol) were gifts from John Swigov, Bristol-Myers, Syracuse, New York. Pure unlabeled VP-16 and VM-26 were gifts from Bristol-Myers Laboratories, Wallingford, CT, and were dissolved in DMSO. [3H]VP-16 (0.9 Ci/mmol) was purchased from Moravek, Brea, CA. [3H]AZP (45.2 Ci/mmol) was from Amersham, United Kingdom. DNR was purchased from Farmitalia (Milan, Italy), VCR was from Eli Lilly (Giessen, Germany), VER was from Knoll (Ludwigshaven, Germany), and Tween 80 was from Sigma (St. Louis, MO). All other chemicals were of reagent grade.

Drug Influx, Efflux, and Accumulation. These were performed as previously described (11). Briefly, cells were washed 4 times in ice-cold Ringer’s solution and suspended at 2 x 10⁶ cells/ml at 37°C in standard medium, which is a phosphate buffer containing 57.0 mM NaCl, 5.0 mM KCl, 1.3 mM MgSO₄, 9 mM NaH₂PO₄, 51 mM Na₂HPO₄ (pH 7.45) to which 5% (v/v) dialyzed calf serum was added (11). Glucose (10 mM) or NaN₃ (10 mM) was added as indicated. The concentration of DMSO did not exceed 1% (v/v), which does not influence cell viability or DNR transport. Nuclear binding was performed as described in Ref. 10.

Experiments were terminated by adding 2.5 ml cell suspension to an ice-cold Ringer’s solution and suspended at 3 x 10⁶ cells/ml at 37°C in glucose-enriched medium, which is a phosphate buffer containing 57.0 mM NaCl, 5.0 mM KCl, 1.3 mM MgSO₄, 9 mM NaH₂PO₄, 51 mM Na₂HPO₄ (pH 7.45) to which 5% (v/v) dialyzed calf serum was added (11). Glucose (10 mM) or NaN₃ (10 mM) was added as indicated. The concentration of DMSO did not exceed 1% (v/v), which does not influence cell viability or DNR transport. Nuclear binding was performed as described in Ref. 10.

Binding to Plasma Membrane Vesicles. Purification of plasma membrane vesicles from EHR2 and EHR2/DNR cells has previously been described (9). A rapid filtration assay was used to determine [3H]VP-16 binding to membrane vesicles (9). Inhibition of [3H]VP-16 binding to plasma membrane vesicles from EHR2/DNR cells by unlabeled VP-16 was as described for anthracyclines in Ref. 9.

Photoaffinity Labeling with AZP. This was performed as previously described (17). Inhibition of AZP photoaffinity labeling of Pgp by drug was assessed by fluorography and photodensitometry. VP-16 and VM-26 were added in DMSO to a 2% (v/v) final DMSO concentration.

Clonogenic Assay. Drug toxicity was assessed by colony formation in soft agar on a feeder layer containing sheep RBCs as described before (18). Both 3-h and 3-week continuous drug incubations were used.

RESULTS

Drug Accumulation. Fig. 1 shows that the steady-state accumulation of [14C]VP-16 in EHR2/DNR cells in glucose-enriched medium is about half of wild-type levels. This was confirmed using the [3H]VP-16 isotope (not shown). Incubation
with NaN₃ in medium without glucose resulted in an increased accumulation of approximately 40 pmol/10⁶ cells in both sublines, an increase which was rapidly reversed by addition of 10 mM glucose (Fig. 1). Because the volume of an EHR2 cell is approximately 1500 µm³ (19), and a suspension of 2 × 10⁶ cells/ml was used, the packed cell mass is approximately 3 µ, leading to a maximal rapidly exchangeable intracellular drug concentration (total intracellular minus nonexchangeable drug) of approximately 45 µM in EHR2 cells in medium containing NaN₃ when medium VP-16 is 5 µM (a cell to medium ratio of 9:1 at steady state) (Fig. 1). The corresponding ratio for EHR2/DNR (cell volume 1071 µm³ (19)) cells in medium containing glucose is only 2.6:1.

VP-16 Efflux. Efflux in drug-free medium containing glucose in the first 30 min (Fig. 2) is similar for both cell lines with a retention at 37°C of approximately 5 pmol/10⁶ cells, while efflux at 4°C was very slight. The retention at 30 min was similar when medium VP-16 was 10 µM, indicating saturation of nonexchangeable drug-binding sites (not shown). The initial efflux after subtraction of nonexchangeable drug at 37°C is rapid when both cell lines are loaded to similar drug levels with a t½ of 32–35 s as calculated from the semilogarithmic plots shown in Fig. 3, bottom, and shows no difference in NaN₃- or glucose-enriched medium (Fig. 3). In Fig. 3, bottom, the initial efflux of all 4 curves is shown to follow first-order kinetics with

![Fig. 1. Accumulation of [14C]VP-16 in EHR2 (A) and EHR2/DNR (B) cells. Medium contained 5 µM VP-16. O, medium contained 10 mM NaNO₃; Δ, medium contained 10 mM glucose; •, addition of 10 mM glucose to medium containing 10 mM NaNO₃.](image1)

![Fig. 2. Efflux and retention of [14C]VP-16 in EHR2 (A) and EHR2/DNR (B) cells. Cells were loaded in 5 µM VP-16 for 30 min at 30°C, washed twice in ice-cold Ringer's solution, and transferred to drug-free medium containing 10 mM glucose at 37°C (•) or at 4°C (O).](image2)

![Fig. 3. Initial efflux of [14C]VP-16 in drug-free medium, with proportional (top) and with logarithmic ordinate (bottom) of the same experiments. EHR2 cells were loaded in 5 µM and EHR2/DNR cells in 10 µM VP-16 for 30 min, washed twice in ice-cold Ringer's solution, and transferred to drug-free medium containing 10 mM glucose at 37°C (O) or at 4°C (O).](image3)

![Fig. 4. Initial influx of [14C]VP-16 in EHR2 (A) and EHR2/DNR (B) cells in the presence of 10 mM NaN₃ (O) or 10 mM glucose (•). VP-16 in medium was 5 µM.](image4)

![Fig. 5. Binding of [3H]VP-16 to plasma membrane vesicles from EHR2 (C) and EHR2/DNR (D) cells in rapid filtration assay at 37°C. VP-16 in medium was 0.5 µM and contained 5 mM ATP and MgCl₂. Points, means; bars, ±SD.](image5)
correlation coefficients of >0.994 and corresponding P values of <0.0001. Q10 values for VP-16 efflux at 1 min in the 25–35°C range were 2.14 for EHR2 and 1.48 for EHR2/DNR cells. When increasing concentrations of VER were added, the percentages of inhibition of VP-16 efflux, after loading to equimolar intracellular VP-16 levels, at 5, 25, and 50 μM VER was 20, 67, and 70, respectively, in EHR2 and 8, 33, and 51 in EHR2/DNR cells, respectively (not shown). Thus, VER had less inhibitory effect on VP-16 efflux in the MDR line compared to wild-type cells.

VP-16 Influx. VP-16 influx during the first minute is shown in Fig. 4. Using the method of least squares, we determined that the initial influx in medium containing NaN3 during the first 30 s is 44 pmol VP-16/10^6 cells/min in EHR2 cells and 23 pmol VP-16/10^6 cells/min in EHR2/DNR. The values for initial influx in glucose-enriched medium are 31 and 5 pmol VP-16/10^6 cells/min for EHR2 and EHR2/DNR, respectively, corresponding to 71 and 22% of values in NaN3-containing medium. When influx was measured at 1 μM VP-16 in medium, the corresponding results were 5.1 and 0.5 pmol VP-16/10^6 cells/min, respectively (not shown). Q10 values for VP-16 influx in the first 30 s in medium containing NaN3 in the 25–35°C range are 1.46 for EHR2 and 1.22 for EHR2/DNR cells.

Nuclear Binding. The binding of VP-16 to isolated nuclei is approximately 20% higher in EHR2 compared to EHR2/DNR cells (Table 1), a result which can be explained by the hypotetraploid EHR2 nuclei (15) being larger than the hyperdiploid EHR2/DNR. When 5 mM ATP was added for 1 min, nuclei from both cell lines bound an additional 23% VP-16.

Binding to Plasma Membrane Vesicles. No difference was found between binding of [3H]VP-16 to plasma membrane vesicles from EHR2 and binding of [3H]VP-16 to plasma membrane vesicles from EHR2/DNR cells (Fig. 5). However, when plasma membrane vesicles from EHR2/DNR were labeled with 100 nM [3H]VCR as described in Ref. 9, unlabeled VP-16 inhibited VCR binding by 45% at 100-fold molar excess (10 μM VP-16) (not shown).

Photoaffinity Labeling. VP-16 inhibits AZP labeling of Pgp by 30% at 100-fold and by 56% at 250-fold molar excess, while the more lipophilic analogue VM-26 inhibits labeling by 68% at 100-fold and 80% at 250-fold molar excess (Fig. 6). In comparison, VCR at 100-fold molar excess inhibits photo-labeling in vesicles from the same cell line by 60% (17).

Competitive Drug Accumulation. Coincubation of VP-16 with typical MDR modulators such as Tween 80 and VER in EHR2/DNR cells had a weak effect on VP-16 accumulation compared to DNR (13) and VCR (14) in which 10–25 μM VER is sufficient to restore drug levels in EHR2/DNR cells to wild-type levels. Both DNR and VM-26 increased VP-16 accumulation in EHR2 only, while the highly lipophilic anthracycline aclarubicin had a weak effect on VP-16 accumulation in both cell lines, and VCR was also more effective in EHR2 cells (Fig. 7). Conversely, the cellular accumulation of DNR, which is transported by the MDR efflux mechanism (11), is increased by coinubcation with other MDR drugs, such as VCR, which are also actively transported (12). This is confirmed in Fig. 8, which also shows that VP-16 up to 100 μM (20-fold molar excess in medium) had no effect on DNR accumulation, while VM-26 at high concentrations was able to increase DNR accumulation.

Clonogenic Assay. It is apparent from Fig. 9 that EHR2/DNR cells are approximately 50-fold cross-resistant to VP-16 when comparing 50% inhibitory values. VER had an equal, weakly modulating effect on VP-16 toxicity in both EHR2 and EHR2/DNR cells. This was also seen when using 3-h incubations (not shown).

DISCUSSION

Changes in drug transport are believed to be the cause of the MDR phenotype, and their elucidation is important for the
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various drugs included. Cells transfected with Pgp complementary DNA are cross-resistant to VP-16, although at relatively low (5- to 10-fold) levels, indicating that Pgp itself can play a role in resistance toward VP-16 (20, 21). Transport studies have not yet been conducted in these transfected cell lines. A recent report concerning an mdr1-transfected KB cell line described only very low (1.5-fold) resistance to VP-16 and no selective modulation by VER (22). This was in contrast to 67-fold cross-resistance to vincristine with full modulation by VER (22). However, transgenic mice expressing the mdr1 gene have VP-16 resistant bone marrow (23).

The decrease in steady-state DNR accumulation in the EHR2/DNR cell to about one-fifth of EHR2 levels is considered to be due to 3 factors, namely, active efflux, decreased influx, and decreased intracellular binding (11). The transport characteristics of VP-16 found in the present study of rapid influx and efflux, a small compartment of nonexchangeable drug, and low Q,0 values agree with those described for L1210 cells (24). The change in VP-16 accumulation effected by deprivation of cellular energy levels is similar to that described by Seeber et al. (25) for another strain of DNR-resistant Ehrlich ascites cells, but because it is the same in both EHR2 and EHR2/DNR cells (Fig. 1), it cannot be ascribed to a MDR efflux mechanism. A similar increase in VP-16 accumulation in wild-type cells in NaN3 medium was also described by Yalowich and Ross (26). The steady-state accumulation of VP-16 in EHR2/DNR cells is only approximately half of that in EHR2 cells, which is in agreement with results in MDR MCF (7) and KB (27) cells, but also in non-Pgp containing altered topoisomerase II-multidrug resistance sublines of KB cells (27). Attempts to find differences between EHR2 and EHR2/DNR in both initial (Fig. 3) and late (Fig. 2) VP-16 efflux have failed, which is in contrast to the increased efflux observed for DNR in EHR2/DNR (11). No difference in VP-16 efflux was described in MDR MCF cells compared to wild type (7), while Danks et al. (2) described a decrease in VP-16 efflux in MDR
CEM cells. We are unaware of any study which has shown the expected increased VP-16 efflux from MDR cells. This is in contrast to the large number of reports of increased efflux of anthracyclines and Vinca alkaloids from MDR cells (1). Differences between nonexchangeable drug pools (Fig. 2) and nuclear binding of VP-16 (Table 1) are also too slight to explain the difference in steady-state accumulation of VP-16. The main intracellular binding site for VP-16 has been shown to be the nucleus (24). However, ATP increased the VP-16 binding to nuclei (Table 1), which is interesting because ATP increases VM-26-induced DNA cleavage by topoisomerase II (28), and the observation might reflect an increased interaction of VP-16 with topoisomerase II or topoisomerase-II-DNA complexes. To explain the decreased steady-state VP-16 accumulation in EHR2/DNR cells, we are left with the decrease in initial influx which is markedly dependent on glucose co-cubation but otherwise appears to be a passive process with Q10 values <1.5 and nonspecific because the analogue VM-26 is without influence on VP-16 accumulation (Fig. 7). Danks et al. (2) reported a similar 10-fold reduced initial VP-16 influx in MDR CEM/VBL cells.

In subcellular assays, an effect of VP-16 on vincristine binding to plasma membrane vesicles of EHR/DNR cells similar to that reported by Gosland et al. (29) was found. VP-16 at high molar excess weakly inhibits AZP labeling of Pgp (Fig. 6). Others have reported that VP-16 did not (27, 30) or only weakly (8) inhibited photoaffinity labeling of Pgp, with VM-26 being more efficient than VP-16 (30), which is also in accordance with our results (Fig. 6). However, VP-16 itself does not differentially bind to plasma membrane vesicles (Fig. 5). It is therefore a question of whether the above-mentioned indirect methods of assaying VP-16 influence on Pgp simply reflect effects by VP-16 on Pgp lipid environment inducing conformational changes in Pgp. These in turn could lead to reduced binding of primary ligand, in much the same way as Tween 80, which also inhibits AZP photolabeling of Pgp (31), is presumed to do. In this respect, it follows that the more lipophilic analogue VM-26 is more efficient than VP-16 in inhibiting photolabeling of Pgp and is also, at high concentrations, able to increase DNR accumulation in EHR2/DNR cells, indicating damage to Pgp function (Fig. 8). Another, not mutually exclusive, explanation is that VP-16 is a low-affinity substrate for Pgp and that passive efflux from the EHR2/DNR cell is so rapid that any contribution by Pgp-mediated active efflux is rendered undetectable. This agree with the observation by Baguley et al. (32) that 4'- (9-acridinylamino)methanesulfon-m-anisidine analogues with poor diffusion were, in general, more susceptible to transport-related MDR. Furthermore, it is apparent that the membrane transport of VP-16 in the wild-type cell, as also described by Yalowich (26), is sensitive to changes in the metabolic state of the cell (Fig. 1) as well as to membrane-perturbing drugs such as VER (Fig. 7), a condition which is not found for DNR and VCR (11, 12). These factors could thus explain the equivocal relationship of VP-16 to the MDR phenotype. Accordingly, VER does increase VP-16 cytotoxicity in EHR2/DNR cells (Fig. 9) but to a lesser degree than found for VCR (14) and to the same extent as for EHR2 cells. A weak VER modulation of VP-16 resistance on other MDR lines (8, 33, 34) as well as on bone marrow toxicity in mdrl transgenic mice (35) has also been reported. Thus, addition of VER to VP-16 may not be a selective advantage in elimination of MDR cells, a result which could obviously be of clinical importance.

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