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Sustained Intracellular Retention of Dolastatin 10 Causes Its Potent Antimitotic Activity

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ABSTRACT

Dolastatin 10 is a highly cytotoxic antimitotic peptide in phase II clinical trials. Its cytotoxicity has been as much as 50-fold greater than that of vinblastine, despite quantitatively similar effects of the two drugs on tubulin polymerization. We compared uptake and efflux of radiolabeled dolastatin 10 and vinblastine in human Burkitt lymphoma CA46 cells to gain an understanding of the greater cytotoxicity of the peptide. In the Burkitt cells, dolastatin 10 was 20-fold more cytotoxic than vinblastine (IC_{50} values, 50 pM and 1.0 nM). When drug uptake at 24 h was compared at IC_{50} values of the two drugs, the intracellular concentrations were almost identical (50–100 nM). The accumulation factor observed for dolastatin 10 was 900 to 1800 versus 60 to 100 for vinblastine. The two drugs showed very divergent uptake kinetics, however. Vinblastine and dolastatin 10 reached maximum intracellular concentrations after 20 min and 6 h, respectively. Depletion of cellular ATP content did not alter the uptake of either drug, indicating passive uptake of both. When drug-preloaded cells were transferred to drug-free medium, there was no loss of dolastatin 10 for at least 2 h, whereas vinblastine exited the cells rapidly (approximate intracellular half-life, 10 min), with less than 10% of the initial drug remaining in the cells after the 2-h incubation. The potency of dolastatin 10 probably derives from its tenacious binding to tubulin, a property that in cells becomes translated into prolonged intracellular retention of the drug. Optimal clinical use of dolastatin 10 may require administration by infusion rather than by bolus.

Dolastatin 10 is a potently cytotoxic peptide, with four of its five subunits derived from modified amino acids. It was originally isolated from the sea hare *Dolabella auricularia* (Pettit et al., 1987), a shell-less mollusk, but it was subsequently totally synthesized (Pettit et al., 1989). Dolastatin 10 causes the accumulation of cells arrested in mitosis and the disappearance of cellular microtubules (Bai et al., 1990b, 1992). The apparent target is the protein tubulin, the major component of microtubules. Characteristics of its interaction with tubulin include a potent inhibition of assembly (IC_{50} value, 1.2 μM), noncompetitive inhibition of the binding of vinblastine to tubulin, and inhibition of nucleotide exchange (Bai et al., 1990a,b). Studies with [^{3}H]dolastatin 10 showed tight binding of the peptide to tubulin, but attempts to demonstrate formation of a tubulin-dolastatin 10 complex were unsuccessful. The smallest distinct species at low drug concentrations seemed to contain two $\alpha\beta$-tubulin molecules and probably two dolastatin 10 molecules. At higher drug concentrations, massive polymers of aberrant morphology (probably tightly coiled spirals) form (Bai et al., 1995).

Dolastatin 10 inhibits the growth of various cancer cell lines at subnanomolar concentrations (Pettit et al., 1987; Bai et al., 1990b; Beckwith et al., 1993; Turner et al., 1998), and the drug is active against human sarcoma, melanoma, and ovarian xenografts in nude mice (Pettit et al., 1987; Waud et al., 1993; Turner et al., 1998). In phase I studies in human subjects, the dose-limiting toxicity of dolastatin 10 was against bone marrow (Bagniewski et al., 1997), and the drug is currently in phase II clinical trials.

In our previous comparative studies of dolastatin 10 and vinblastine, we found the drugs had similar potencies as inhibitors of tubulin assembly, but the peptide was 20- to 50-fold more potent than vinblastine as an inhibitor of cell growth. The experiments described in this study were undertaken to gain insight into possible reasons for this difference. The most obvious possibility is a major difference in cellular accumulation of the two drugs.

Vinblastine and other vinca alkaloids have been shown to accumulate in cells (Lengsfeld et al., 1982; Gout et al., 1984; Ferguson and Cass, 1985; Jordan et al., 1991; Van Belle et al., 1991; Pierre et al., 1992; Singer and Himes, 1992; Breier et al., 1994; Dhamodharan et al., 1995; El Hafny et al., 1997), and studies with both vincristine (Breier et al., 1994) and vinblastine (Ferguson and Cass, 1985; Van Belle et al., 1991)

ABBREVIATIONS: p-gp 170, P-glycoprotein p170 (the multidrug transporter protein); MDR, multidrug resistant.

have indicated that accumulation of these drugs is by passive diffusion. Singer and Himes (1992) did note a close correlation among vinca alkaloids between efficiency of drug accumulation and degree of cytotoxicity. Moreover, Singer and Himes (1992) extrapolated their data to the IC_{50} values, concluding that at these initial extracellular concentrations the intracellular drug concentrations should be far lower than the total cellular tubulin concentration.

Our strategy was to directly examine drug effects primarily at the IC_{50} values. We have thus compared uptake and accumulation of [^{3}H]vinblastine and [^{3}H]dolastatin 10 at equitoxic concentrations. Efflux studies, however, were performed at the IC_{50} value for vinblastine. We found a good correlation between drug potency and the extent of intracellular drug accumulation. Thus, a substantially higher proportion of dolastatin 10 compared with vinblastine accumulates in cells. This results primarily from apparent entrapment of the peptide within cells, whereas substantial efflux of vinblastine occurs.

Materials and Methods

Materials. [^{3}H]dolastatin 10 (5.4 Ci/mmol) was prepared as described previously (Bai et al., 1995), and [^{3}H]vinblastine (15.5 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). The methanol solvent for both drugs was removed by evaporation at room temperature under a gentle stream of air, and the residues were dissolved in dimethyl sulfoxide (stock solutions at 10 μM). Monoclonal antibody C219 against P-glycoprotein p170 (p-gp 170) was generously provided by Dr. M. S. Poruchynsky, National Cancer Institute. Horseradish peroxidase-conjugated goat anti-mouse IgG was obtained from Pierce (Rockford, IL). Human Burkitt lymphoma CA46 cells were obtained from the American Type Culture Collection (Manassas, VA). These cells were used in all experiments, except as noted. A cellular volume of 0.651 pl (Magrath et al., 1980) was used to calculate intracellular concentrations, with no correction made for nuclear volume. For analysis of p-gp 170 content in CA46 cells, two additional Burkitt lines were used as controls. These were the parental EW-36 line (Cherney et al., 1997) and the derived multidrug-resistant (MDR) line EW-36VCR60 (Mickley et al., 1998). Both lines were generously provided by Dr. T. Fojo, National Cancer Institute.

Cell Culture and Drug Transport Studies. In all experiments, cells were grown in suspension culture in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 μM L-glutamine, 10 μg/ml gentamicin sulfate, and 0.1% (v/v) dimethyl sulfoxide (solvent for all drugs). Incubation was at 37° in a humidified 5% CO_2 atmosphere. For determination of IC_{50} values, an increase in cell number after 24 h was the parameter measured. In these studies, the initial inoculum was 3.5 to 4.0 $\times$ 10^5 cells/ml. Cell were grown in 25-cm^2 flasks containing 5 ml of medium.

The EW-36VCR60 cells were maintained in the same medium supplemented with 65 nM vincristine. One passage without vincristine was performed before using these cells for cytotoxicity experiments.

For the 24-h endpoint measurement of [^{3}H]dolastatin 10 or [^{3}H]vinblastine accumulation, cells were seeded at 1.4 $\times$ 10^5 cells/ml in 75 ml of medium in 75-cm^2 flasks. After 24 h, cell density was 3.2 $\times$ 10^5 cells/ml, and cells were treated with 50 pM [^{3}H]dolastatin 10 or 1.0 nM [^{3}H]vinblastine (the IC_{50} values; see below). After 24 h, cell suspensions were split into three aliquots, and cells were harvested in 50-ml polypropylene conical tubes by centrifugation for 5 min at 330*g*. Cells were resuspended in PBS (pH, 7.4) and recentrifuged. The supernatant was removed, and the bottom of each tube containing the cells was cut from the tube and placed in a scintillation vial. NaOH (0.5 ml of 0.5 M solution) was added to lyse the cells, followed 1 h later by 10 ml of scintillation cocktail.

Drug uptake kinetics was determined over 1-min, 2-h, and 24-h periods. Cells (3.8 to 4.5 $\times$ 10^5 cells/ml) were treated with [^{3}H]dolastatin 10 or [^{3}H]vinblastine at the indicated concentrations. Cells were separated from medium by centrifugation through a cushion of silicone oil (Monks et al., 1985). A 13-ml aliquot of cell suspension was placed in a 15-ml polypropylene conical tube containing 1 ml of silicone oil (Versilube F50 from General Electric Silicone Products Division, Waterford, NY) and centrifuged at 3200*g* for 5 min at room temperature. Time points correspond to incubation times plus the time at which the centrifuge reached 100 to 150*g* (approximately 10 sec). The medium phase, the medium-oil interface, and most of the oil were removed sequentially. The bottom of the tube containing the cell pellet was cut and processed for counting as described. For time points up to 2 h, aliquots of cell suspension were incubated in tubes over oil. For longer time points, aliquots were withdrawn from the tissue culture flask, after even resuspension of the cells, and placed in tubes over oil shortly before centrifugation.

Drug efflux kinetics was determined over a 2-h period. Cells (4.9 $\times$ 10^5 cells/ml) were treated with 1.0 nM [^{3}H]dolastatin 10 or [^{3}H]vinblastine for 1 h in a 125 cm^2 flask (110-ml cell suspension). Cells were harvested by centrifugation and resuspended in fresh medium without drug. Aliquots (13 ml) were processed over silicone oil as described at the indicated time points.

ATP depletion was achieved by supplementing the medium with 15 mM NaN_3 and 50 mM 2-deoxy-D-glucose. For uptake experiments, cells were incubated for 15 min before treatment with 1.0 nM drug. For efflux experiments, drug-loaded cells were resuspended in the azide/2-deoxy-D-glucose-supplemented medium. For efflux experiments in the presence of verapamil, drug-loaded cells were resuspended in medium containing 10 μM verapamil.

Cellular Tubulin Content. Cells were harvested by centrifugation, washed with PBS, and lysed for 30 min at 4° in a buffer containing 0.1 M 4-morpholineethanesulfonate (pH 6.6 with NaOH in 1 M stock solution), 20 μg/ml aprotinin, 10 μg/ml leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride, and 1% (v/v) NP-40. Extracts were centrifuged in an Eppendorf 5417C centrifuge at 14,000 rpm for 1 min at room temperature. The protein content of the supernatants was determined by the Lowry procedure. Tubulin in these extracts was quantitated with a competitive enzyme-linked immunosorbent assay method (Thrower et al., 1991). Purified bovine brain tubulin (Hamel and Lin, 1984) was used to coat 96-well, high-binding capacity microplates and was also used as the tubulin standard. For the coating procedure, 100 μl of a tubulin solution at 6 μg/ml was placed in each well, and the plate was left at room temperature for 1 h. For the generation of the standard tubulin curve, a tubulin solution at 0.2 mg/ml was used.

Cellular p-gp 170 Content. The Western blot technique was used to measure cellular p-gp 170 levels. Cell extracts were prepared as described for measuring tubulin content, except that the lysis buffer was the P-MER buffer obtained from Pierce supplemented with the protease inhibitor cocktail "Complete" obtained from and used as recommended by Boehringer-Mannheim. Extract proteins (40 μg/lane) were electrophoresed on 8% acrylamide Tris-glycine polyacrylamide gels obtained from Novex (pH 8.6). Proteins were electrotransferred to a polyvinylidene difluoride membrane. The membrane was soaked for 1 h at room temperature in a solution containing Tris-buffered saline (25 mM Tris-HCl at pH 7.4, 137 mM NaCl, and 3 mM KCl; from Quality Biological), 0.1% Tween 20, and 5% dry milk (Solution A), followed by a brief wash in Tris-buffered saline containing 0.1% Tween 20 (Solution B). The membrane was incubated overnight at 4° in the primary C219 antibody directed against p-gp 170 (the stock antibody solution was diluted 1/5,000 in Solution A). The membrane was washed three times for 5 min in Solution B. The membrane was then incubated for 1 h at room temperature in Solution B containing horseradish peroxidase-conjugated secondary antibody (goat antimurine IgG) at a 1/50,000 dilution. The membrane was washed three times in Solution B for 5 min. The membrane was placed in chemiluminescent SuperSignal West Dura Extended Duration Signal solution for horseradish peroxidase

from Pierce for 5 min at room temperature. The membrane was exposed for 30 sec to Biomax-ML film from Kodak, as recommended by Pierce.

Results

Inhibition of CA46 Burkitt Lymphoma Cell Growth. In our initial studies we determined effects of the radiolabeled preparations of dolastatin 10 and vinblastine on the growth of CA46 Burkitt lymphoma cells. The [^{3}H]dolastatin 10 was 20-fold more potent than the [^{3}H]vinblastine. IC_{50} values for the two drugs were 50 ± 10 (S.D.; n = 4) pM and 1.0 ± 0.3 (S.D.; n = 2) nM, respectively, for inhibition of growth after a 24-h incubation. The values obtained for the nonradiolabeled drugs were identical, within experimental error.

Cellular Accumulation of [^{3}H]dolastatin 10 and [^{3}H]vinblastine. Our initial drug uptake study was measurement of intracellular drug content after 24 h of growth at the IC_{50} values (Table 1). Despite the 20-fold greater addition of vinblastine to the medium, virtually identical amounts of [^{3}H]dolastatin 10 and [^{3}H]vinblastine accumulated in the cells. Dolastatin 10 accumulated 19-fold more extensively than vinblastine, with only 3% of added vinblastine entering cells compared with 68% of the added dolastatin 10. This finding supports the idea that both drugs have the same intracellular target, assumedly tubulin (see *Discussion*), consistent with abundant morphological (mitotic arrest, disappearance of microtubules) and biochemical (effects on tubulin assembly etc.) observations.

Uptake Kinetics. On observing such extensive uptake of dolastatin 10 by the Burkitt cells, we hypothesized that it must have resulted from an active transport process, especially considering that the initial extracellular concentration was so low (50 pM). We also thought that the uptake of dolastatin 10 would be rapid. Studies of uptake kinetics and with cells depleted of ATP disproved both assumptions.

In the uptake kinetic studies, cells were pelleted through silicone oil before determination of cell-associated drug (Monks et al., 1985), in contrast with the initial direct centrifugation studies described above. Experiments at the IC_{50} values of dolastatin 10 and vinblastine were performed over a 24-h period (Fig. 1), and the results showed that dolastatin 10 uptake was far slower than that of vinblastine. The intracellular concentration of vinblastine had already reached an apparent maximum by 2 h, and the vinblastine concentration was substantially higher than the dolastatin 10 intracellular concentration at this time. In contrast, the dolastatin 10 concentration in cells increased over about 6 h before it, too, leveled off and then declined. In these experiments, the maximum concentration of vinblastine in the cells was 0.079 pmol/10^6 cells at 2 h, with a decline to 0.044 pmol at the 24-h point. The maximum dolastatin 10 concentration in cells was 0.061 pmol/10^6 cells at 6 h and declined to 0.031 pmol at 24 h. However, cell number was increasing during the experiment, and the percent drug in cells varied with time. The vinblastine in cells was 4.2% at 2 h and 2.4% at 24 h, and there was a somewhat smaller proportional decrease in the percentage of dolastatin 10 in the cells, from about 55% to 41%. This could indicate some cell lysis in these drug-treated cultures. Note that the 24-h values for intracellular drug were lower for both vinblastine and dolastatin 10 when the cells were pelleted through oil as opposed to harvested by direct centrifugation (Table 1). Usually this is interpreted to indicate removal of nonspecifically adherent drug. Possibly, however, the oil extracts drug specifically bound to membrane tubulin (Rubin et al., 1982), which could play a role in the cytotoxicity of these agents.

Drug uptake kinetics was next examined in detail over a 2-h period at the IC_{50} values, confirming the more rapid uptake of vinblastine, compared with dolastatin 10 (Fig. 2). Rapid vinblastine uptake has been reported for other cell lines (Gout et al., 1984; Ferguson and Cass, 1985; Pierre et al., 1992; El Hafny et al., 1997), but in some cases uptake of this drug is slow (cf. Van Belle et al., 1991). In our experiments, a plateau in intracellular vinblastine concentration was reached by 20 min, whereas there was a steady increase in the intracellular dolastatin 10 concentration over the 2-h time course. The actual difference in the uptake rates closely reflected the 20-fold difference in initial extracellular drug concentrations, with net uptake of vinblastine being 5.3 fmol/min/10^6 cells versus 0.3 fmol/min/10^6 cells for dolastatin 10 (rates measured between the 10- and 20-min time points).

Dolastatin 10 Uptake Is Not an Active Transport Process. Because the initial rate of dolastatin 10 uptake versus that of vinblastine seemed to reflect primarily the initial extracellular drug concentration, it seemed unlikely that an active transport process accounted for the more extensive intracellular accumulation of the peptide. Additional confirmation was obtained by examination of dolastatin 10 and vinblastine uptake at 1.0 nM and 50 pM, respectively, for comparison with the previous studies with dolastatin 10 at 50 pM and vinblastine at 1.0 nM. The data at the two concentrations of both drugs are presented in Fig. 3 as percentage of the added drug that enters cells over the first 2 h of

TABLE 1

Accumulation of dolastatin 10 or vinblastine in cells

Cells were treated for 24 h with 50 pM [^{3}H]dolastatin 10 or 1.0 nM [^{3}H]vinblastine (the IC_{50} values). Each experiment was performed twice, with triplicate values obtained with both drugs, except that the silicone oil experiment with vinblastine was performed three times.

Drug Added	Intracellular Drug	Intracellular Drug Concentration	Accumulation Factor[a]	Drug Initially Added to Medium in Cells
	pmol/10^6 cells ± S.D.	*nM*		*%*
Expt. I: Direct centrifugation				
Dolastatin 10	0.060[b]	92	1840	68
Vinblastine	0.063 ± 0.003	97	97	3.4
Expt. II: Centrifugation through oil				
Dolastatin 10	0.031 ± 0.001	46	920	41
Vinblastine	0.044 ± 0.01	61	61	2.4

S.D., standard deviation.
[a] The accumulation factor was calculated by dividing the intracellular drug concentration at 24 h by the initial concentration of the drug in the medium.
[b] The same value was obtained in both experiments.

incubation. For the first 15- to 20-min equivalent percentages of both drugs at both concentrations entered the cells, consistent with a diffusion-controlled mechanism. After 20 min, however, net additional accumulation of vinblastine was minimal, whereas that of dolastatin 10, at both concentrations, continued at virtually the same rate until the last time point.

An additional experiment further supported passive diffusion as the primary mode of drug entry into the Burkitt cells. Within a wider range of concentrations of both dolastatin 10 and vinblastine, there was no evidence for saturation of a transport mechanism (Fig. 4). When the rate of uptake of dolastatin 10 and vinblastine were measured within 90 sec of drug addition to cells at concentrations up to 10 nM, rate for both drugs varied only as a function of drug concentration.

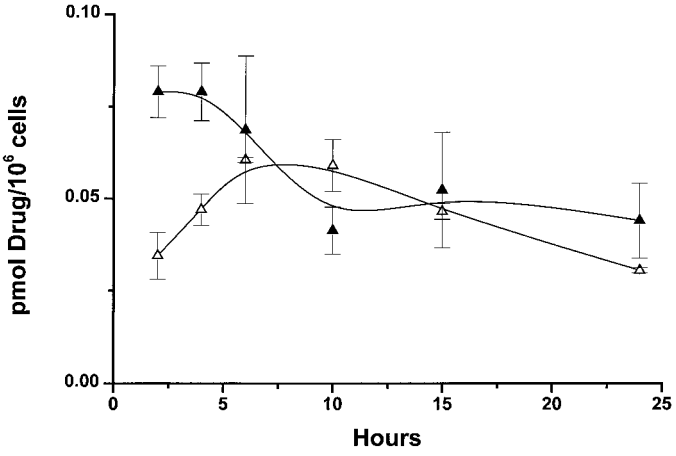

Fig. 1. Uptake of dolastatin 10 (△) and vinblastine (▲) over 24 h in Burkitt cells treated at the IC_{50} value for each drug. The initial media concentrations were 50 pM for [^{3}H]dolastatin 10 and 1.0 nM for [^{3}H]vinblastine. The dolastatin 10 experiment was performed twice and the vinblastine experiment three times (standard deviations indicated).

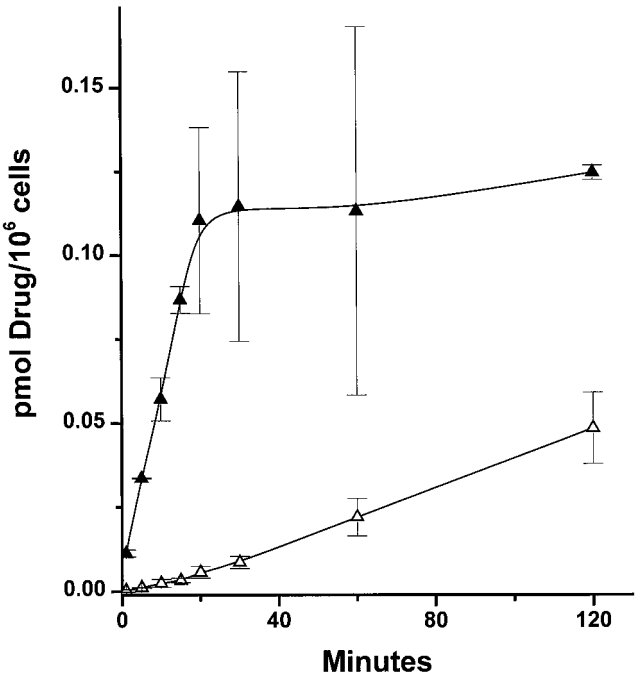

Fig. 2. Uptake of dolastatin 10 (△) and vinblastine (▲) over 2 h in Burkitt cells treated at the IC_{50} value for each drug. The experiment was performed twice (standard deviations indicated).

The absence of an active transport mechanism for dolastatin 10 accumulation was additionally confirmed in an ATP depletion experiment, performed with drug at 1.0 nM (Fig. 5). Cells were pretreated with sodium azide and 2-deoxy-D-glucose for 15 min before drug addition. There was virtually no difference in uptake kinetics of dolastatin 10 from the kinetics observed without inhibitors of ATP generation. With vinblastine, however, intracellular drug concentration reached a modestly higher level than had occurred in the absence of azide and 2-deoxy-D-glucose. The difference may be within experimental error, but could also result from an ATP-dependent efflux of vinblastine (see below) that was inhibited by interfering with cellular production of ATP.

Efflux Kinetics. Cells were pretreated for 1 h with 1.0 nM [^{3}H]dolastatin 10 or [^{3}H]vinblastine, and drug efflux was followed (Fig. 6). The higher dolastatin 10 concentration was used to obtain sufficient radiolabel to follow in the efflux phase of the experiment, and we preferred a short treatment time to minimize effects because of poor viability of the cells. No efflux of dolastatin 10 was detected over the 2 h of the experiment, whereas almost 90% of the intracellular vinblastine was lost over the same period. After an initial lag of approximately 10 min, vinblastine efflux seemed to follow first-order kinetics for approximately 1 h, with a half-life of approximately 10 min. A similar rapid efflux of vinblastine has been reported for many cell lines (Lengsfeld et al., 1982; Gout et al., 1984; Ferguson and Cass, 1985; Pierre et al., 1992; El Hafny et al., 1997).

When cells were placed in ATP-depleting conditions for 2 h, after incubation for 1 h in the presence of radiolabeled drug

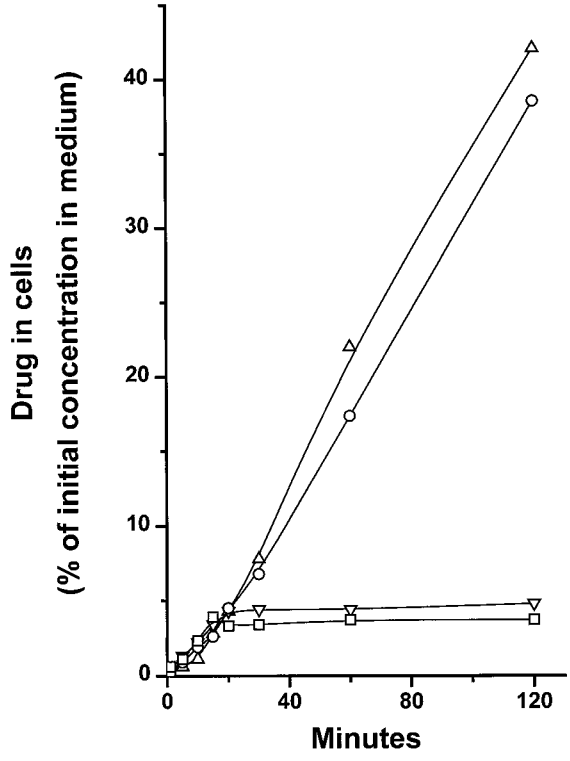

Fig. 3. Proportion of dolastatin 10 and vinblastine taken up over 2 h in Burkitt cells relative to the initial drug concentration in the medium. Symbols as follows: ○, 50 pM [^{3}H]dolastatin 10; △, 1.0 nM [^{3}H]dolastatin 10; □, 50 pM [^{3}H]vinblastine; ▽, 1.0 nM [^{3}H]vinblastine. The experiments were performed twice, and the 50 pM dolastatin 10 data and 1.0 nM vinblastine data are the same data presented in Fig. 2.

at 1.0 nM, their retention of dolastatin 10 was unaffected, whereas there was a doubling in the retained vinblastine from about 9% to 18% of the level present before the efflux phase of the experiment (Table 2). Loss of vinblastine from the cells was minimally prevented by verapamil, and this agent was significantly less effective than the azide/2-deoxy-D-glucose treatment in preventing vinblastine loss (Table 2).

These findings with verapamil and azide/2-deoxy-D-glucose treatment made it very unlikely that the P-glycoprotein multidrug transporter (Gottesman and Pastan, 1993) was substantially involved in the loss of vinblastine from the CA46 Burkitt cells. To confirm this more directly, we examined the CA46 cells for expression of p-gp 170 in comparison with other Burkitt lines whose MDR status was well established (the negative EW-36 line and the highly positive EW-36VCR60 line [Mickley et al., 1998]). As shown in Fig. 7, the amounts of p-gp 170 in the CA46 and EW-36 lines were below detectable levels, whereas there was abundant p-gp 170 protein in the EW-36VCR60 line. For EW-36 cells the IC_{50} values obtained for vinblastine, vincristine, and dolastatin 10 were 4.0, 1.0, and 0.1 nM, respectively; and for EW-36VCR60 cells, 53, 248, and 0.4 nM (unpublished observations).

From these studies we conclude that the substantially higher proportion of added dolastatin 10 that accumulates in Burkitt cells as compared with vinblastine results from the absence of efflux of the peptide, whereas the intracellular vinca alkaloid concentration reaches an equilibrium based on passive diffusion, both into and out of the cells, combined with an active efflux process different from MDR-1.

Discussion

We found that dolastatin 10 accumulated 15- to 19-fold more extensively than vinblastine at equitoxic concentrations (the IC_{50} values) in Burkitt lymphoma cells and therefore conclude that the 20-fold greater cytotoxicity of dolastatin 10 results from its more extensive intracellular accumulation. Because ATP depletion did not greatly affect uptake of either drug, their entry into cells probably results from passive diffusion through the plasma membrane. Supporting this, we found that the octanol/water partition coefficients for dolastatin 10 (1.4) and vinblastine (1.3) at physiological pH were nearly identical.

The major difference we observed between the drugs was a rapid efflux of vinblastine (Lengsfeld et al., 1982; Gout et al., 1984; Ferguson and Cass, 1985; Pierre et al., 1992; El Hafny et al., 1997) in contrast to no detectable efflux of dolastatin 10. Vinblastine efflux, additionally, was reduced under conditions of ATP depletion, suggesting involvement of an active mechanism. Verapamil had little effect on intracellular vinblastine, and p-gp 170 was not detected in the CA46 cells. Therefore, another unknown transport mechanism seems to be responsible for the apparent active efflux of vinblastine that we observed.

Dolastatin 10 and vinblastine, at their IC_{50} values, reached similar intracellular concentrations after 24 h, supporting the conclusion from morphological and biochemical findings that their intracellular target is tubulin. To define this more quantitatively, we measured total cellular protein in the Burkitt cells, obtaining a value of 62 pg of protein/cell, and determined that tubulin was 5.0% of cellular protein.

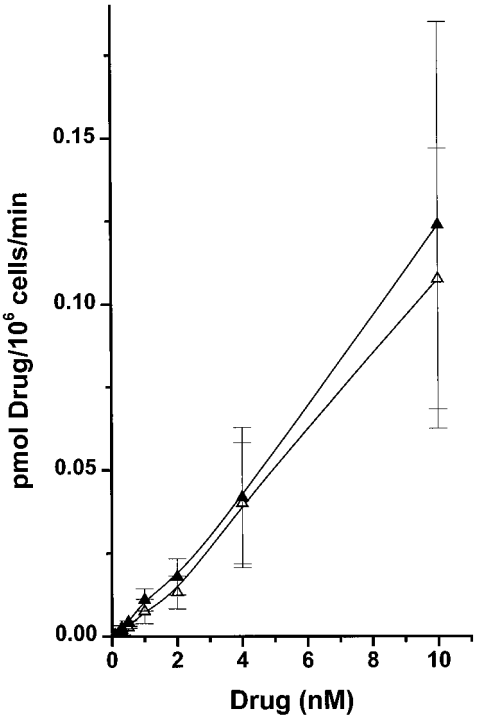

Fig. 4. Initial uptake rates of [^{3}H]dolastatin 10 (△) and [^{3}H]vinblastine (▲) by Burkitt cells as a function of initial drug concentration in the medium. For each data point the indicated drug concentration was added to cells suspended in medium and rapidly mixed without disturbing the silicone oil cushion, and the tube was centrifuged. Cells and drug in the medium were in contact for 60–90 sec. The experiment was performed three times (standard deviations indicated).

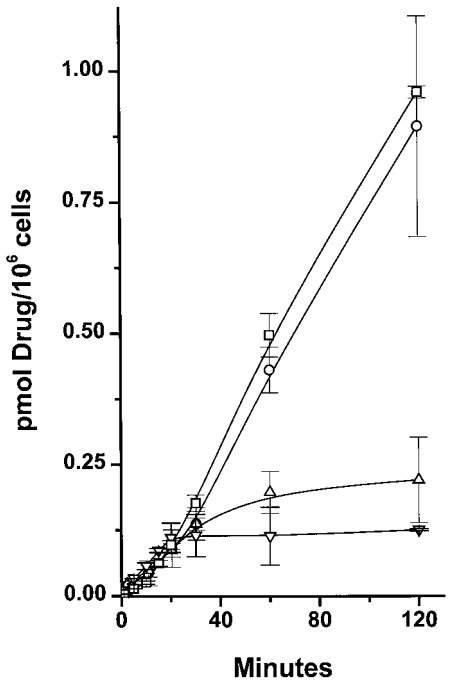

Fig. 5. ATP depletion has negligible effects on dolastatin 10 and vinblastine uptake by Burkitt cells. The medium contained the following: ○, 1.0 nM [^{3}H]dolastatin 10; ▽, 1.0 nM [^{3}H]vinblastine; □, 1.0 nM [^{3}H]dolastatin 10, NaN_3, and 2-deoxy-D-glucose; or △, 1.0 nM [^{3}H]vinblastine, NaN_3, and 2-deoxy-D-glucose. The data without NaN_3 and 2-deoxy-D-glucose are the same as shown in Figs. 2 (vinblastine) and 3 (dolastatin 10), and the experiment with NaN_3 and 2-deoxy-D-glucose was performed three times. Standard deviations are indicated for all data.

Moreover, there was no decline in tubulin levels during 24 h of treatment with either vinblastine, in agreement with Jordan et al. (1991), or dolastatin 10 at the IC_{50} values. Taking cell volume as 0.651 pl (Magrath et al., 1980) and ignoring the nucleus, CA46 cells contain 48 μM total tubulin, so that approximately one molecule of dolastatin 10 or vinblastine for 500-1000 tubulin $\alpha\beta$-dimers inhibits cell growth by 50%. Expressed another way, assuming 2/3 of tubulin is in polymer, the average microtubule is 5 μm in length (the cell radius), and each microtubule is anchored at the microtubule-organizing center, the concentration of microtubule (+)-ends in the untreated cell would be ~4 nM and tubulin at (+)-ends (13 protofilaments) ~50 nM. This is similar to intracellular concentrations of the drugs (50–100 nM) that we observed at the IC_{50} values and consistent with the hypothesis that interference with dynamic instability of microtubules rather than disassembly is primarily responsible for the inhibitory effects of microtubule depolymerizing agents on cell growth (Wilson and Jordan, 1995). Furthermore, effects on dynamic instability by vinblastine in biochemical systems (Jordan and Wilson, 1990) and cells (Dhamodharan et al., 1995) have been observed at concentrations similar to the intracellular concentration we obtained at the IC_{50} value for CA46 cells.

We find vinblastine to be more potent than did Singer and Himes (1992), but our vinblastine:tubulin ratio is almost identical with that obtained from the data of Jordan et al. (1991). Singer and Himes (1992) derived an intracellular vinblastine concentration in B16 melanoma cells at the IC_{50} value from the accumulation factor determined at 1 μM drug (initial extracellular concentration) and concluded that at the IC_{50} value the B16 melanoma cells would have a vinblastine:tubulin ratio of about 1:150–200. Jordan et al. (1991) reported that the tubulin concentration of HeLa cells was 2.0 mg/ml and that near the IC_{50} value the intracellular vinblastine concentration was 38 nM, yielding a drug:tubulin ratio of 1:500.

Our comparison of dolastatin 10 and vinblastine suggests that equitoxic concentrations of microtubule assembly inhibitors in the culture medium lead to intracellular drug concentrations that have equivalent effects on the microtubule cytoskeleton and, consequently, on the fate of the cell. The study of Jordan et al. (1991) in HeLa cells with vinblastine and vincristine supports this concept. Vincristine and vinblastine do not show major differences in their interactions with tubulin (Himes, 1991), and, at initial extracellular drug concentrations close to the IC_{50} and IC_{90} values, intracellular drug concentrations were the same (30–40 and 150–160 nM, respectively). [The actual data (Jordan et al., 1991) follows. For vinblastine, the IC_{50} value was 0.45 nM and the extrapolated IC_{90} value was about 3 nM; for vincristine, 1.8 nM and about 7 nM, respectively. Jordan et al. (1991) reported 38 nM intracellular vinblastine with an initial extracellular concentration of 0.6 nM, and 33 nM intracellular vincristine with 1.8 nM extracellular drug. With 2.6 nM extracellular vinblastine, the intracellular concentration was 152 nM; with 10 nM extracellular vincristine, the intracellular drug was 163 nM.]

What accounts for the dramatic retention of dolastatin 10 as compared with vinblastine by CA46 cells? The difference in cellular accumulation can be explained in terms of the interactions of the two drugs with tubulin. Although their quantitative effects on assembly reactions are similar, there are major differences in their binding to tubulin and/or aberrant tubulin polymers. With both drugs it is difficult to document binding to the $\alpha\beta$-tubulin dimer in the absence of an associated formation of spiral polymers. Both polymers

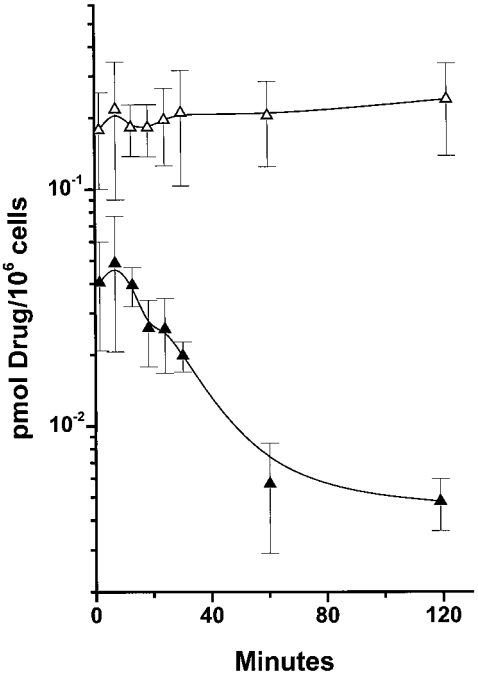

Fig. 6. Dolastatin 10, but not vinblastine, is tenaciously retained by Burkitt cells. Cells were preloaded with radiolabeled drug during a 1-h incubation with either 1.0 nM [^{3}H]dolastatin 10 (△) or 1.0 nM [^{3}H]vinblastine (▲). Cells were harvested by centrifugation and resuspended in media without drug. At the indicated times, cells in aliquots of each reaction mixture were centrifuged through oil. The experiment was performed three times (standard deviations indicated).

TABLE 2
Effect of ATP depletion and verapamil on efflux of dolastatin 10 and vinblastine from Burkitt CA46 cells
The % relative to value before efflux phase is shown in parentheses.

Drug	Before Efflux Phase[a]	No Addition[b]	+ NaN_3/2dG[c]	+ Verapamil[d]
	pmol drug / 10^6 cells ± S.D.[e]			
Dolastatin 10	0.27 ± 0.04 (100)	0.28 ± 0.01 (104)	0.28 ± 0.03 (104)	0.30 ± 0.05 (111)
Vinblastine	0.028 ± 0.006 (100)	0.0026 ± 0.002 (9.3)	0.0050 ± 0.003 (18)	0.0035 ± 0.002 (13)

S.D., standard deviation.
[a] Cells were incubated for 1 h in medium containing 1.0 nM [^{3}H]dolastatin 10 or [^{3}H]vinblastine. Aliquots were removed, and the cells were centrifuged through silicone oil to obtain the values shown in this column. For the efflux phase of the experiment, the remainder of each culture was divided into three portions, with the cells harvested by centrifugation.
[b] Cells were resuspended in medium and incubated for 2 h. Cells were harvested by centrifugation through silicone oil.
[c] Cells were resuspended in medium containing NaN_3 and 2-deoxy-D-glucose (2dG) and incubated for 2 h. Cells were harvested by centrifugation through silicone oil.
[d] Cells were resuspended in medium containing verapamil and incubated for 2 h. Cells were harvested by centrifugation through silicone oil.
[e] The experiment was performed twice, and in each experiment data points were based on triplicate samples.

and smaller oligomeric intermediates can be resolved from heterodimer on HPLC gel permeation columns, but preservation of vinblastine-induced structures requires drug in the column equilibration buffer (Singer et al., 1988). Preservation of dolastatin 10-induced structures does not require drug in the column buffer (Bai et al., 1995), and an unbound dolastatin 10 peak only appeared when drug:tubulin > 1.0. Moreover, the apparent K_a value obtained for dolastatin 10 (3.8×10^7 M^{-1}; Bai et al., 1995) is 21-fold higher than that obtained for vinblastine (1.8×10^6 M^{-1}; Safa et al., 1987) under comparable reaction conditions. [Literature values for the apparent K_a for vinblastine vary from 2×10^4 to 6×10^6 M^{-1}, and the variation seems to depend primarily on reaction conditions (Himes, 1991).] This difference in apparent K_a values is almost identical with the difference in the accumulation factors obtained at 24 h.

The much more rapid uptake of vinblastine compared with dolastatin 10 suggested that cell kill with the vinca alkaloid would require a shorter treatment time, although vinblastine efflux after medium exchange or possible differences in minimum drug exposure time for onset of apoptosis (cf. Mooberry et al., 1997) could complicate data interpretation. Other lymphoma cell lines showed irreversible cytotoxicity after 4–8 h of exposure to dolastatin 10 (Beckwith et al., 1993), in good agreement with our observation that maximum uptake of the peptide at its IC_{50} value occurred at 6 h. When we examined drug exposure time for the Burkitt cells at the IC_{90} values (determined from the 24-h continuous exposure studies), we found that 4 h of exposure to dolastatin 10 and 24 h of exposure to vinblastine were required for maximum effects on subsequent cell growth (data not presented). Thus, differences in efflux and uptake rates between the two drugs, together with difference in the strength of their interactions with tubulin, probably play roles in the rapidity with which they kill cells. Our data suggest that for clinical studies dolastatin 10 would be optimally delivered as a slow infusion over several hours rather than by bolus administration, and that still longer infusion times would enhance the efficacy of vinblastine.

The depsipeptides cryptophycins 1 and 52 have many similarities to dolastatin 10 in their effects on tubulin, including formation of structures stable to gel filtration (Kerksiek et al., 1995; Bai et al., 1996; Smith and Zhang, 1996; Moore, 1997). For Burkitt CA46, EW-36, and EW-36VCR60 cells we obtained IC_{50} values, respectively, of 20, 12, and 13 pM for cryptophycin 1 (unpublished observations). Cryptophycin 52 has been reported to be similar to cryptophycin 1 in its interactions with cells and tubulin (Moore, 1997). One would therefore predict similar findings to those reported here for dolastatin 10, and there have been two recent cellular uptake studies with cryptophycin 52 (Chen et al., 1998; Panda et al., 1998).

Chen et al. (1998), using a very high cryptophycin 52 concentration (1.5 μM) in a leukemia cell line, observed no detectable drug efflux (similar to our finding), maximal uptake by 20 min (versus the 6 h we found for dolastatin 10), and saturable uptake, suggesting an active transport process (opposite to our finding with dolastatin 10 concentrations up to 10 nM).

Panda et al. (1998), studying HeLa cells treated with 11 pM cryptophycin 52, the IC_{50} value, found that the intracellular drug concentration at 20 h was 8 nM, with an accumulation factor of 730 (somewhat lower than we found with dolastatin 10). Because the 2.0 mg/ml tubulin content of HeLa cells (Jordan et al., 1991) is less than half that of Burkitt cells, the data of Panda et al. (1998) yield a drug:tubulin ratio of 1:2500 at the IC_{50} value, 2.5 to 5-fold lower than our ratio for dolastatin 10 and 3- to 5-fold lower than our ratio for vinblastine. Panda et al. (1998) interpreted their results in terms of an exceptionally tight binding of cryptophycin 52 to microtubule ends with consequent inhibition of dynamic instability. A direct comparison of dolastatin 10 with cryptophycin 52 should provide additional insights into the mechanism of action of these potent peptide antimitotic drugs.

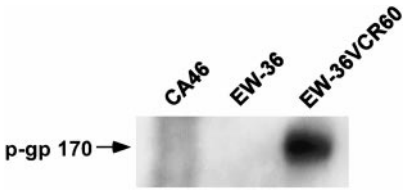

Fig. 7. Expression of p-gp 170 in CA46, EW-36, and EW-36VCR60 Burkitt cell lines. Equal amounts (40 μg) of total cell extract from each cell line was loaded onto the polyacrylamide gel. Western blot analysis for p-gp 170 was performed with the monoclonal antibody C219 as described in detail in the text.

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