Cancer Research

Structural Basis for Induction of Peripheral Neuropathy by Microtubule-Targeting Cancer Drugs

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Abstract

Peripheral neuropathy is a serious, dose-limiting side effect of cancer treatment with microtubule-targeting drugs. Symptoms present in a "stocking-glove" distribution, with longest nerves affected most acutely, suggesting a length-dependent component to the toxicity. Axonal transport of ATP-producing mitochondria along neuronal microtubules from cell body to synapse is crucial to neuronal function. We compared the effects of the drugs paclitaxel and ixabepilone that bind along the lengths of microtubules and the drugs eribulin and vincristine that bind at microtubule ends, on mitochondrial trafficking in cultured human neuronal SK-N-SH cells and on axonal transport in mouse sciatic nerves. Antiproliferative concentrations of paclitaxel and ixabepilone significantly inhibited the anterograde transport velocity of mitochondria in neuronal cells, whereas eribulin and vincristine inhibited transport only at significantly higher concentrations. Confirming these observations, anterogradely transported amyloid precursor protein accumulated in ligated sciatic nerves of control and eribulin-treated mice, but not in paclitaxel-treated mice, indicating that paclitaxel inhibited anterograde axonal transport, whereas eribulin did not. Electron microscopy of sciatic nerves of paclitaxel-treated mice showed reduced organelle accumulation proximal to the ligation consistent with inhibition of anterograde (kinesin based) transport by paclitaxel. In contrast, none of the drugs significantly affected retrograde (dynein based) transport in neuronal cells or mouse nerves. Collectively, these results suggest that paclitaxel and ixabepilone, which bind along the lengths and stabilize microtubules, inhibit kinesin-based axonal transport, but not dynein-based transport, whereas the microtubule-destabilizing drugs, eribulin and vincristine, which bind preferentially to microtubule ends, have significantly less effect on all microtubule-based axonal transport. *Cancer Res; 76(17); 5115–23. ©2016 AACR.*

Introduction

Microtubule-targeting drugs, among the most widely used chemotherapeutic agents, are effective in treatment of a variety of cancers. A common dose-limiting side effect of their use is peripheral neuropathy, primarily involving the sensory nervous system, although motor and autonomic systems can also be affected (1). Symptoms such as paresthesia, hyperalgesia, and allodynia first appear in a stocking-glove distribution, suggesting that nerves are affected most acutely in a length-dependent manner (2). Microtubules are critical for proper nerve function. They serve as tracks along which proteins, RNA, mitochondria, and other organelles that are synthesized in the cell bodies are transported by motor proteins to the nerve terminals, where synaptic vesicles carry out nerve actions requiring high ATP levels. Kinesin moves cargoes along microtubules anterogradely, from the nerve cell body to the axon terminal, and dynein moves cargoes along microtubules retrogradely, from the axon terminal to the nerve cell body. Thus, the longest neurons are particularly vulnerable to disruptions in axonal transport. As axonal length appears to be a significant factor in chemotherapy-induced peripheral neuropathy, many have hypothesized that inhibition of microtubule-based axonal transport may be an important mechanism of peripheral neuropathy (3). To investigate the causes of peripheral neuropathy induced by microtubule-targeted drugs, we examined the effects of four drugs on anterograde and retrograde transport in cultured human neuronal cells and in mouse sciatic nerves.

There are two major subtypes of microtubule-targeted drugs, the taxanes and epothilones that bind initially along microtubule surfaces or sides before becoming internalized into microtubules (4–6) and the vinca alkaloids and halichondrins that bind only to microtubule ends at their lowest effective concentrations (see Discussion also; refs. 5, 7–9). The first group is sometimes referred to as microtubule stabilizers because by binding along the microtubule lattice, they stabilize microtubule dynamic instability and inhibit microtubule depolymerization. At sufficiently high concentrations, the microtubule stabilizers also induce polymerization of microtubules. The second group including drugs such as vinca alkaloids and eribulin (a halichondrin) bind primarily to microtubule ends and are often referred to as microtubule

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destabilizers because at high concentrations, they block the addition of new tubulin subunits to microtubule ends, thus leading to microtubule depolymerization. However, their most potent activity is stabilization and inhibition of microtubule dynamic instability, which occurs with binding of low numbers of drug molecules at microtubule ends (7–9). Despite sharing the ability to suppress microtubule dynamic instability, we found that these two classes of drugs exert very different effects on microtubule-based transport.

We previously compared the effects of the four drugs on organelle transport in squid axoplasm and on kinesin-based gliding motility in reassembled bovine brain microtubules *in vitro* and found that very high (micromolar) concentrations of the four drug types had very different effects on transport (10).

In the clinic and in animal systems, different microtubule-targeted drugs induce varying levels of neurotoxicity (11, 12). For example, in mice treated for 2 weeks with the MTDs of the microtubule surface–binding drugs paclitaxel or ixabepilone, Wozniak and colleagues (12) observed significantly greater decreases in caudal and digital nerve conduction velocity and amplitude than were induced by the microtubule end–binding drug eribulin. The slight changes in nerve conduction velocity induced by eribulin were accompanied by less axonopathy in the sciatic nerve and less cellular damage in dorsal root ganglia as compared with paclitaxel or ixabepilone (12). This finding closely correlates with the clinical incidence of peripheral neuropathy with the three drugs, with paclitaxel and ixabepilone inducing a higher incidence of neuropathy in patients than eribulin (13–16). In contrast, vincristine induces a high clinical incidence of neuropathy, despite having a mechanism of action somewhat similar to that of eribulin (see Discussion; refs. 7, 17, 18).

In this study, we directly compared the effects of microtubule side-binding and end-binding drugs on microtubule-dependent transport in two experimental systems, cultured human neuronal cells and mice after ligation of their sciatic nerves. We compared the effects of four drugs on mitochondrial trafficking in neurites of SK-N-SH human neuroblastoma cells, at concentrations that impaired cancer cell proliferation equivalently for the four drugs. At antiproliferative paclitaxel or ixabepilone concentrations (at both IC_{50}s and $3 \times IC_{50}$s), anterograde mitochondrial transport velocity decreased significantly, with no significant effect on retrograde transport. In contrast, vincristine and eribulin did not significantly alter either retrograde or anterograde mitochondrial transport at the IC_{50} or at the $3 \times IC_{50}$ concentrations, although they did inhibit anterograde transport at higher concentrations that can induce microtubule depolymerization.

To confirm these results, we also examined paclitaxel's and eribulin's effects on axonal transport in ligated sciatic nerves of mice. After nerve ligation, paclitaxel, at its MTD in mice, markedly reduced anterograde (kinesin based) transport, whereas eribulin had no effect on transport. Neither drug affected retrograde (dynein based) transport. Together, the results suggest that the microtubule drugs that bind along microtubule lengths more strongly inhibit axonal transport than microtubule end–binding drugs.

Materials and Methods

Reagents

Eribulin mesylate was synthesized and provided by Eisai, Inc. Ixabepilone was provided by Eisai, Inc. Vincristine sulfate and paclitaxel were purchased from Sigma-Aldrich. For *in vitro* experiments, all four drugs were stored as 10 mmol/L stock solutions in 100% DMSO at −20°C. Retinoic acid, 30 mg/mL (Sigma), and MitoTracker Green FM, 1 mg/mL (Life Technologies), were also dissolved in 100% DMSO and stored at −20°C.

Potencies for inhibition of human cancer cell proliferation

Potencies for inhibition of human cancer cell proliferation by eribulin, vincristine, paclitaxel, and ixabepilone were determined to examine drug effects on mitochondrial transport at concentrations that paralleled their antitumor potencies. Cell lines purchased from and authenticated by the ATCC were passaged for no more than 6 months and were grown as follows: MDA-MB-435 human melanoma cells, DU145 human prostate cancer cells, and HeLa human cervical cancer cells were grown in DMEM with 10% FBS (Atlanta Biologics), MCF7 human breast cancer cells in DMEM with 15% FBS, H460 human lung cancer cells in RPMI1640 with 10% FBS, and OVCAR3 human ovarian cancer cells in RPMI1640 with 20% FBS. Cells were incubated with a range of drug concentrations for 72 hours (MDA-MB-345, H460, and MCF7) or 96 hours (DU145, HeLa, and OVCAR3), and inhibition of cell proliferation was measured using a sulforhodamine B assay (19). Mean IC_{50} values ± SE were 0.8 ± 0.2 nmol/L eribulin, 2.8 ± 0.8 nmol/L vincristine, 11 ± 5.6 nmol/L paclitaxel, and 7.5 ± 2.6 nmol/L ixabepilone. Mitochondrial transport was determined at the IC_{50} values and $3 \times IC_{50}$ values as well as some higher concentrations. The $3 \times IC_{50}$ concentrations used were 3 nmol/L eribulin, 8 nmol/L vincristine, 30 nmol/L paclitaxel, and 30 nmol/L ixabepilone or 2.18 ng/mL, 6.6 ng/mL, 25.6 ng/mL, and 15.18 ng/mL, respectively. These concentrations represent approximately 1% to 10% of the preclinical C_{max} exposure following one of a cumulative 6-dose MTD regimen in mice (20) and approximately 1% to 6% of the clinical C_{max} concentrations previously described in patients [3,650–8,530 ng/mL for paclitaxel (21, 22); 127–528 ng/mL for eribulin (23); 252 ng/mL for ixabepilone (24); and 82.4 to 412 ng/mL for vincristine (25)].

Mitochondrial trafficking

SK-N-SH human neuroblastoma cells were purchased from ATCC and subcultured for less than 6 months. SK-N-SH cells were cultured in Eagle's Essential Medium supplemented with 2 mmol/L L-glutamine and 10% FBS and seeded at 70,000 cells per well onto glass coverslips (coated with collagen type I overnight at 4°C) in 6-well plates in 2 mL medium with 10% FBS and 6 μg/mL retinoic acid and allowed to differentiate for 4 days. After differentiation, cells were incubated for 4 hours with drug or DMSO as a control, and then mitochondria were labeled with 50 nmol/L MitoTracker Green FM for 45 minutes in the dark at 37°C. Excess dye was removed, and cells were washed once with PBS and placed into a live cell chamber with recording medium [cell medium with 10% FBS, 15 mmol/L HEPES pH 7.2, and oxyrase (Oxyrase)]. Live cells were imaged on a Nikon E800 microscope with a heated stage (38°C) and 60× objective. Images were acquired every 5 seconds for 3 minutes. Mitochondrial movement in neurites was tracked from time-lapse images by using MT-LHAP software developed by Emin Oroudjev using IgorPro software version 6.2.2.2; the software can be downloaded at http://www.igorexchange.com/node/1767. A movement event was defined as having a minimum velocity of 1 μm/minute and moving a total distance of at least 2 μm, with no more than a 5-second pause (one

Table 1. Mitochondrial trafficking velocity

Drug	Drug concentration nmol/L	Anterograde velocity μm/min	Retrograde velocity μm/min
Control	0	14.2 ± 0.4	16.3 ± 0.6
Eribulin	***3*** *(3 × IC_{50})*	***13.8 ± 1.1***	***20.0 ± 2.5***
	6	12.7 ± 1.0	15.9 ± 1.8
	10	9.5 ± 0.9[a]	13.5 ± 1.5
Vincristine	3	14.6 ± 1.3	17.2 ± 1.2
	8 *(3 × IC_{50})*	***14.1 ± 0.9***	***16.1 ± 1.5***
	10	9.6 ± 0.7[b]	17.4 ± 1.7
Paclitaxel	3	12.5 ± 0.7	15.3 ± 1.2
	10	10.6 ± 0.8[c]	15.2 ± 1.3
	30 *(3 × IC_{50})*	***9.5 ± 0.6***[b]	***13.5 ± 1.2***
	100	10.6 ± 0.7[b]	12.4 ± 1.2[a]
Ixabepilone	3	14.5 ± 1.0	18.1 ± 1.4
	10	10.7 ± 0.7[c]	16.7 ± 1.2
	30 *(3 × IC_{50})*	***10.4 ± 0.8***[c]	***14.3 ± 1.3***

NOTE: Drug concentration dependence for inhibition of mitochondrial trafficking velocity. Velocities of mitochondria at the 3 × IC_{50} cancer cell antiproliferative concentrations (3 nmol/L eribulin, 8 nmol/L vincristine, 30 nmol/L paclitaxel, and 30 nmol/L ixabepilone) are shown in bold italics. Number of measurements was between 36 and 269 mitochondria for drug-treated cells and between 324 and 400 for controls.
[a]$P < 0.05$.
[b]$P < 0.001$.
[c]$P < 0.01$.

frame) within the event. Results are mean ± SE for ≥13 cells and ≥26 movement events per condition (N = total number of movement events for each condition, see Tables 1 and 2). Data were analyzed for statistical significance by one-way ANOVA using GraphPad Prism 5 software. Comparisons to control were analyzed for significance by Dunnett *post hoc* test. For all statistical data, $P \leq 0.05$ was considered statistically significant.

Axonal transport in mouse sciatic nerve

Female BALB/c mice (approximately 8–10 weeks) were obtained from Harlan Laboratories Inc. and maintained with free access to food and water. All procedures were performed as per protocols approved by the Institutional Animal Care and Use Committee at Johns Hopkins University (Baltimore, MD). Paclitaxel (LC Laboratories) or eribulin was administered intravenously as three injections every other day for 2 weeks with a 2 day rest between weekly cycles at 30 mg/kg and 1.75 mg/kg, respectively. These are previously determined MTDs (12). For mouse experiments, eribulin was dissolved in 100% DMSO to produce a 10 mg/mL stock solution, which was stored at −80°C in single-use aliquots. Each administration day, the stock solution was thawed and diluted with saline to a final concentration of 0.25 mg/mL in 2.5% DMSO/saline yielding dosing solutions in a 10 mL/kg volume. Paclitaxel was dissolved in 100% ethanol at 10% of the final volume. An equal volume of Cremophor (10% of the final volume) was then added and mixed by vortexing for 10 minutes. Immediately prior to injection, ice-cold saline was added to the final volume (80% of the final volume), and the solution was maintained on ice during dosing. Dosing solutions were made fresh daily and dosed in a volume of 10 mL/kg. Based upon previous studies, the estimated C_{max} concentration following a single dose of a cumulative 6-dose MTD regimen in mice are 95,000 and 203 ng/mL of paclitaxel and eribulin, respectively (20).

Sciatic nerve ligations and axonal transport studies in mice were performed as described previously (26). In brief, one hour after the last dose administration, mice were anesthetized with isoflurane and the left sciatic nerve exposed at the mid-thigh level and tightly ligated at two points approximately 2 to 3 mm apart using ethilon 6–0 nylon suture. Wounds were closed and the mice left to recover in their home cage. After 24 hours, mice were anesthetized and the ligated sciatic nerves were exposed and dissected. Segments (2-mm thick) were collected from the proximal and distal sides of the ligations. Tissues from 5 mice per group were pooled, frozen, and stored at −80°C for three independent experiments. For Western blot analysis, the pooled tissues were homogenized in lysis buffer containing protease inhibitors, and equal amounts of protein were loaded onto a Novex 8% to 16% Tris-glycine gel (Life Technologies). Proteins were probed with polyclonal rabbit APP antibody (Millipore) and dynein antibody (Santa Cruz Biotechnology). GAPDH antibody (Sigma) was used a loading control.

Table 2. Mitochondrial trafficking run length

Drug	Drug concentration nmol/L	Anterograde length μm	Retrograde length μm
Control	0	5.2 ± 0.2	4.8 ± 0.3
Eribulin	***3*** *(3 × IC_{50})*	***4.1 ± 0.3***	***4.9 ± 0.6***
	6	4.9 ± 0.6	3.9 ± 0.5
	10	3.8 ± 0.3	3.4 ± 0.3
Vincristine	3	5.0 ± 0.5	4.2 ± 0.3
	8 *(3 × IC_{50})*	***4.8 ± 0.3***	***3.8 ± 0.3***
	10	3.7 ± 0.3	3.4 ± 0.2
Paclitaxel	3	4.3 ± 0.3	4.3 ± 0.8
	10	4.4 ± 0.05	4.6 ± 0.05
	30 *(3 × IC_{50})*	***3.8 ± 0.2***[a]	***4.2 ± 0.5***
	100	4.0 ± 0.2[a]	3.5 ± 0.2
Ixabepilone	3	4.4 ± 0.4	4.4 ± 0.4
	10	4.0 ± 0.3	4.7 ± 0.4
	30 *(3 × IC_{50})*	***4.1 ± 0.3***	***3.5 ± 0.3***

NOTE: Drug concentration dependence for inhibition of mitochondrial trafficking run length. Run lengths of mitochondria at the 3 × IC_{50} cancer cell antiproliferative concentrations (3 nmol/L eribulin, 8 nmol/L vincristine, 30 nmol/L paclitaxel, and 30 nmol/L ixabepilone) are shown in bold italics. Number of measurements was between 26 and 120 mitochondria for drug-treated cells and between 324 and 400 mitochondria for controls.
[a]$P < 0.05$.

Sciatic nerve morphology

For morphologic studies, mice received the full 6-dose MTD regimen (30 and 1.75 mg/kg per dose i.v.) for paclitaxel and eribulin, respectively, and were then anesthetized with 10% chloral hydrate and euthanized by transcardial perfusion with PBS, followed by 4% paraformaldehyde and 2% glutaraldehyde in 0.1 mmol/L phosphate buffer, pH 7.4, for 10 to 15 minutes. Segments (2-mm thick) proximal and distal to the ligations were dissected and post-fixed in osmium tetroxide and embedded in Epon. Sections (70 nm-thick) were cut and processed for electron microscopy, and images were acquired using a high-performance Zeiss LIBRA 200 transmission electron microscope. Tissues from 2 mice per group were imaged and analyzed for one experiment.

Results

Determination of equally effective antiproliferative drug concentrations for comparison of drug effects on mitochondrial trafficking

We wanted to compare the effects of four clinically used drugs, eribulin, vincristine, paclitaxel, and ixabepilone, on mitochondrial trafficking in human neuroblastoma SK-N-SH

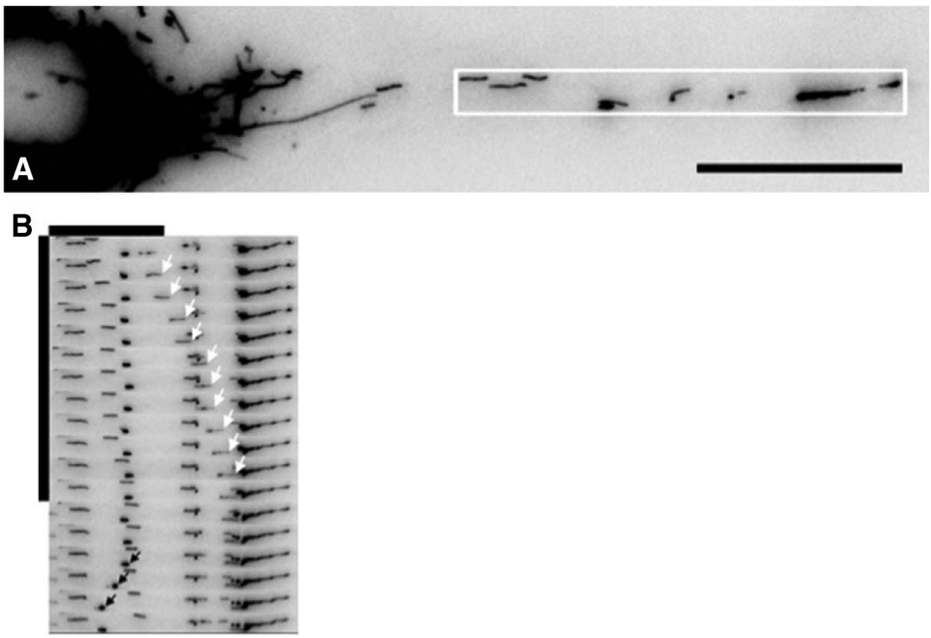

Figure 1.
Images of a control SK-N-SH cell with mitochondria labeled by MitoTracker Green, showing anterograde and retrograde mitochondria movement within a neurite. **A,** cell body and neurite of a control cell with labeled mitochondria. Box, region of interest expanded in kymograph in **B**. Scale bar, 50 μm. **B,** kymograph of neurite area containing mitochondria shown in the box in **A**. Time-lapse images were acquired every 5 seconds. Mitochondria move in anterograde (white arrows) and retrograde (black arrows) directions. Horizontal bar, 50 μm; vertical bar, 1 minute.

neurites. The four drugs vary widely in their clinical doses. The recommended clinical dose for eribulin is 1.4 mg/m^2; for vincristine, 1.4 mg/m^2; for ixabepilone 40 mg/m^2; and for paclitaxel 135–175 mg/m^2; refs. 21–23, 26). To determine the appropriate drug concentrations for examining the effects on peripheral neuropathy in SK-N-SH cells, we first determined the concentration dependence for each drug for inhibition of cell proliferation in 6 human tumor cell lines (MDA-MB-435, H460, MCF7, DU145, HeLa, and OVCAR3). The concentrations that inhibited cell proliferation by 50% (IC_{50}) after 72- or 96-hour incubation (Materials and Methods) were averaged for each drug for all 6 cell lines. Thus, the neurotoxic effects of the drugs on mitochondrial transport were measured at the 3 × IC_{50} antiproliferative concentration of each drug, which was 3 nmol/L eribulin, 8 nmol/L vincristine, 30 nmol/L paclitaxel, and 30 nmol/L ixabepilone.

Effects of eribulin, vincristine, paclitaxel, and ixabepilone on mitochondrial trafficking in human neuronal cells

Mitochondrial velocity and run lengths were determined in differentiated SK-N-SH human neuroblastoma cell neurites by quantitative live cell microscopy of mitochondria labeled with MitoTracker Green mitochondrial dye. SK-N-SH cells were differentiated for 4 days to allow neurites to extend and then incubated with drug at a range of concentrations, including the 3 × IC_{50} antiproliferative concentrations for 4 hours. Mitochondria were then labeled and time-lapse digital images of mitochondrial movement were acquired every 5 seconds for 3 minutes. Figure 1A shows MitoTracker Green–labeled mitochondria in an SK-N-SH cell neurite. In Fig. 1B, a kymograph shows the time sequence of mitochondrial anterograde and retrograde movement for the mitochondria enclosed in the boxed region of Fig. 1A.

Effects on mitochondrial velocity

The mean mitochondrial velocities induced by the four drugs are shown in Table 1 for the entire range of concentrations and in Fig. 2 for the 3 × IC_{50} antiproliferative concentrations. Anterograde velocity in control cells was 14.2 ± 0.4 μm/minute (Table 1;Fig. 2A). The microtubule surface–binding drugs, paclitaxel and ixabepilone, at both the IC_{50} and the 3 × IC_{50} antiproliferative concentrations (10 and 30 nmol/L, respectively, for both drugs) significantly reduced the anterograde velocity. At the 3 × IC_{50} it was reduced by 33% and 27%, respectively ($P < 0.01$), to 9.5 ± 0.6 μm/minute and 10.4 ± 0.8 μm/minute, respectively. In contrast, the 3 × IC_{50} antiproliferative concentrations of the end-binding drugs eribulin (3 nmol/L) and vincristine (8 nmol/L) did not significantly affect anterograde velocities, which were 13.8 ± 1.1 and 14.1 ± 0.9 μm/minute, respectively. However, as the drug concentration was increased above the 3 × IC_{50} concentration, anterograde mitochondrial trafficking velocity became sensitive to all the four drugs. When the concentration of eribulin or vincristine was raised to 10 nmol/L, higher than the 3 × IC_{50} antiproliferative concentrations, both end-binding drugs also significantly inhibited anterograde mitochondrial transport. Higher drug concentrations than those shown in the tables were not used because they resulted in significant loss of neurites, with few moving mitochondria in the remaining neurites. The data in Table 1 also show that the microtubule surface–binding drugs, paclitaxel and ixabepilone, are exceptionally potent inhibitors of anterograde transport. Anterograde transport velocity was also inhibited by paclitaxel or ixabepilone at the relatively low concentration of 10 nmol/L, lower than the 3 × IC_{50} concentration of 30 nmol/L.

Inhibitory effects on mitochondrial retrograde velocity were minimal

At the 3 × IC_{50} antiproliferative concentrations, none of the four drugs altered retrograde velocity in a statistically significant manner (Fig. 2B). Mean retrograde velocity for mitochondria in controls was 16.3 ± 0.6 μm/minute. After incubation with eribulin, vincristine, paclitaxel, or ixabepilone, the retrograde velocities were 20.0 ± 2.5, 16.1 ± 1.5, 13.5 ± 1.2, and 14.3 ± 1.3 μm/minute, respectively. However, at a very high concentration

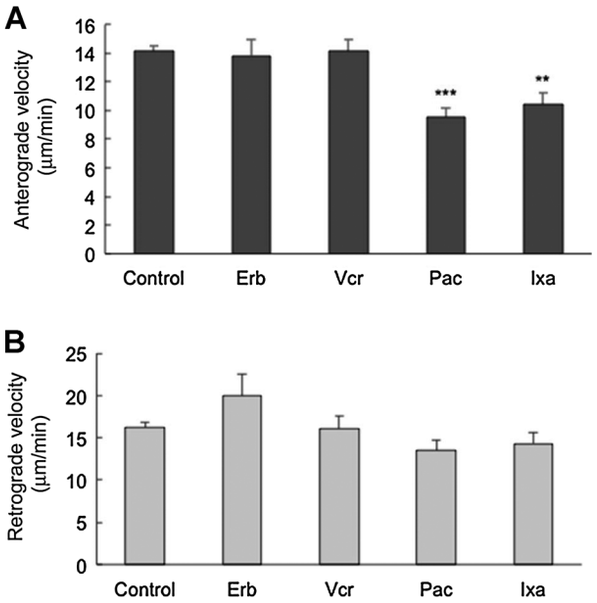

Figure 2.

Paclitaxel and ixabepilone at the 3 × IC_{50} antiproliferative concentration for each drug significantly reduced anterograde mitochondrial trafficking velocity in cultured SK-N-SH human neuronal cell neurites, whereas none of the drugs significantly affected retrograde velocity. Mitochondria were labeled with MitoTracker Green, and time-lapse images were acquired every 5 seconds for 3 minutes. Data are the average velocity of mitochondrial movement events, as described in Materials and Methods. **A,** drug effects on anterograde mitochondrial trafficking velocity (μm/minute). **B,** drug effects on retrograde mitochondrial trafficking velocity (μm/minute). Mitochondrial velocity was measured at the 3 × IC_{50} antiproliferative value for each drug: eribulin (Erb), 3 nmol/L; vincristine (Vcr), 8 nmol/L; paclitaxel (Pac), 30 nmol/L; and ixabepilone (Ixa), 30 nmol/L. Error bars, SE; **, $P < 0.01$; ***, $P < 0.001$.

(100 nmol/L, 10 × IC_{50}), paclitaxel reduced the retrograde velocity by 19% ($P < 0.05$).

Effects on mitochondrial run length

Drug effects on the distance a mitochondrion traveled in a continuous movement event (the run length) are shown in Table 2 for the range of concentrations tested and in Fig. 3 for the 3 × IC_{50}. Only paclitaxel (30 and 100 nmol/L, 3 × IC_{50} and 10 × IC_{50}) reduced anterograde run length significantly, by 27% ($P < 0.05$), from a run length of 5.2 ± 0.2 μm in control cells to 3.8 ± 0.2 and 4.0 ± 0.2 μm, respectively. Anterograde run length was reduced slightly, but not statistically significantly, by eribulin (to 4.1 ± 0.3 μm), vincristine (to 4.8 ± 0.3 μm), and ixabepilone (to 4.1 ± 0.3 μm). Retrograde run lengths were not reduced significantly by any of the drugs at any concentration tested (Fig. 3B).

The results indicate that at drug concentrations that approximate the dosages necessary to effectively inhibit cancer cell proliferation in human cells, the microtubule surface–binding drugs (drugs binding along the lengths of microtubules), paclitaxel and ixabepilone, significantly inhibit mitochondrial transport velocity down the axon (anterograde). Only paclitaxel also inhibits the run length at the concentrations that could be tested. Thus, both actions have the potential to significantly inhibit neuronal function by reducing ATP generation in the distal regions of axons and at nerve termini.

The data in Table 1 also show that paclitaxel and ixabepilone are exceptionally potent inhibitors of anterograde transport. Anterograde transport was also inhibited by paclitaxel or ixabepilone at a concentration of 10 nmol/L, lower than the 3 × IC_{50} concentration (30 nmol/L). Above 10 nmol/L paclitaxel, there was no further increase in inhibition of anterograde velocity, and above 30 nmol/L paclitaxel, there was no further increase in reduction of anterograde run length (Table 2), suggesting a maximal effect.

Paclitaxel, but not eribulin, inhibits axonal transport in mouse sciatic nerves

In mouse sciatic nerves, paclitaxel inhibited anterograde axonal transport at the MTD. Nerve ligations were performed as depicted in Fig. 4A. Amyloid precursor protein (APP) and dynein accumulation were monitored as markers of anterograde and retrograde transport, respectively. Figure 4B shows a representative Western blot of APP accumulation in ligated nerves of the vehicle controls and of the paclitaxel- and eribulin-treated mice. Paclitaxel reduced APP accumulation by 39% in the sciatic nerve segments proximal to the ligation, indicating impaired anterograde axonal transport [compare lanes 4 (control) and 8 (paclitaxel) in Fig. 4B and C]. In contrast, in eribulin-treated mice, APP accumulation was not reduced in proximal nerve segments relative to vehicle-treated control mice (compare lanes 2 and 6, Fig. 4B and C). APP did not accumulate in the nerve segments distal to the ligation site, consistent with APP being a specific marker for

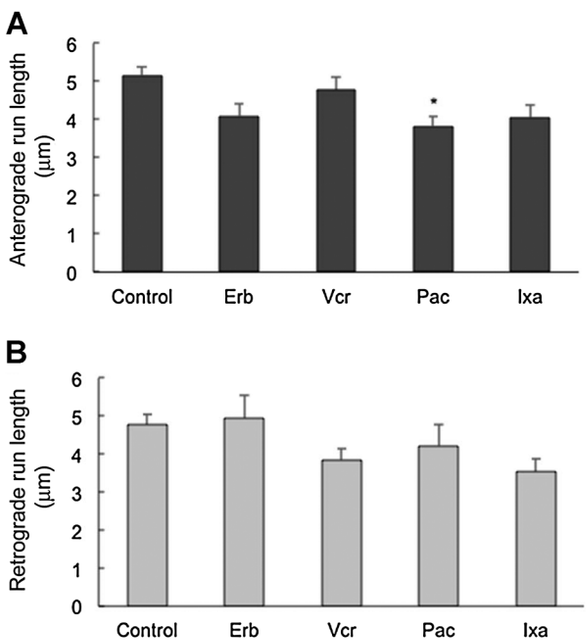

Figure 3.

Effects of drugs on anterograde (**A**) and retrograde (**B**) mitochondrial run lengths in cultured human SK-N-SH neuronal cell neurites at the 3 × IC_{50} antiproliferative drug concentrations. Only paclitaxel significantly inhibited the anterograde mitochondrial run length. None of the drugs significantly affected retrograde run length. Bars represent mean mitochondrial run lengths per movement event measured at the 3 × IC_{50} antiproliferative value for each drug: eribulin (Erb), 3 nmol/L; vincristine (Vcr), 8 nmol/L; paclitaxel (Pac), 30 nmol/L; and ixabepilone (Ixa), 30 nmol/L. Error bars, SE; *, $P < 0.05$.

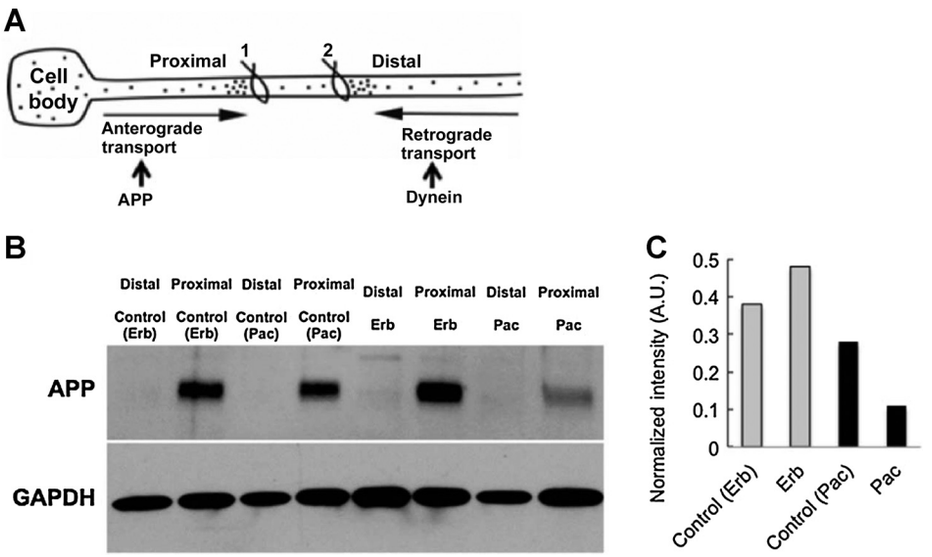

Figure 4.

Effects of paclitaxel and eribulin on anterograde axonal transport of APP in mouse sciatic nerve. As compared with vehicle-treated control animals, paclitaxel-treated mice exhibited less APP staining in the 2-mm nerve segment proximal to the ligation, indicating reduced anterograde axonal transport, whereas APP staining in eribulin-treated mice was not reduced. **A,** schematic showing location of ligation points on sciatic nerve. APP was the marker for anterograde transport; dynein marked retrograde transport. **B,** representative Western blot from one of three separate experiments showing APP content in proximal and distal ligated nerve sections of vehicle control-treated mice as compared with eribulin (Erb)- and paclitaxel (Pac)-treated mice. APP accumulation in nerve sections proximal to the ligation site was reduced in paclitaxel-treated mice (0.11 adjusted intensity units, lane 8) as compared with control (0.28 adjusted intensity units, lane 4); eribulin-treated mice showed no reduction in APP accumulation (0.48 adjusted intensity units, lane 6) as compared with vehicle control (0.38 adjusted intensity units, lane 2). **C,** densitometric quantitation of Western blot in **B** showing APP accumulation in proximal nerve sections. A.U., arbitrary units. APP signal intensity was normalized to GAPDH. Erb, eribulin (gray bars); Pac, paclitaxel (black bars). Paclitaxel was administered in a Cremophor vehicle, which is toxic to neurons (45); thus, the controls for paclitaxel and eribulin are not comparable. Data are representative of three independent experiments.

anterograde transport. Neither paclitaxel nor eribulin had any appreciable effect on retrograde transport. Dynein accumulation in the segment distal to the ligation was similar with paclitaxel or eribulin treatment and with their respective controls (data not shown).

Figure 5 shows electron micrographs of ligated sciatic nerve axons of control, eribulin-, and paclitaxel-treated mice. Control mice (Fig. 5A) and eribulin-treated mice (Fig. 5B) showed similar levels of axoplasmic accumulation consisting of organelles, neurofilaments, and microtubules in the proximal segments of the sciatic nerves, whereas after paclitaxel treatment, axoplasms were conspicuously clear in proximal nerve segments. Thus, transmission electron microscopy indicated a greater transport of intraaxonal organelles into the proximal neuronal segment after eribulin treatment as compared with little evidence of transport after paclitaxel treatment. These results agree with the results of ligation experiments shown in Fig. 4 and indicate little to no inhibition of axonal transport in eribulin-treated animals. Electron microscopy of the sciatic nerve segments distal to the ligation sites in mice treated with MTDs (a full 6-dose regimen consisting of 30 and 1.75 mg/kg per dose i.v. for paclitaxel or eribulin, respectively) were similar to controls, indicating that neither drug affected retrograde transport (Fig. 5C).

Discussion

To ask whether peripheral neuropathy induced by microtubule-targeted drugs might arise from inhibition of motor-driven transport along neuronal microtubules, we examined the effects of four microtubule-targeted drugs, eribulin, paclitaxel, ixabepilone, and vincristine, on anterograde and retrograde transport in two experimental systems, in neurites of differentiated SK-N-SH human neuroblastoma cells and in ligated sciatic nerves of mice.

In SK-N-SH cells, at both the IC_{50} and the $3 \times IC_{50}$ antiproliferative drug concentrations that were averaged over six human cancer cell lines, only paclitaxel and ixabepilone (which bind along microtubule lengths), but not eribulin and vincristine (which bind at their lowest effective concentrations to microtubule ends), significantly inhibited anterograde axonal transport of mitochondria (Figs. 2 and 3). As shown in Tables 1 and 2, eribulin and vincristine inhibited only when they were used at concentrations that exceeded the $3 \times IC_{50}$.

Similarly, in ligated sciatic nerves of mice treated with MTDs (a full 6-dose MTD regimen, 30 and 1.75 mg/kg per dose i.v.) for paclitaxel or eribulin, respectively, only paclitaxel, but not eribulin, inhibited anterograde axonal transport of APP (Fig. 4). These findings are supported by electron microscopy of the ligated mouse sciatic nerve axons, which showed that paclitaxel reduced accumulation of organelles, microtubules, and neurofilaments in regions proximal to the ligation (Fig. 5), further indicating that paclitaxel inhibits anterograde microtubule-based transport. In contrast, the microtubule end–binding drug eribulin had no apparent effect on mouse nerve microtubule-based transport at the MTD, as evidenced by levels of sciatic nerve organelle density similar to that of control animals. Thus, both in neurites of cultured human neuronal cells and in mouse sciatic nerves, at the drug concentrations used, only anterograde (kinesin based) transport was significantly affected by any of the drugs with little

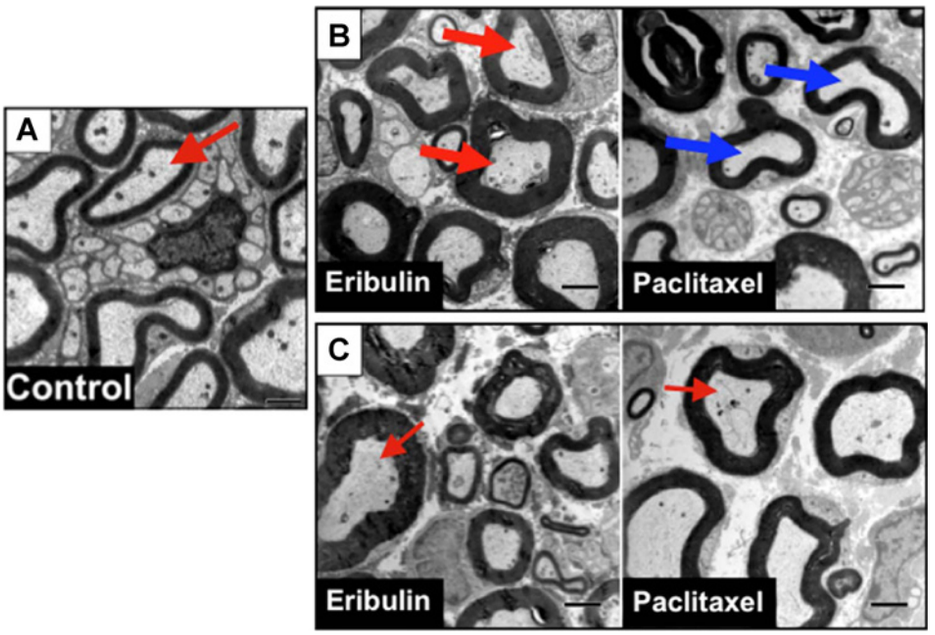

Figure 5.

Effects of paclitaxel and eribulin on accumulation of intraaxonal organelles in ligated sciatic nerve. Mice received a full 6-dose MTD regimen (30 and 1.75 mg/kg per dose i.v.) for paclitaxel or eribulin, respectively. **A,** sciatic nerve of control mouse (no drug) showing accumulation of organelles, microtubules, and neurofilaments (red arrows) proximal to the ligation site. **B,** in the 2-mm nerve segment proximal to the ligation site, sections of an eribulin-treated mouse showed more accumulation of organelles and filaments (red arrows) as compared with paclitaxel-treated mice (blue arrows), indicating that that paclitaxel induced greater inhibition of anterograde transport, while eribulin did not. **C,** distal to the ligation site, no difference was observed in quantity of intraaxonal organelles between eribulin- and paclitaxel-treated mice and control mice (not shown), suggesting no difference between the two drugs in effects on retrograde transport. Scale bars, 1 (**A**) and 2 μm (**B** and **C**).

or no effect on retrograde (dynein based) transport. Taken together, results from two experimental systems indicate that a significant cause of peripheral neuropathy by drugs that bind along the lengths of microtubules appears to involve inhibition of kinesin-driven anterograde transport.

The importance of microtubule-based mitochondrial transport

Neurons are highly polarized cells with their nuclei located in the cell body, far from the distal termini of the axons (as far as a meter away), where energy and materials must be supplied for neuronal viability and function (27). The transport of new materials anterogradely along the axon to its terminus and the retrograde return and recycling of aged or dysfunctional mitochondria and other materials from the axon terminus to the cell body are essential for neuronal viability and function. Mitochondria travel enormous distances from the cell body to the nerve terminus, requiring days to reach the terminus (28, 29). Their transport is crucial for the maintenance of ATP production along the axon to support transport of materials, including proteins and RNA, from their site of production in the cell body to the nerve terminus. Their mitochondria also produce large amounts of ATP that must be readily available for pumping out ions that are released into the nerve cell interior upon opening of synaptic nerve vesicles (30). Even a resting neuron consumes as many as 4.7 billion ATP molecules per second (31), thus small interruptions or perturbations of mitochondrial transport can have major effects on neuronal activity.

Paclitaxel binds along the length of the microtubule, as does ixabepilone (32–35). Although paclitaxel ultimately resides on the inside surface of the microtubule, it significantly alters the overall conformation of the tubulin to which it is bound, enhancing pi–pi interactions, increasing hydrogen bonds, stiffening the protofilaments, and even affecting the conformation of α-tubulin and changing the number of protofilaments in the microtubule from 13 to 12 (6).

Kinesin I, the anterogradely moving motor protein, moves hand-over-hand toward the plus ends of microtubules, taking highly regular and straight steps and moving along single protofilaments (36). It seems reasonable that perturbation of the microtubule surface structure and conformation by paclitaxel and other drugs that act along microtubule lengths can interfere with the highly regular movement of kinesin-driven anterograde transport. In direct support of this idea, Peck and colleagues (37) found that different forms of the microtubule-associated protein tau changed the kinesin-based gliding movement of microtubules *in vitro* that were stabilized by 20 μmol/L paclitaxel.

Why is anterograde transport inhibited by taxanes and ixabepilone at 3 × IC_{50} dose levels, whereas retrograde transport is not inhibited?

The anterograde motor kinesin I and the retrograde motor dynein move along microtubule surfaces differently. In contrast to the straight and highly regular manner in which kinesin I moves along single protofilaments, dynein can step sideways, forward, backward, and take steps of varying sizes along microtubules.

Dynein walks in a significantly more uncoordinated or wandering, sometimes called "drunken", fashion than kinesin (38), moving on more than one protofilament. It seems reasonable that perturbation of microtubule surfaces by paclitaxel and ixabepilone may not affect retrograde movement due to the more adaptable movement of dynein. Dynein may be able to step around drug-induced changes in conformation or flaws in the microtubule surface and continue travel unabated. In addition, high concentrations of drugs that bind along microtubule surfaces can induce microtubule bundling in cells (39), another factor that may inhibit microtubule-mediated anterograde transport by kinesin due to disorganization of axonal microtubule tracks, whereas dynein might more easily switch tracks within a microtubule bundle.

Lack of transport inhibition by end-binding drugs at 3 × IC_{50} antiproliferative drug concentrations

Vincristine and eribulin at 3 × IC_{50} did not significantly inhibit either anterograde or retrograde transport in the systems investigated in this work. At their lowest effective concentrations, the two drugs act by binding strictly at the plus ends of the microtubules. At these concentrations, both drugs inhibit steady-state growth at plus ends of microtubules, without causing appreciable microtubule depolymerization (5, 7, 8, 40). It is easy to imagine how such end-binding actions may not inhibit kinesin and dynein transport along the surface of microtubules, because these drugs do not act on the microtubule surfaces, where the motors are located. However, relatively high concentrations of end-binding drugs do depolymerize microtubules ($\geq$10 nmol/L), and such depletion of microtubules at nerve endings may reduce the microtubule density, resulting in the dying back of neuronal processes. In HeLa cells, microtubule depolymerization was detectable at concentrations of vinblastine (a drug similar in mechanism and potency to vincristine), at concentrations as low as 6 nmol/L, and complete depolymerization occurred at 100 nmol/L vinblastine (40). In this work, the higher concentrations of 10 nmol/L eribulin or 10 nmol/L vincristine did inhibit mitochondrial trafficking velocity (Table 1). Such inhibition is more likely due to partial microtubule depolymerization, rather than direct inhibition of motor transport along existing microtubules.

Microtubule-targeted drugs can affect other neuronal functions that may lead to peripheral neuropathy via other mechanisms

Axonal termini are not stable unchanging structures, but they continually sense the environment and rapidly alter their structures accordingly. For such behavior, unimpaired microtubule dynamics are necessary, and inhibition of dynamic instability, and possibly treadmilling (5) behavior, could also play roles in the induction of peripheral neuropathy by microtubule-targeted drugs. There is also evidence to support the idea that microtubule-targeted drugs, such as paclitaxel and vinorelbine (a drug closely related in structure and activity to vincristine), may contribute to peripheral neuropathy by binding directly to the tubulin found in mitochondrial membranes (41–44). Such binding can lead to opening of the permeability transition pore, to mitochondrial swelling, and to cell death (41–44). Thus, inhibition of mitochondrial transport by drug-binding along microtubule surfaces, as shown here, may be only one of the several mechanisms by which microtubule-targeted drugs induce peripheral neuropathy.

Disclosure of Potential Conflicts of Interest

J.A. Smith reports receiving a commercial research grant from Eisai Research Institute. S. Feinstein reports receiving a commercial research grant from EISAI, has received speakers bureau honoraria from Genentech, and is a consultant/advisory board member for EISAI. M.A. Jordan is a consultant/advisory board member for Eisai, Inc. No potential conflicts of interest were disclosed by the other authors.

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