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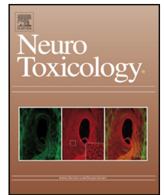

Effects of eribulin, vincristine, paclitaxel and ixabepilone on fast axonal transport and kinesin-1 driven microtubule gliding: Implications for chemotherapy-induced peripheral neuropathy

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ABSTRACT

Chemotherapy-induced peripheral neuropathy (CIPN) is a serious, painful and dose-limiting side effect of cancer drugs that target microtubules. The mechanisms underlying the neuronal damage are unknown, but may include disruption of fast axonal transport, an essential microtubule-based process that moves cellular components over long distances between neuronal cell bodies and nerve terminals. This idea is supported by the "dying back" pattern of degeneration observed in CIPN, and by the selective vulnerability of sensory neurons bearing the longest axonal projections. In this study, we test the hypothesis that microtubule-targeting drugs disrupt fast axonal transport using vesicle motility assays in isolated squid axoplasm and a cell-free microtubule gliding assay with defined components. We compare four clinically-used drugs, eribulin, vincristine, paclitaxel and ixabepilone. Of these, eribulin is associated with a relatively low incidence of severe neuropathy, while vincristine has a relatively high incidence. In vesicle motility assays, we found that all four drugs inhibited anterograde (conventional kinesin-dependent) fast axonal transport, with the potency being vincristine = ixabepilone > paclitaxel = eribulin. Interestingly, eribulin and paclitaxel did not inhibit retrograde (cytoplasmic dynein-dependent) fast axonal transport, in contrast to vincristine and ixabepilone. Similarly, vincristine and ixabepilone both exerted significant inhibitory effects in an *in vitro* microtubule gliding assay consisting of recombinant kinesin (kinesin-1) and microtubules composed of purified bovine brain tubulin, whereas paclitaxel and eribulin had negligible effects. Our results suggest that (i) inhibition of microtubule-based fast axonal transport may be a significant contributor to neurotoxicity induced by microtubule-targeting drugs, and (ii) that individual microtubule-targeting drugs affect fast axonal transport through different mechanisms.

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1. Introduction

Microtubules are a major component of the cytoskeleton. They are dynamic polymers composed of tubulin dimers arranged end to end in linear protofilaments that align to form a hollow cylinder. In addition to playing essential roles in migration and mitosis (Jordan and Wilson, 2004), microtubules are critical for intracellular transport, serving as tracks for "motor" proteins that carry vesicular cargo. Neurons are especially dependent on intracellular transport because of their complex elongated cellular architecture. In neuronal axons, the motor protein conventional kinesin transports various cargoes from the cell body toward distal terminals (anterograde fast axonal transport), supplying essential new materials for synapse function and maintenance (Morfini et al., 2011). On the other hand, cytoplasmic dynein transports cargo in the opposite direction (retrograde fast axonal transport), clearing toxic components from nerve terminals (*e.g.*, misfolded proteins, damaged organelles), and delivering survival factors to the cell body. The importance of fast axonal transport to neuronal function is highlighted by multiple lines of evidence linking deficits in this process to neurodegeneration (Millecamps and Julien, 2013; Morfini et al., 2009; Perlson et al., 2010).

Many cancer chemotherapeutic agents target microtubule structure and function (Jordan and Wilson, 2004), and a frequently

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dose-limiting side effect of these drugs is chemotherapy-induced peripheral neuropathy (CIPN). Sensory neurons are preferentially affected, and symptoms typically appear first in distal extremities (Argyriou et al., 2012; Carlson and Ocean, 2011; Windebank and Grisold, 2008), indicating increased vulnerability of neurons with the longest axons. In affected neurons, the fine axonal fibers innervating the skin are lost (Pachman et al., 2011; Siau et al., 2006), consistent with a "dying back" pattern of degeneration (Argyriou et al., 2012). The incidence of severe neuropathy varies among different microtubule targeting drugs, with vincristine ranking among the highest (Carlson and Ocean, 2011). In contrast, eribulin has a relatively low incidence of severe neuropathy (Cortes et al., 2012).

The mechanisms by which microtubule-targeting drugs cause neuropathy are unknown. Mitochondrial dysfunction, changes in gene expression and membrane excitability, and inflammation have all been proposed (Jaggi and Singh, 2012; Pachman et al., 2011; Xiao et al., 2011). However, none of these mechanisms explains the selective vulnerability of long sensory neurons. An alternative hypothesis consistent with the "dying back" pattern of degeneration produced by microtubule-targeting drugs is that these drugs disrupt fast axonal transport (Argyriou et al., 2012; Komlodi-Pasztor et al., 2011; Windebank and Grisold, 2008). This hypothesis is supported by studies in cultured cells (Carbonaro et al., 2012; Shemesh and Spira, 2010; Theiss and Meller, 2000) and sciatic nerves (Nakata and Yorifuji, 1999; Sahenk et al., 1987). However, in these experimental systems it is difficult to distinguish effects on fast axonal transport from effects on other cellular processes.

Here, we directly measured effects of four clinically important microtubule-targeting drugs on fast axonal transport using two simplified experimental systems. These drugs, eribulin, vincristine, paclitaxel and ixabepilone, represent four distinct drug classes (*i.e.* halichondrins, *Vinca* alkaloids, taxanes, and epothilones) and induce peripheral neuropathy to varying extents. Using vesicle motility assays in isolated squid axoplasm, all four drugs inhibited anterograde transport, with varying potencies. In contrast, retrograde axonal transport was not affected by eribulin or paclitaxel, but was inhibited by vincristine and ixabepilone. In an independent, cell-free assay, the drugs displaying the strongest inhibitory effects in the axoplasm assay, vincristine and ixabepilone, also inhibited kinesin-1-driven gliding of microtubules assembled from purified bovine brain tubulin. In contrast, eribulin and paclitaxel had no significant effect on microtubule gliding. Our results indicate that inhibition of fast axonal transport may contribute significantly to the neurotoxicity induced by microtubule-targeting drugs, and that eribulin's lower incidence of severe peripheral neuropathy may result, at least in part, from its milder effects on fast axonal transport relative to other microtubule-targeting drugs and from the low doses at which eribulin is clinically effective.

2. Methods

2.1. Proteins and chemicals

Drugs sources were as follows: eribulin mesylate (Eisai Inc.), vincristine sulfate (Sigma), paclitaxel (Molecular Probes for vesicle motility assays in axoplasm; Sigma for microtubule gliding assays), and ixabepilone (Bristol-Myers Squibb). Stocks were prepared at 10 mM concentrations in DMSO. For microtubule gliding assays, tubulin was isolated from bovine brain and purified by phosphocellulose chromatography (Miller and Wilson, 2010). Rhodamine-labeled tubulin was prepared as described (Peck et al., 2011). Guanosine-5′-[(α,β)-methyleno]triphosphate sodium salt (GMPCPP) was purchased from Jena Bioscience. The recombinant kinesin construct used in microtubule gliding assays, K560-His, was a kind gift from Dr. Ron Vale at the University of California, San Francisco. It corresponds to the first 560 amino acids of human conventional kinesin (KIF5B, GenBank Accession NP_004512.1) fused to a 6$\times$-histidine tag (located at the carboxy terminus) to facilitate nickel-based affinity purification. It was expressed in bacteria and purified as described (Peck et al., 2011).

2.2. Vesicle motility assays in isolated squid axoplasm

Axoplasm from squid (*Loligo pealii*) giant axons was extruded from its plasma membrane as previously described (Brady et al., 1985). All drugs were diluted for perfusion in 5 mM ATP-supplemented axoplasm buffer (175 mM potassium aspartate, 65 mM taurine, 35 mM betaine, 25 mM glycine, 10 mM HEPES, 6.5 mM $MgCl_2$, 5 mM EGTA, 1.5 mM $CaCl_2$, 0.5 mM glucose, pH 7.2). The volume of a single axoplasm was $\sim$5 μl, and each axoplasm was perfused with 20 μl of buffer containing the drug of interest. Therefore, drug concentration was diminished approximately 20% by the volume of the extruded axoplasm. Motility was analyzed using a Zeiss Axiomat microscope equipped with a 100$\times$, 1.3 N.A. objective and differential interference contrast optics. Organelle velocities were measured by matching calibrated cursor movements to the speed of vesicles moving in the axoplasm (Morfini et al., 2006), where a "match" between vesicle and cursor speed required agreement of two observers. With each axoplasm, a baseline measurement was taken of anterograde and retrograde fast axonal transport velocities prior to perfusion with drug. Following perfusion with drug, axonal transport velocities were collected over a 50 min period as described (Morfini et al., 2007).

With the exception of 10 μM ixabepilone, which was tested in a single axoplasm due to its strong effects, all drug concentrations were tested in 3–4 axoplasms. Velocity measurements of anterograde and retrograde fast axonal transport from each assay condition were pooled and plotted as a function of time. Curves were fitted in GraphPad Prism using an equation for one-phase exponential decay. To compare fast axonal transport among axoplasms of different experimental conditions, data collected between 30 and 50 min post perfusion were pooled (avg. $N = 26$, low $N = 4$, high $N = 38$) and then analyzed by a one-way ANOVA followed by Tukey's post-test (GraphPad Prism statistical software). All data are expressed as mean $\pm$ SEM.

2.3. Immunofluorescence microscopy-based visualization of microtubules in squid axoplasm

Squid axoplasms were extruded onto a microscope slide as described above, and a PAP pen (Sigma) was used to draw a hydrophobic oval surrounding the axoplasm. ATP-supplemented axoplasm buffer with or without drug was prepared as for vesicle motility assays, except that the total volume was increased to 30 μl to ensure that the entire axoplasm was covered. Therefore, final drug concentrations in this assay were slightly higher than in the vesicle motility assay described above. Following a 50 min incubation, the axoplasm buffer was carefully removed with a pipette and replaced with 50 μl of 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.0 (Electron Microscopy Sciences). After 1 h incubation, axoplasms were washed three times with PBS, and then blocked for 1 h in PBS + 0.1% Triton X100 (PBT) containing 1% IgG-free BSA (Jackson Immunoresearch). Axoplasms were subsequently labeled with the anti-tubulin antibody DM1A (1:500; Sigma) followed by FITC-conjugated goat anti-mouse secondary antibody (1:1000, Cappel), and then mounted using ProLong Gold antifade reagent (Invitrogen). Axoplasms were imaged at 20$\times$ magnification on a BX60 Olympus microscope equipped with a MacroFire camera (Optronics).

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