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RESEARCH REPORT

Vincristine and bortezomib cause axon outgrowth and behavioral defects in larval zebrafish

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Abstract Peripheral neuropathy is a common side effect of a number of pharmaceutical compounds, including several chemotherapy drugs. Among these are vincristine sulfate, a mitotic inhibitor used to treat a variety of leukemias, lymphomas, and other cancers, and bortezomib, a 26S proteasome inhibitor used primarily to treat relapsed multiple myeloma and mantle cell lymphoma. To gain insight into the mechanisms by which these compounds act, we tested their effects in zebrafish. Vincristine or bortezomib given during late embryonic development caused significant defects at both behavioral and cellular levels. Intriguingly, the effects of the two drugs appear to be distinct. Vincristine causes uncoordinated swimming behavior, which is coupled with a reduction in the density of sensory innervation and overall size of motor axon arbors. Bortezomib, in contrast, increases the duration and amplitude of muscle contractions associated with escape swimming, which is coupled with a preferential reduction in fine processes and branches of sensory and motor axons. These results demonstrate that zebrafish is a convenient *in vivo* assay system for screening potential pharmaceutical compounds for neurotoxic side effects, and they provide an important step toward understanding how vincristine and bortezomib cause peripheral neuropathy.

Key words: behavior, chemotherapy, development, neuropathy, proteasome inhibitor

Introduction

Peripheral neuropathies, characterized by degeneration or dysfunction of peripheral nerves, are collectively one of the most frequently reported neurological disorders (MacDonald *et al.*, 2000). Causes of peripheral neuropathy are many and include genetics, disease, trauma, and toxic chemical exposure (Dyck

and Thomas, 2005). Peripheral neuropathy can be caused by a number of pharmaceutical compounds, including many with chemotherapeutic applications (Hilkins and van den Bent, 1997; Windebank and Grisold, 2008).

Although far from being the only chemotherapy drugs to cause peripheral neuropathy, microtubule disruptors and proteasome inhibitors are of particular interest due to their high rates of neuropathic side effects. Vincristine sulfate is a microtubule polymerization inhibitor used to treat various leukemias, Hodgkin's and non-Hodgkin's lymphomas, and a number of other cancers (McLeod and Penny, 1969;

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Kempin et al., 1982). A naturally occurring compound of the Vinca alkaloid class, vincristine, disrupts mitotic spindle formation by binding α/β tubulin heterodimers, thereby competitively inhibiting their incorporation into growing microtubules (Himes et al., 1976; Haskins et al., 1981; Gigant et al., 2005). Vincristine causes a high incidence of dose-dependent peripheral neuropathy in human patients (Rosenthal and Kaufman, 1974; Legha, 1986). This is likely due to the disruption of axonal microtubule networks and the impairment of transport along these scaffolds, ultimately leading to axonal degeneration (Topp et al., 2000; Silva et al., 2006).

Bortezomib is used primarily to treat relapsed and refractory multiple myeloma (Montagut et al., 2005; San Miguel et al., 2006) and mantle cell lymphoma (Leonard et al., 2006; Kane et al., 2007). Peripheral neuropathy is the primary dose-limiting side effect of bortezomib, and like vincristine, this side effect occurs in a large fraction of patients (Cavaletti and Nobile-Orazio, 2007; Chaudhry et al., 2008; Delforge et al., 2010). The mechanism by which bortezomib induces peripheral neuropathy is unknown, but it may be due to dysregulation of calcium homeostasis, neurotrophin function, or both (Argyriou et al., 2008).

Despite their neurotoxicity, vincristine and bortezomib are important therapies. It is therefore critical to understand the mechanisms by which these compounds cause peripheral neuropathy *in vivo* with the goal of discovering ways in which these debilitating side effects can be prevented (Cavaletti and Nobile-Orazio, 2007). Zebrafish have long been a favored model system to study vertebrate development due to their small size, rapid development, ease of handling, embryonic transparency, amenability to genetic manipulation, and low maintenance costs. Increasingly, the zebrafish system has been used to study the biology of cancer (Stoletov and Klemke, 2008; Frazer et al., 2009; Smith et al., 2010) and other diseases (Berman et al., 2003; Taylor et al., 2004; Hsu et al., 2007; Kari et al., 2007). Because zebrafish embryos and larvae are permeable to many small molecules when added to normal growth medium (Khersonsky et al., 2003; Peterson et al., 2004; Murphey et al., 2006; Choi et al., 2007), they can be used to rapidly assay the effects of potential chemotherapy drugs (Mizgirev and Revskoy, 2010). We have taken advantage of these features of the zebrafish system to investigate the cellular and behavioral effects of vincristine and bortezomib during late embryonic development. Our findings demonstrate that both vincristine and bortezomib cause sensorimotor defects and that these defects are correlated with underlying changes in sensory and motor axon outgrowth and/or maintenance. The cellular and behavioral effects of these drugs appear to be distinct,

consistent with the fact that these drugs have different cellular targets.

Materials and Methods

Fish care

Zebrafish were bred and maintained using standard procedures and in accordance with Williams College animal and welfare assurance certificate A3133-01. AB* wild-type or HB9::EGFP (Flanagan-Steet et al., 2005) fish were used for all studies. AB* were obtained from the Zebrafish International Resource Center (Eugene, OR, USA), and HB9::EGFP were obtained from the laboratory of Dr. Joshua Sanes at Harvard University. Embryos were raised in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄) containing 0.00016% methylene blue and staged as equivalent hours post-fertilization (hpf) at 28.5°C (Kimmel et al., 1995).

Drug application

Vincristine sulfate was obtained from MP Biomedicals (Solon, OH, USA), and bortezomib was obtained from Chemietek (Indianapolis, IN, USA). We chose to test vincristine concentrations between 25 and 100 μ M based on studies in *Xenopus laevis* (Ubbels et al., 1983; Ton and Parnig, 2005) and bortezomib between 10 and 50 μ M based on its relative effective dose in cell culture (Montagut et al., 2005). Vincristine was dissolved in sterile, distilled water to 25 mM. This stock solution and dimethyl sulfoxide (DMSO) were diluted directly into E3 medium containing 1% DMSO. DMSO has been shown to be a safe carrier solvent in zebrafish embryos at up to 1.5% (Hallare et al., 2006), and 1% DMSO does not have any effect on cell cycle progression or motor axon outgrowth in our hands. Bortezomib was dissolved in 85% DMSO in water to a concentration of 4.16 mM. This stock was diluted directly into E3 containing 1% DMSO. Manually dechorionated 24 hpf embryos were transferred into Petri dishes containing each solution and placed at 28.5°C for 24 h. All experiments were performed on age-matched siblings. Larvae were washed three times in E3 prior to analysis.

Behavioral analysis

Each drug was washed out as described above. Ten larvae each were tested for a response to five stimuli each to the head and to the tail using a probe. To preclude any pre-stimulus modulation of the response (Burgess and Granato, 2007), we waited a minimum of 5 s between each stimulus.

Except in the case of 50 μ M bortezomib (where only two larvae mounted an escape response), the

escape responses of a minimum of five 50 hpf larvae from each sample were recorded. A touch stimulus was applied to the trunk using a dulled 0.2 mm insect pin attached to a surgical blade holder, and responses recorded using a high-speed digital camera (Fastec Imaging, San Diego, CA, USA) mounted to a 35 mm lens (Nikon, Melville, NY, USA) collecting at a rate of 500 frames per second (fps). Body bend angle, or the angle of deviation of the tail, was measured with the assistance of specialized software (McKeown et al., 2012), recorded for each frame, and plotted using MICROSOFT EXCEL. These data were used to determine body bend amplitude, frequency, and contraction velocity. Statistical tests were performed using GRAPHPAD software.

Immunohistochemistry

Forty-eight hours post-fertilization larvae were anesthetized in 0.016% tricaine and fixed in fresh 4% formaldehyde (Ted Pella, Redding, CA, USA) in phosphate buffered saline (PBS) for 2 h at room temperature. After fixation, larvae were incubated for 1–2 h in distilled water, dehydrated through a methanol series, and stored at -20°C in 100% methanol. Larvae were then either transferred to acetone for 10 min at -20°C and rehydrated through methanols, or directly rehydrated through a methanol series and treated with 0.1% collagenase in PBS for 45 min at room temperature. Larvae were incubated overnight at 4°C in one or more of the following primary antibodies: anti-phospho-histone H3 (Ser10) (H3P; Millipore, Billerica, MA, USA; 1:100), znp-1 (Developmental Studies Hybridoma Bank, Iowa City, IA, USA; 1:100), anti-acetylated tubulin (AT; Sigma, St. Louis, MO, USA; 1:800), or F59 (Developmental Studies Hybridoma Bank; 1:100). After washing, larvae were incubated overnight at 4°C in one or more of the following secondary antibodies: Alexa 633 goat-anti-rabbit for H3P (Invitrogen, Grand Island, NY, USA; 1:200), horseradish peroxidase (HRP)-conjugated goat-anti-mouse for znp-1 (Invitrogen; 1:200), and Alexa 488 goat-anti-mouse for AT and F59 (Invitrogen; 1:200). After washing, HRP was visualized using the Alexa 546 Tyramide Signal Amplification kit (Invitrogen). Stained larvae were then transferred to glycerol and mounted using Vectashield (Vector Labs, Burlingame, CA, USA).

Image capture and image analysis

Image stacks were obtained using a Zeiss Axiolmager epifluorescent compound microscope with ApoTome (AT and F59) or a Nikon C1 laser scanning confocal microscope (H3P and znp-1) equipped with $\times 20$ air (N.A. 1.4), $\times 40$ oil (N.A. 1.3), or $\times 60$ water (N.A. 2.0) objectives. For quantitation of H3P-positive cells,

images were segmented, and number of particles was determined automatically using AUTOQUANT software (Media Cybernetics, Bethesda, MD, USA). Means were derived from a minimum of three samples per concentration. Sensory neuron processes were quantified in IMAGE J using a previously published method (Grider et al., 2006). Briefly, 8-bit images were converted into binary images to calculate pixel density. Total pixel number for each image was recorded and used for statistical analysis. For axon arbor surface areas, individual axonal arbors were manually isolated and cropped. Images were then segmented, and AUTOQUANT software was used to automatically determine the total surface area of staining. Means were derived from a minimum of three arbors from a minimum of three different samples. Fisher's exact tests were performed using GRAPHPAD software. ANOVA, Mann-Whitney *U*-tests, and Fisher's exact tests were carried out using the calculators at <http://faculty.vassar.edu/lowry/VassarStats.html>.

Results

Vincristine and bortezomib cause an increase in M-phase cells

To establish that vincristine and bortezomib suppress cellular proliferation in zebrafish larvae, we stained for phosphorylated (ser10) histone H3 (H3P), suggestive of M-phase arrest (Murphey et al., 2006). Vincristine caused a statistically significant dose-dependent increase in the number of H3P-positive cells (Figs. 1A–D and 1E) ($p < 0.0001$, one-way ANOVA). These effects were statistically significant at all doses (Figs. 1A'–C'). No increase in mortality was observed with any dose of vincristine, although larvae treated with 100 μM vincristine began to exhibit morphological defects, primarily ventral curvature of the trunk and tail (Fig. 1D').

Bortezomib caused a small, but statistically significant, increase in H3P-positive cells suggestive of M-phase arrest (Figs. 2A–D and 2E) ($p < 0.05$, one-way ANOVA). Unlike with vincristine, H3P staining in bortezomib-treated larvae was not evenly dispersed throughout the embryo but was concentrated in the dorsal aorta, caudal vein, or both, suggesting that vascular or hematopoietic cells may be disproportionately affected. The magnitude of arrest was much less than that for vincristine, but due to the fact that H3P-positive cells were so densely packed in larvae treated with 50 μM bortezomib, the extent of cell cycle arrest was likely underestimated due to limitations in the method of quantitation. No increase in mortality was observed with bortezomib, although some morphological defects, such as dorsal tail curvature

Figure 1. Vincristine effects on cell cycle progression. (A–D) Lateral views of the trunk at 48 hours post-fertilization (hpf). Area shown in (A–D) is represented by boxed area in (A'). (A) Control staining for histone H3 phosphate (H3P), an indicator of M-phase arrest. (B–D) Vincristine causes a dose-dependent increase in the number of H3P-positive cells ($p < 0.0001$, one-way ANOVA). (A'–D') Images of live 48 hpf larvae. (A') Control larva. (B' and C') Morphology is normal in larvae treated with 25–50 μM vincristine. (D') One hundred micromolar vincristine causes some ventral curvature of the axis. p Values: pair-wise Student's *t*-tests with Bonferroni correction. Scale bars = 200 μm.

and slight developmental delays, were observed at 25 and 50 μM (Figs. 2A'–D').

Vincristine and bortezomib reduce touch responsiveness

Because the primary dose-limiting side effect of both vincristine and bortezomib is sensory neuropathy, we tested their effects on touch responsiveness. The touch response in larval zebrafish relies upon stimulation of either the trigeminal sensory neurons in the head or Rohon-Beard cells in the trunk and tail (Clarke et al., 1984; Sagasti et al., 2005). To test whether these drugs might differentially affect trigeminal and Rohon-Beard sensory neurons, larvae were scored for responses to stimuli to either head or tail. Vincristine caused a dose-dependent decrease in the number of larvae that responded to stimuli to both head and tail (Table 1). The effect of vincristine was statistically significant for both head and tail stimuli ($p = 0.016$ and 0.005 , respectively, Fisher's exact test) at 100 μM. There was no significant difference between responses to head and tail stimuli at any

dose, suggesting that vincristine impairs trigeminal and Rohon-Beard neurons similarly.

Bortezomib also impaired responses to touch in a dose-dependent manner. At 50 μM, the highest dose tested, only 3 of 10 larvae responded to all five stimuli to the head, and only 5 of 10 responded to all five stimuli to the tail (Table 1). The effect on response to head stimulation was statistically significant at 50 μM. While its effect on the responses to head stimulus was statistically significant at 50 μM ($p = 0.01$, Fisher's exact test) and its effect on responses to tail stimulus was not, the difference between the two effects was not sufficient to suggest that bortezomib affects trigeminal neurons any differently than Rohon-Beard neurons.

Vincristine and bortezomib disrupt escape swimming behavior

Because vincristine and bortezomib are also known to cause motor neuropathy in mammalian systems, we examined the effects of vincristine or bortezomib on swimming behavior associated with the escape response. Larval escape response is

Figure 2. Bortezomib effects on cell cycle progression. (A–D) Lateral views of the trunk at 48 hours post-fertilization (hpf). Areas shown represented by boxed area in (A'). (A) Control H3P staining. (B–D) Bortezomib causes a dose-dependent increase in the number of H3P-positive cells ($p < 0.05$, one-way ANOVA). The H3P-positive cells appear to be concentrated in the dorsal aorta and/or caudal vein (brackets in (D)). (A'–D') Images of live 48 hpf larvae. (A') Control larva. (B') Morphology is completely normal in larvae treated with 10 μM bortezomib. (C') Twenty-five micromolar bortezomib-treated larvae occasionally show subtle defects, including dorsal curvature of the axis. (D') Fifty micromolar bortezomib causes some curvature of the axis in all larvae. These larvae also appear to be developmentally delayed. Scale bars = 200 μm.

Table 1. Summary of touch responses in drug-treated zebrafish larvae at 48 hpf.

Drug	Number with 100% response*		Mean number of responses ± SEM†	
	Head	Tail	Head	Tail
Vincristine (μM)				
0	10/10	10/10	5.0 ± 0.00	5.0 ± 0.00
50	8/10	7/10	4.6 ± 0.31	4.5 ± 0.31
100	5/10 ($p = 0.016$)‡	4/10 ($p = 0.005$)‡	3.9 ± 0.41 ($p = 0.03$)§	4.2 ± 0.25 ($p = 0.01$)§
Bortezomib (μM)				
0	9/10	9/10	4.9 ± 0.10	4.9 ± 0.10
25	7/10	5/10	4.7 ± 0.13	3.4 ± 0.64
50	3/10 ($p = 0.01$)‡	5/10 (N.S.)‡	3.8 ± 0.33 ($p = 0.01$)§	3.6 ± 0.54 (N.S.)§

hpf, hours post-fertilization; N.S., not statistically significant; SEM, standard error of the mean.

*Ten larvae for each condition were stimulated five times on the head and five times on the tail, and the presence or absence of a response was recorded.

†Five is the maximum possible.

‡Fisher's exact test.

§Mann-Whitney *U*-test.

often initiated in response to sensory input; however, sensation is not a prerequisite for escape swimming (Granato et al., 1996; Ribera and Nusslein-Volhard, 1998), so any changes observed may be attributable

to effects on motor circuitry. At 2 dpf, control larvae exhibited classic escape responses, consisting of one or two rapid, large-amplitude body bends ("C-bends") away from the stimulus, a counter bend

Figure 3. Vincristine and bortezomib effects on escape swimming behavior. Select frames from movies taken at 500 fps. Times in milliseconds. (A) Normal larva exhibits one strong C-bend (20 ms) followed by smaller amplitude curls, which propel it away from the site of stimulation. Note that frames 120, 140, 160, and 180 represent successive curls to the same side, indicating that the period of coiling is approximately 20 ms. (B) Fifty micromolar vincristine-treated larva exhibits a single C-bend (20 ms) followed by a pause and then uncoordinated coiling that fails to propel the larva away from the site of stimulus. (C) Twenty-five micromolar bortezomib-treated larva exhibits continuous high-angle C-bend-like contractions that fail to propel it away from the site of stimulus.

for reorientation, then lower-amplitude undulations that propel the larva away from the stimulus (Figs. 3A and 4A; Movie S1). Larvae treated with 50 μ M vincristine usually exhibited a typical C-bend; however, these were followed by irregular, lower-amplitude body bends that generally failed to propel larvae away from the stimulus (Figs. 3B and 4B; Movie S2). Larvae treated with 100 μ M vincristine also typically performed C-bends at the outset of their response, but these were followed by highly irregular, sometimes

unilateral, body bends. In addition, the frequency of undulations was less than controls (Fig. 4C).

Bortezomib-treated larvae also demonstrated abnormal escape responses. Larvae treated with 25 μ M bortezomib responded to touch with repetitive, high-amplitude C-bends. These high-amplitude bends persisted until the termination of the response, rather than transitioning to lower-amplitude undulations, and they usually failed to propel the larvae away from the stimulus (Figs. 3C and 4D; Movie S3). The mean

Figure 4. Kinematic analysis of escape behavior. Body bend angle, the angle of deviation of the tail from a neutral position, is plotted for each treatment as a function of time. Different larvae are indicated by differently colored traces. (A) Normal larvae typically exhibit one or two high-amplitude “C-bends,” characterized by a body bend angle of 150° or more. The initial C-bends are followed by rapid, low-amplitude bends of approximately 55° . (B) Larvae treated with $50\ \mu\text{M}$ vincristine typically exhibited normal C-bends, followed by uncoordinated contractions, some of which were increased in amplitude relative to controls (e.g., orange trace) and others that were increased in duration (e.g., green trace) relative to controls. (C) Larvae treated with $100\ \mu\text{M}$ vincristine exhibited normal C-bends followed by uncoordinated contractions. In one case, the contractions were strictly unilateral (green trace). (D) The mean tail curl amplitude of larvae treated with $100\ \mu\text{M}$ vincristine was increased relative to controls, but this difference was not significant ($p = 0.15$, Mann-Whitney *U*-test). (E) Larvae treated with $25\ \mu\text{M}$ bortezomib exhibited continuous high-amplitude C-bend-like contractions. (F) The contractions were significantly higher in amplitude than propulsive body bends exhibited by control larvae ($p = 0.007$, Mann-Whitney *U*-test).

body bend amplitude during the “swimming” phase of the response was significantly greater than controls (Fig. 4F; $p = 0.007$, Mann-Whitney *U*-test). While the contraction amplitude was significantly increased, the contraction velocity was decreased (compare slopes of traces in Figs. 4A and 4E).

At $50\ \mu\text{M}$ bortezomib, the highest dose tested, larvae were generally unable to respond to stimulus. The two larvae that exhibited any sort of response were

so abnormal as to preclude meaningful quantitative analysis (data not shown).

Vincristine and bortezomib reduce sensory innervation density

Because vincristine- and bortezomib-treated larvae displayed reduced touch responses, we examined sensory axon projections in larvae treated with either drug. In this experiment, we observed significant

Figure 5. Vincristine and bortezomib effects on sensory axon density. (A–H) Lateral views of 48 hours post-fertilization larvae stained with anti-acetylated tubulin, which labels all axons. Only the sensory axons innervating the skin were imaged. (A) Imaged area is indicated by box. (E) Control for both vincristine and bortezomib treatments. (I) Taken together, the treatments caused a statistically significant alteration in sensory axon density ($p < 0.0001$ one-way ANOVA for all treatments). At 100 μM , the effect of vincristine was statistically significant ($p = 0.0059$, Bonferroni post-hoc), as was the effect of bortezomib at 50 μM ($p = 0.0015$, Bonferroni post-hoc). (J) Both vincristine and bortezomib showed apparent dose-dependent effects on development of the lateral line. Scale bar = 50 μm .

reductions in the density of sensory axons in the skin ($p < 0.0001$, one-way ANOVA for experiment). Individually, the effects of 100 μM vincristine (Figs. 5A–D and 5H) and 50 μM bortezomib (Figs. 5E–H and 5I) were statistically significant ($p = 0.0059$ and 0.0015, respectively). Bortezomib seemed to have a greater effect on fine processes than on large-diameter axons (Fig. 5H). Both drugs also caused a dose-dependent reduction and, in some cases, loss of the lateral line (Figs. 5D, 5H, and 5J).

As migration of the lateral line is frequently used as a marker of larval development (Kimmel et al., 1995), reduction/loss of the lateral line could indicate a developmental delay. However, we observed little, if any, concordance between reduction in the lateral line and sensory innervation density, suggesting that a developmental delay does not explain the observed decrease in sensory innervation density.

It should be noted that the effects of both drugs on sensory axons was quite variable, and the morphologies observed for the highest dose of each ranged from completely normal to severely disrupted (Fig. S1).

Vincristine and bortezomib reduce motor axon arbors

Because treated larvae also exhibited abnormal escape behaviors, we analyzed motor axon morphology in treated larvae. For this analysis, we focused on the arbors of caudal primary (CaP) neurons, which extend ventrally and medially out of the spinal cord to the ventral-most axial muscle, then turn laterally and dorsally to innervate the exterior portion of the muscle. The main trunks of these primary motor axons are established by 24 hpf but continue to grow and, importantly, develop branches between 24 and 48 hpf

Figure 6. Vincristine and bortezomib effects on motor axon morphology. (A–H) High-magnification lateral views of 48 hours post-fertilization larvae stained with znp-1, which labels motor neurons and their axons. The CaP motor axon extends ventrally from the spinal cord and hooks around the ventral edge of the muscle to project dorsally and laterally to the outside of the axial muscle. (A) Control motor axon staining for vincristine treatment. (B–D) Vincristine treatment significantly decreased the surface area of motor axon arbors ($p < 0.0001$, one-way ANOVA). (I) This effect was individually significant at all doses (p values indicated on graph). (E) Control staining for bortezomib treatment. (F–H) Bortezomib significantly decreased motor axon arbor area ($p = 0.0006$, one-way ANOVA). (J) This effect was individually significant only at 50 μM (p value indicated on graph). Interestingly, bortezomib appeared to decrease the area of branches, while the main axon trunk remained intact (e.g., arrowhead in (H)). p Values: pair-wise Student's t -tests with Bonferroni correction. Scale bar = 50 μm .

(Myers, 1985; Myers et al., 1986). Vincristine caused a statistically significant, dose-dependent reduction in the areas of motor axon arbors ($p < 0.0001$, one-way ANOVA) (Figs. 6A–D and 6I). Vincristine appeared to alter the integrity of both the main trunk of the axon and branches but did not have any effect on the distance traveled by the leading edge of the axon, suggesting that the effect of vincristine was primarily on axons, not due to a developmental delay. This finding suggests that vincristine may affect axon outgrowth, maintenance, or both.

Bortezomib also caused a statistically significant, dose-dependent decrease in the areas of motor axon arbors ($p = 0.0006$, ANOVA) (Figs. 6E–H and 6J). Interestingly, bortezomib seemed to affect branches

to a greater extent than the primary axon trunk, similar to its effect on sensory axons (Fig. 6H).

Effects of vincristine and bortezomib on muscle morphology

Because defects in muscle structure and function could generate some of the neural and behavioral abnormalities caused by vincristine or bortezomib, we also examined axial muscle morphology in larvae treated with the intermediate and highest doses of each drug. At intermediate doses (50 μM vincristine and 25 μM bortezomib), the axial muscles appear normal (Figs. 7A, 7B, 7D, and 7E). At the highest dose of each drug (100 μM vincristine and 50 μM bortezomib), however, individual fibers begin to separate

Figure 7. Vincristine and bortezomib effects on muscle morphology. Lateral views of 48 hours post-fertilization larvae stained for F59, slow muscle myosin. (A) Muscle morphology in control larva for vincristine treatment. (B) Muscle morphology appears normal at 50 μ M vincristine. (C) Muscle begins to look disorganized at 100 μ M vincristine. (D) Control larva for bortezomib treatment. (E) Muscle morphology appears normal at 25 μ M bortezomib. (F) Muscle begins to look disorganized at 50 μ M bortezomib. Black spots in (C) and (F) are pigment-containing melanophores in the overlying skin.

from one another, resulting in subtle disorganization of the myotome (Fig. 7C and 7F).

Discussion

The primary dose-limiting side effect of several important chemotherapeutic compounds, including vincristine and bortezomib, is peripheral neuropathy. A high priority has therefore been placed on developing ways to minimize the neuropathic side effects while retaining the therapeutic properties of these drugs (Rosenthal and Kaufman, 1974; Cavaletti and Nobile-Orazio, 2007). To achieve this goal, it is essential to establish model systems that can be used to help elucidate the mechanisms by which these compounds exert their effects *in vivo*. The zebrafish system has been shown to be a useful model for a number of neurological disorders (Oprea et al., 2008; Boon et al., 2009; Kabashi et al., 2011), and vincristine has recently been shown to be effective in a zebrafish model of acute lymphoblastic leukemia (Mizgirev and Revskoy, 2010). The work described here demonstrates that zebrafish can be used to study the neurotoxic effects of chemotherapy drugs. Although we focus on late embryonic/early larval development, the cell biological mechanisms by which these compounds disrupt axon outgrowth and cause peripheral neuropathy may be similar. Indeed, vincristine has been demonstrated to disrupt both assembly and stability of microtubules (Himes et al., 1976), and our research has shown that it affects both nascent and established zebrafish axons equally (N.Benaich, unpublished data). Although

the variability in our sensory axon data suggests that this particular assay may not lend itself to large-scale screens, any of the other assays employed here could certainly be used.

Not surprisingly, both vincristine and bortezomib impaired touch responses in zebrafish, consistent with the side effects of these drugs in humans, which frequently includes sensory defects (Bradley et al., 1970; Richardson et al., 2003). The reduction in sensory innervation that we observed was similar in magnitude and may account for the reduced touch responses. What is perhaps surprising is that the sensory effects of these drugs were not even greater given the fact that sensory impairment in humans is the most common side effect of these drugs. This difference may be due to differences between the physiology of zebrafish and humans or it may simply reflect differences between our assay systems.

Although their effects on the touch response were similar, the effects of vincristine and bortezomib on escape swimming differed in that vincristine resulted in uncoordinated swimming, while bortezomib caused an increase in the amplitude of contractions. The discoordination caused by vincristine is consistent with broad disruption of neural pathways that coordinate this behavior. It is interesting to note that although these larvae are uncoordinated, the left–right alternating pattern of muscle contractions remains largely intact, suggesting that defects in pathways that govern reciprocal inhibition (Saint-Amant and Drapeau, 1998; Drapeau et al., 2002; Brustein et al., 2003; Buss et al., 2003) do not primarily underlie the lack of coordination.

By contrast, the repetitive, high-amplitude body bends caused by bortezomib appear to result from a failure of muscle contraction to propagate smoothly down the body axis; rather, all of the muscles along one side of the body appear to contract simultaneously, although the left–right alternation of the contractions appears again unaffected. This unusual behavior could be caused by any number of mechanisms, but it is worth noting that it is similar to that observed for the *techno trousers* mutant, a loss-of-function mutant in the glial glutamate transporter *slc1a2b/EAAT2* (K. A. McKeown and G. B. Downes, unpublished data).

Differences in behaviors caused by the two drugs are undoubtedly related to differences in their biochemical mechanisms. Vincristine disrupts microtubules, so it is therefore not surprising that it causes defects in sensory and motor (and possibly other) axons and subsequent discoordination of the escape response. Because inhibiting the proteasome likely alters many cellular pathways, the mechanisms by which bortezomib affects axons is less clear. One of the mechanisms by which it suppresses cancer progression is through enhanced inhibition of the anti-apoptotic transcription factor NF- κ B (Hideshima et al., 2002). However, the specific mechanisms underlying its anti-proliferative and neurotoxic effects are not well understood (Argyriou et al., 2008). Recent studies have shown how the proteome of cancer cell lines is altered by bortezomib (Chauhan et al., 2008; Loeffler-Ragg et al., 2009); a complementary approach could be useful to discover how the neuronal proteome is altered in order to better understand the mechanisms of bortezomib-induced peripheral neuropathy. On the other hand, although bortezomib is reportedly quite specific for the proteasome (Lightcap et al., 2000), recent *in vitro* studies and patient trials have shown that the novel proteasome inhibitor carfilzomib does not cause neurotoxicity (Arastu-Kapur et al., 2011; Khan and Stewart, 2011), suggesting that bortezomib's neurotoxic side effects may be independent of its effects on the proteasome. It remains to be seen whether carfilzomib or other proteasome inhibitors act similarly in the zebrafish system.

It is conceivable that the effects of vincristine or bortezomib on behavior are due to generalized toxicity to the embryo. However, the ranges of vincristine (25–100 μ M) and bortezomib (10–50 μ M) used here were not substantially different from the range of concentrations (10–33 μ M) typically used for large-scale small-molecule screens in the zebrafish (Kaufman et al., 2009; Rihel et al., 2010), and they are at least an order of magnitude lower than the concentrations of known compounds used in a recent screen for cell cycle inhibitors (Murphey et al., 2006). Furthermore, generalized toxicity would be expected

to result in impaired growth of the embryo; yet nearly all treated embryos were normal in size except at the highest dose of each drug. This, coupled with our observation that treated larvae appear morphologically indistinguishable from controls at doses at which behavioral and cellular phenotypes are observed, again suggests against general toxicity as a mechanism for the observed defects.

Alternatively, the axon defects we observed could be indirectly due to effects on other tissues, independent of toxicity. For example, the axon degeneration observed in hereditary peripheral neuropathies is commonly secondary to myelin defects (Berger et al., 2002). In addition, the defects in sensory (Paulus et al., 2009) and/or motor (Lefebvre et al., 2004) axons seen at the highest doses of each drug could be secondary to the effects on muscle. (Conversely, the observed muscle fiber disorganization could be a consequence of failure of axons to properly innervate muscle.) We also cannot rule out the possibility that the effects of the drugs are due, at least in part, to changes in neuronal physiology independent of the increase in excitability that results from increased sodium conductance. These possibilities are all worth testing.

Many aspects of the mechanisms of cancer development (Langenau and Zon, 2005; Yee and Pack, 2005; Feitsma and Cuppen, 2008; Stoletov and Klemke, 2008; Marques et al., 2009), cancer treatment (Mizgirev and Revskoy, 2010), neuronal development (Hutson and Chien, 2002), and neuromuscular disease (Boon et al., 2009) are conserved between humans and zebrafish. The results described here demonstrate that two important chemotherapy drugs, vincristine and bortezomib, cause neurodevelopmental defects in zebrafish larvae. As the mechanisms underlying these defects are likely to share features with peripheral neuropathy in human patients, these results provide a foundation for further analysis of the effects of these and other drugs using zebrafish as a model system.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Movie S1. Normal zebrafish larval escape response. High-speed video recording of a typical control larva at approximately 50 hours post-fertilization exhibiting escape response to touch. Larva responds to touch with one or two high-amplitude body bends (C-bends), followed by low-amplitude left–right alternating contractions that propel the larva away from the site of stimulus. Video recorded at 500 frames per second (fps) and displayed at 30 fps, or approximately $\times 17$ slower than real time.

Movie S2. Escape response of vincristine-treated larva. High-speed video recording of a typical larva treated with 50 μM vincristine. Larva responds to touch with an initial C-bend, but this is followed by irregular, uncoordinated contractions. Video recorded at 500 frames per second (fps) and displayed at 30 fps, or approximately $\times 17$ slower than real time.

Movie S3. Escape response of bortezomib-treated larva. High-speed video recording of a typical larva treated with 25 μM bortezomib. The larva responds to touch by performing continuous C-bend-like contractions. Video recorded at 500 frames per second (fps) and displayed at 30 fps, or approximately $\times 17$ slower than real time.

Figure S1. Variations in sensory axon density. Lateral views illustrating the range of sensory axon phenotypes within each treatment group. “High density” and “low density” refer to samples within each treatment group with the highest and lowest innervation density, respectively. “Reduced lateral line” shows an example within treatment group with reduced or absent lateral line. Note that overall density of sensory axons is often normal in larvae lacking the lateral line (E, H, K). VCR, vincristine. Bort, bortezomib.

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