Paclitaxel-induced epithelial damage and ectopic MMP-13 expression promotes neurotoxicity in zebrafish

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Paclitaxel is a microtubule-stabilizing chemotherapeutic agent that is widely used in cancer treatment and in a number of curative and palliative regimens. Despite its beneficial effects on cancer, paclitaxel also damages healthy tissues, most prominently the peripheral sensory nervous system. The mechanisms leading to paclitaxel-induced peripheral neuropathy remain elusive, and therapies that prevent or alleviate this condition are not available. We established a zebrafish in vivo model to study the underlying mechanisms and to identify pharmacological agents that may be developed into therapeutics. Both adult and larval zebrafish displayed signs of paclitaxel neurotoxicity, including sensory axon degeneration and the loss of touch response in the distal caudal fin. Intriguingly, studies in zebrafish larvae showed that paclitaxel rapidly promotes epithelial damage and decreased mechanical stress resistance of the skin before induction of axon degeneration. Moreover, injured paclitaxel-treated zebrafish skin and scratch-wounded human keratinocytes (HEK001) display reduced healing capacity. Epithelial damage correlated with rapid accumulation of fluorescent-conjugated paclitaxel in epidermal basal keratinocytes, but not axons, and up-regulation of matrix-metalloproteinase 13 (MMP-13, collagenase 3) in the skin. Pharmacological inhibition of MMP-13, in contrast, largely rescued paclitaxel-induced epithelial damage and neurotoxicity, whereas MMP-13 overexpression in zebrafish embryos rendered the skin vulnerable to injury under mechanical stress conditions. Thus, our studies provide evidence that the epidermis plays a critical role in this condition, and we provide a previously unidentified candidate for therapeutic interventions.

MMP-13 | degeneration | regeneration | Taxol | epidermis

Paclitaxel is a widely used chemotherapeutic agent in the treatment of cancer. Although paclitaxel arrests tumor growth through stabilizing microtubules, it also causes variable peripheral neuropathy in patients. A lack of understanding of the underlying mechanisms hinders therapeutic discovery, and commonly used mammalian models have not provided conclusive evidence about the etiology of this condition. To overcome this, we developed a larval zebrafish model that permits the analysis of paclitaxel neurotoxicity in living animals. This study identifies that keratinocyte damage and ectopic expression of matrix-metalloproteinase 13 (MMP-13) contributes to paclitaxel-induced peripheral neuropathy in zebrafish. We further show that inhibition of MMP-13 improves skin defects and prevents paclitaxel neurotoxicity. Thus, this study offers a previously unidentified avenue for potential therapeutic interventions.

Significance

Paclitaxel is a widely used chemotherapeutic agent in the treatment of cancer. Although paclitaxel arrests tumor growth through stabilizing microtubules, it also causes variable peripheral neuropathy in patients. A lack of understanding of the underlying mechanisms hinders therapeutic discovery, and commonly used mammalian models have not provided conclusive evidence about the etiology of this condition. To overcome this, we developed a larval zebrafish model that permits the analysis of paclitaxel neurotoxicity in living animals. This study identifies that keratinocyte damage and ectopic expression of matrix-metalloproteinase 13 (MMP-13) contributes to paclitaxel-induced peripheral neuropathy in zebrafish. We further show that inhibition of MMP-13 improves skin defects and prevents paclitaxel neurotoxicity. Thus, this study offers a previously unidentified avenue for potential therapeutic interventions.

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and cell culture (17). Therefore, perturbations of the skin environment by paclitaxel treatment could promote axon degeneration, yet no studies to date have examined this possibility. We have established a zebrafish in vivo model to study paclitaxel’s neurotoxic effects in live animals. These studies demonstrate that paclitaxel promotes epidermal damage and neurotoxicity and induces keratinocyte-specific up-regulation of matrix-metalloproteinase 13 (MMP-13, collagenase 3). Pharmacological inhibition of MMP-13 rescues paclitaxel-induced neurotoxicity, making it a previously unidentified therapeutic candidate.

**Results**

**Paclitaxel Induces Neurotoxicity in the Zebrafish Caudal Fin.** To assess peripheral neuropathy in adult zebrafish, we administered up to 0.133 mg/kg paclitaxel in DMSO by i.p. injections on 4 consecutive days. Because paclitaxel preferentially affects the distal extremities in mammals, we analyzed the equivalent distal caudal fin in zebrafish. Immunofluorescence staining 1 d after the last injection (day 4) using anti-acetylated tubulin (Fig. 1 B and C and Movies S1 and S2) and Neurofilament 160 (Fig. S1) antibodies revealed a selective loss of fine cutaneous fibers and axons projecting along the bony rays within the distal, but not proximal, fin regions. Three distinct neuronal populations innervate the caudal fin. DRG axons project into the distal fin, whereas motor axons innervate neuromasts along the bony rays. Because primarily fine cutaneous axons were lost in the distal-most fin region, we conclude that paclitaxel treatment primarily affects DRG axons. To further corroborate this, we also examined temporal changes in the touch response, which we expected to be attenuated if cutaneous axons are lost (Fig. 1A). This showed that paclitaxel-treated animals needed significantly more stimulations at the distal fin before a twitching response was evoked compared with controls (Fig. 1D). We next determined the effects of paclitaxel on swimming behavior, given that in mammals high doses of paclitaxel have been associated with motor deficits. Using an automated tracking device, we measured daily 1-h swimming distances, which did not show significant differences (Fig. 1E). These findings indicate that paclitaxel specifically damages DRG axons within the distal caudal fin.

We next investigated axon degeneration in zebrafish larvae using in vivo imaging to obtain a higher temporal resolution. In larval fish [up to 26 dpf postfertilization (dpf)], the skin consists of two layers: the superficial periderm of ectodermal origin and the epidermal basal cell layer (18, 19). The epidermis is separated from the underlying rudimentary dermis by a basement membrane. DRG neurons are not functional until ~4 wk when the epidermis stratifies, and initially axons of unmyelinated Rohon-beard (RB) neurons with analogy to mammalian C-fibers (20) innervate the skin and arborize between both layers. To assess RB axon degeneration in transgenic Tg(isl2b:GFP) (21) (Fig. 2B) larvae with fluorescently labeled sensory neurons, we incubated them in 22 μM paclitaxel starting at 2 dpf. This showed that a 96-h treatment (2–6 dpf) resulted in significant axon degeneration (Fig. 2 A and C–E). Incubated larvae had a slightly decreased caudal fin diameter (Fig. S2), which was not caused by increased apoptosis (Fig. S3), suggesting that paclitaxel slows developmental growth. We next assessed the touch response (Fig. 2F), which was significantly reduced starting 1 d after treatment began. In contrast, no defects were seen in locomotor activity (Fig. 2G). We next analyzed paclitaxel-dependent axon damage following injections into the cardinal vein once daily on 3 consecutive days using 10 μM paclitaxel (Fig. 2H), as this concentration has also been used in various mammalian models (22). Microinjections similarly induced axon degeneration (Fig. 2I) and reduced touch sensitivity (Fig. 2J). Both were prominent after the last injection and rapidly recovered thereafter. It is noteworthy that at 11 dpf both control and paclitaxel-injected larvae harbored fewer axon branches, likely due to the onset of programmed RB neuron death (23). Collectively, these findings show that paclitaxel also induces RB neurotoxicity in larval fish without affecting locomotor activity.

**Paclitaxel Damages the Fin Epithelium Before Onset of Axon Degeneration.** We noticed that the morphology of the caudal fin-fold in paclitaxel-injected larvae was altered as early as 1 h after injection (Fig. 3 A–C and Fig. S4). Caudal fins had a disheveled appearance and were often injured due to mechanical stress during handling of larvae (Fig. 3D). Scanning electron microscopy (SEM) showed an increased number of microtears in the distal caudal fin following 3 h of paclitaxel treatment (Fig. 3 E and F). This phenotype worsened in larvae treated with paclitaxel for 96 h, evident by delamination of keratinocytes from both layers and exposure of collagen-rich actinotrichia in the mesenchyme beneath. Also the adult skin displayed paclitaxel-induced morphological changes assessed with a green fluorescent ceramide membrane stain. The cells appeared disorganized and rounded compared with the cuboidal shape of control cells (Fig. S5). These findings indicate that paclitaxel damages the skin epithelium, making the skin less resistant to mechanical stress and prone to injury.

To further investigate the role of mechanical stress in paclitaxel-induced epithelial damage, we assessed the formation of...
reactive oxygen species (ROS) in the caudal fin of mechanically stressed animals using a H$_2$O$_2$-selective sensor. Three-hour paclitaxel treatment followed by gentle pipetting led to more widespread ROS/H$_2$O$_2$ formation compared with control animals (Fig. 3G). Intriguingly, adjacent wounds remained devoid of ROS/H$_2$O$_2$, suggesting that stress-related ROS formation may be regulated by different mechanisms than injury-induced ROS. Given the stress responses, we next examined the NF-kB stress response pathway in a transgenic Tg(NF-kB:EGFP) reporter strain (24), which shows NF-kB activation in keratinocytes (Fig. S6). NF-kB was activated in keratinocytes by paclitaxel but not vehicle treatment under both unstressed and mechanically stressed conditions (Fig. 3H and I). Because NF-kB is known to be regulated by H$_2$O$_2$ (25), we also assessed the relationship between NF-kB activity and ROS/H$_2$O$_2$ formation with the superoxide scavenger dihydroliphenyliodion (DP1) and apocynin, a bona fide NOX inhibitor, both of which attenuated NF-kB activity (Fig. 3F). These findings suggest that NF-kB activation in keratinocytes is in part mediated by paclitaxel-induced oxidative stress.

The rapid phenotypic changes in the larval caudal fin suggested that the epithelium might be more susceptible to paclitaxel-induced damage than RB neurons. To test this, we tracked paclitaxel accumulation in the fin epithelium and in RB neurons of transgenic Tg(CREST3:tdTomato) larvae during 12-h time-lapse recordings (Fig. 3J–L) using tubulin tracker, a paclitaxel conjugate to Oregon Green 488, which selectively binds to microtubules with high affinity (K$_d$ ~ 10$^{-4}$ M) and which fluoresces upon cleavage by intracellular esterases. Following normalization, we observed a transient fluorescence increase in the caudal fin within 3 h (Fig. 3J and K), whereas neuronal fluorescence peaked around 5–8 h and was only present in some, but not all, RB neurons (Fig. 3L). To further determine whether tubulin tracker within the caudal fin accumulated in keratinocytes and/or RB axons, we performed colocalization studies in mice either transiently injected with CREST3:tdTomato to label axons in red or animals transgenic for basal keratinocyte-specific dsRed expression [Tg(p63:dsRed)]. Although we did not detect tubulin tracker in axons up to 12 h following injections (Fig. 3M and Movie S3), we found its rapid accumulation in basal keratinocytes (Fig. 3N and Movie S4). Interestingly, only basal but not periderm cells showed tubulin tracker accumulation. Together, these findings indicate that basal keratinocytes are more susceptible to paclitaxel accumulation compared with RB neurons and their cutaneous axons.

Paclitaxel Impairs Cutaneous Axon Regeneration. We previously demonstrated that epithelial keratinocytes stimulate cutaneous axon regeneration through release of H$_2$O$_2$ into the wound environment (15), and our observations showed that H$_2$O$_2$ production is impaired in wounds of paclitaxel-treated larvae (Fig. 3G). We therefore hypothesized that axon regeneration might be impaired, possibly due to perturbed keratinocyte function. To assess this, we first tracked the mean growth of single-labeled RB axons for 12 h following caudal fin amputation during which axons remained in vehicle or paclitaxel solution (Fig. 4A–C). This showed that paclitaxel significantly impaired axon regeneration (Movies S5 and S6). We wanted to further analyze how paclitaxel influences growth cone behavior, as the growth cone core domain of regenerating axons is rich in dynamically unstable microtubules that allow growth and shrinkage of axons (27), which could be stabilized by paclitaxel. Such growth and retraction behavior is also characteristic for RB axons (15). Quantification of total growth and retraction over the course of 12 h revealed that paclitaxel attenuated, but did not abolish, this process (Fig. 4D). Because lack of growth could also relate to defects mediated within the epidermis due to keratinocyte damage, we next assessed paclitaxel’s effects on Wallerian degeneration (WD), for which it was shown in Drosophila and zebrafish that axon debris clearance depends on keratinocytes acting as “nonprofessional” phagocytes (28, 29). Similar to mammals, zebrafish cutaneous axons degenerate by WD when severed (30), a process that is defined by a lag phase during which the severed axons remain intact, an axon fragmentation phase, and a clearance phase during which axon debris is phagocytosed. Paclitaxel did not interfere with the ability of axons to fragment; however, the duration of clearance was altered. The time between fragmentation onset of individual axon branches and complete clearance of axon debris was twice as long for paclitaxel-treated compared with vehicle-treated controls (Fig. 4 A, B, and E). These findings suggest that paclitaxel may exert its effects on axon regeneration through damaging keratinocytes.

MMP-13 Inhibition Partially Rescues Impaired Axon Regeneration. We exploited our zebrafish model to screen for chemical compounds that can restore impaired axon regeneration and debris clearance in the presence of paclitaxel. We used preselected compounds targeting proteins of genes that we found were differentially
regulated in H$_2$O$_2$-treated larval zebrafish following RNAseq analysis (Fig. 4F) (31). Each compound was coadministered with paclitaxel for 12 h during time-lapse recordings. This screen identified one compound, CL-82198 (10 μM) (Fig. 4G), for improving axon regeneration (Fig. 4C). CL-82198 is a MMP-13 inhibitor and displays no activity against MMP-1 or MMP-9 (32). Its efficacy in humans is currently unknown. To confirm MMP-13 as a target, we tested another selective, non-zinc-chelating MMP-13 inhibitor, DB04760 (33) (Fig. 4G), which also significantly rescued axon regeneration (Fig. 4C and Movie S7) and axon debris clearance (Fig. 4 E and H). We next assessed whether MMP-13 inhibition attenuates paclitaxel neurotoxicity by analyzing axon branch density and touch response following 96 h of incubation. Intriguingly, both inhibitors, when coadministered with paclitaxel, prevented axon degeneration (Fig. 4F) and also largely restored the touch response, with DB04760 being more efficient than CL-82198 (Fig. 4F). Only ~30% of larvae treated with paclitaxel + CL-82198 were unresponsive to touch, as opposed to ~50% when treated with paclitaxel (Fig. 4K), suggesting that a subset of animals benefited from this compound. Continuous CL-82198 but not DB04760 coadministration for 4 d showed some adverse effects, evident by a decreased response when stimulated in the head beneath the eyes, which served as the control region. Head stimulation evoked, however, wild type-like responses when either CL-82198 or DB04760 were administered simultaneously for 4 d, showing that a subset of animals benefited from this compound. Continuous CL-82198 but not DB04760 coadministration for 4 d showed some adverse effects, evident by a decreased response when stimulated in the head beneath the eyes, which served as the control region. Head stimulation evoked, however, wild type-like responses when either CL-82198 or DB04760 were administered simultaneously for 4 d, showing that a subset of animals benefited from this compound.
Paclitaxel Induces Ectopic MMP-13 Expression. MMP-13 is expressed at relatively low levels in the uninjured skin epithelium but is up-regulated in response to acute tissue injury (34, 35) where it is essential for proper wound repair (36). On the contrary, increased MMP-13 activity in uninjured tissues can promote injury (37) and cancer metastasis (38), suggesting that precisely controlled levels are essential for tissue homeostasis. We hypothesized that paclitaxel induces ectopic MMP-13 expression within the skin, consistent with the beneficial effects of the inhibitors. To test this, we determined mRNA expression levels of the zebrafish MMP-13 homolog mmp13a with quantitative PCR (qPCR) following 3 h of paclitaxel incubation. Transcript levels were elevated in uninjured paclitaxel but not vehicle-treated larvae and were enhanced upon amputation (Fig. 6A). MMP-13 exists as both an uncleaved (pro-enzyme) and cleaved active form. Various isoforms were reported, including 35, 48, and 54 kDa for the active and 60 and 80 kDa for the proenzyme (39), likely depending on species, age, and tissue types analyzed. Western analysis following 3 h of treatment revealed expected bands at 48 and 54 kDa for the cleaved and 80 kDa for the proenzyme (Fig. 6B). Quantifications revealed that the intermediate 54 kDa, but not 48 kDa, isoform was more abundant in paclitaxel-treated larvae compared with the respective vehicle control groups (Fig. 6C and D).

Given the preferential accumulation of tubulin tracker in basal keratinocytes, we hypothesized that MMP-13 is up-regulated in keratinocytes. To test this, we used whole-mount immunofluorescence staining. In mice, MMP-13 has been detected in dermal fibroblasts of skin wounds (40) and in the leading edge of migratory epithelial cells following corneal injury (41). In zebrafish embryos, mmp13a was detected after caudal fin amputation (35), and we also detected MMP-13 specifically at the amputation wound of larvae (Fig. 6E–E″). Paclitaxel treatment enhanced MMP-13 expression, showing a uniform staining within the caudal fin (Fig. 6F–F″, G, and Movie S8) but not within RB axons (Fig. 6I–K″ and Movie S9). Intriguingly, similar to tubulin tracker, MMP-13 expression was also localized to basal keratinocytes (Fig. 6L–L″). In the adult distal caudal fin, MMP-13 expression was found in the dermis of both vehicle and paclitaxel-treated animals but was specifically up-regulated in basal cells after paclitaxel treatment (Fig. 5D and E). MMP-13 staining...
was adjacent to, but not within, DRG axons (Fig. 5F). Interestingly, although MMP-13 expression showed an even punctate pattern in the basal layer, we also found distinct clusters in both the basal and suprabasal layer (Fig. 5E, arrowheads), which were largely absent in vehicle controls (Fig. 5D, arrowheads). At the surface of the skin, MMP-13 was clustered within dead cells seen after paclitaxel but not vehicle treatment (Fig. 5E, arrows). Collectively, these findings suggest that paclitaxel up-regulates MMP-13 expression in epidermal keratinocytes but not within cutaneous axons.

**MMP-13 Up-Regulation Impairs Epithelial Barrier Function and Reduces Mechanical Stress Resistance.** Increased MMP-13 activity has been linked to defects in epithelial barrier function, such as in the gut epithelium, where it destabilizes tight junctions (TJs) (42). We therefore assessed whether paclitaxel-dependent MMP-13 up-regulation promotes skin barrier defects. We previously showed that H$_2$O$_2$ diffuses into the larval skin, evident by its ability to induce RB axon growth in uninjured animals (15). We hypothesized that barrier defects will enhance diffusion of exogenous H$_2$O$_2$ into the skin. To quantitatively assess this, we generated transgenic Tg(tp63:CAAX-GFP) fish expressing the ratio-metric, genetic H$_2$O$_2$ sensor HyPer in keratinocytes. The submicromolar affinity of HyPer for H$_2$O$_2$ and its insensitivity to other ROS permits the detection of small changes in H$_2$O$_2$ concentrations. We found that the mean HyPer ratio following...
addition of H\(_2\)O\(_2\) to the larval media was ∼1.3-fold (Fig. 7A and D). Three-hour pretreatment with paclitaxel significantly increased this ratio to ∼1.6-fold (Fig. 7B and D). We next coadministered CL-82198, which led to decreased HyPer oxidation below levels observed when treated with DMSO vehicle (Fig. 7C and D). Interestingly, CL-82198 administration alone led to a further reduction, suggesting either that DMSO might induce low-level MMP-13 activity or that some MMP-13 activity is necessary under homeostatic conditions to maintain the skin barrier.

To assess the role of MMP-13 in skin damage, we mechanically stressed larvae overexpressing either a wild-type homolog of MMP-13, mmp13a, or a mutated, nonfunctional control variant (Fig. S9A and B) following mRNA injections into one-cell stage embryos. Mechanical stress at 2 dpf promoted rupturing of the yolk and fins in mmp13-overexpressing larvae, whereas larvae expressing the deletion variant were largely unaffected (Fig. 7E). We next tested if pharmacological MMP-13 inhibition rescued paclitaxel-dependent skin and injury phenotypes. Larvae cotreated with paclitaxel and either CL-82918 or DB04760 showed improved skin morphologies when examined with SEM (Fig. 7G–H' and Fig. S9C) and increased mechanical stress resistance (Fig. 7F). These findings implicate MMP-13 in paclitaxel-induced skin damage.

Increased MMP-13 Activity Impairs Wound Repair. MMP-13 is known to be up-regulated during epidermal wound repair, and we show that paclitaxel further increases MMP-13 expression upon injury (Fig. 6). We therefore assessed the relationship between paclitaxel and MMP-13 in an injury setting. We recorded 12-h time-lapse movies following puncture wounding of the caudal fin in transgenic Tg(p63:CAAX-GFP) larvae in which the plasma membrane of TP63-positive basal keratinocytes is fluorescently labeled. Punctured vehicle controls showed a rapid but distinct healing response, marked by a slight increase in wound diameter within the first 2 h, followed by wound closure around 5 h (Fig. S10A and C). Despite a similar initial wound diameter, wounds in paclitaxel-treated larvae continuously increased and failed to close (Fig. S10B and C), which was largely rescued upon co-administration of CL-82198 and DB04760 (Fig. S10C).

To examine keratinocyte-specific effects, we used an established in vitro scratch assay and the human keratinocyte line HEK001 plated on a collagen matrix. We first assessed H\(_2\)O\(_2\) production following scratch injury. Although control cells at the scratch margin produced H\(_2\)O\(_2\) within ~20 min (Fig. S10D and G), which remained present until scratch wound closure was completed (Fig. S10D and E), paclitaxel-treated keratinocytes showed a dose-dependent reduction in ROS/H\(_2\)O\(_2\) formation during the first ~2 h (Fig. S10D, D', and G). At 12 h, control gaps were nearly closed and few cells produced ROS/H\(_2\)O\(_2\), whereas gaps remained large in paclitaxel-treated wells despite the fact that many cells now produced ROS/H\(_2\)O\(_2\) (Fig. S10E, E', and H). By 24 h, gaps were no longer visible in control wells, whereas paclitaxel impaired closure (Fig. S10F, F', and H). Thus, paclitaxel delays H\(_2\)O\(_2\)/ROS formation and impairs keratinocyte healing.

To determine the role of MMP-13 in scratch wound repair, HEK001 cells were treated with paclitaxel and either CL-82198 or DB04670 for 30 min before scratching. This showed a dose-dependent partial improvement in gap closure (Fig. S10I and J), suggesting that impaired scratch healing is in part mediated by keratinocyte-specific MMP-13 activity. Interestingly, inhibition of MMP-13 in wild-type keratinocytes considerably enhanced scratch repair. To analyze whether closure defects were mediated by cytoskeletal defects induced by paclitaxel treatment, we monitored scratch margin cells over time (Fig. S10K). Although migratory control cells formed lamellipodia at the leading edges, indicating migration, lamellipodia were absent in paclitaxel-treated HEK001 cells. Coadministration of DB04760 (or CL-82198) in contrast restored lamellipodia formation and migration, as did DB04670 treatment alone. These findings indicate that increased MMP-13 activity induced by paclitaxel impairs keratinocyte migration, likely due to excessive collagen degradation.

Discussion
A roadblock in the development of therapies for paclitaxel-induced peripheral neuropathy is the lack of understanding about the underlying mechanisms. Our studies demonstrate that keratinocyte damage, which precedes axon degeneration, underlies paclitaxel...
neurotoxicity in zebrafish and that MMP-13 plays a critical role (Fig. 7I). This finding is intriguing, as paclitaxel-induced axon degeneration in rat models is initially evident within the epidermis (11). Why epidermal keratinocytes are affected, but not axons, is unclear. It is possible that dose-dependent differences in paclitaxel metabolism or uptake play a role. For instance, administration of paclitaxel over four cumulative doses at 2 mg/kg induced terminal arbor degeneration (TAD) in only the intraepidermal DRG axons of rats (11), which could potentially be mediated by keratinocyte-specific damage. In contrast, higher doses (>8 mg/kg) administered to rats induced distinct phenotypes, such as peripheral nerve-specific degeneration and neuronal death (43), which may relate to nerve-specific uptake of paclitaxel. Our model, in which we expose animals to significantly lower paclitaxel concentrations, appears to mimic more closely the TAD phenotype.

The finding that perturbations of skin homeostasis induce neurotoxicity is intriguing given that human patients undergoing chemotherapy with paclitaxel develop various skin phenotypes, such as peripheral nerve-specific degeneration and neuropathic pain in a rat model where DRG neurons show increased expression of MMP-3 (47). Given the general role of MMPs in matrix-degrading enzymes, evidence suggests that general MMP inhibition using the potent MMP inhibitor tetracycline-3 positively influences paclitaxel-induced hyperalgesia in mice (46). MMPs have also been implicated in paclitaxel-induced neuropathic pain in a rat model where DRG neurons show increased expression of MMP-3 (47). Given the general role of MMPs in paclitaxel neurotoxicity, MMP-13’s functions in peripheral neuropathy may not be restricted to zebrafish. The question remains by which mechanisms MMP-13 is up-regulated following paclitaxel treatment. One possibility is that MMP-13 accumulates within the ECM due to reduced protein turnover and altered microtubule functions within keratinocytes. Alternatively, microtubule stabilization alters signaling cascades that promote mmp13a gene expression. These could be induced by mechanical stress-dependent ROS formation. A number of factors favor this model: (i) We observed increased ROS/H2O2 formation upon mechanically stressing paclitaxel-treated zebrafish larvae; (ii) mechanical stress triggers Nox2-dependent “X-ROS” formation in cardiomyocytes and skeletal myofibers (48, 49), and X-ROS formation is exacerbated in skeletal muscle of mice with Duchenne Muscular Dystrophy due to enhanced microtubule stiffness (49); and (iii) our RNAseq analysis shows that H2O2 induces mmp13a expression in larval zebrafish (31).
The question remains how paclitaxel and MMP-13–dependent epidermal perturbations promote axon degeneration. Excessive MMP-13 activity may lead to increased collagen degradation, which could alter the mechanical properties of the skin, given the collagen-rich network within the ECM that is essential to maintain tissue integrity (50). Because the distal fin edges and also the glabrous skin in mammals are frequently exposed to biomechanical stresses, axons in these regions may be more susceptible to damage compared with other body regions. Nociceptors and small-diameter mechanoreceptors in hairy skin have been shown to be modulated by mechanical stress through binding of collagen to integrins alpha 2 and beta 1 (12). Parallel mechanisms in glabrous skin may exist, and disruptions due to increased MMP-13 activity may promote axon degeneration. Alternatively, MMP-13 could function in cellular signaling. In the intestinal epithelium during sepsis and in inflammatory bowel disease, MMP-13 promotes LPS-induced goblet cell depletion, endoplasmic reticulum stress, and TJ destabilization through its role as TNF sheddase, which cleaves pro-TNF into its bioactive form (42). A similar function could promote junction destabilization in keratinocytes following paclitaxel treatment, consistent with reduced skin resistance and barrier function. Further studies are required to explore these possibilities. Interestingly, we observed prominent MMP-13 expression in the dermis of both adult vehicle and paclitaxel-treated animals, yet dermal axons are not affected by MMP-13 activity. One possible explanation is that the dermis contains myelinated axons which do not establish direct contact with the microenvironment, unlike unmyelinated axons in the epidermis (13, 14). This is further evidence that interactions between keratinocytes and unmyelinated axons might play a role in paclitaxel neurotoxicity in zebrafish.

Our studies demonstrated that MMP-13 inhibition with two different chemical inhibitors, CL-82198 and DB04760, significantly reduced paclitaxel neurotoxicity. A number of MMP inhibitors have been developed for the treatment of cancer where MMPs are up-regulated (51). The first generation of inhibitors was designed to chelate the zinc ion in the active site, thereby preventing enzymatic activity (52). Because of the low selectivity of these inhibitors due to sequence conservation within the active site, more selective MMP inhibitors were subsequently developed. CL-82198 belongs to the class of highly selective, non–zinc-chelating compounds and was shown to exhibit specific but weak inhibition of MMP-13 (89% at 10 μg/mL) without activity against MMP-1, 9, and TACE (tumor necrosis factor–α-converting enzyme) (32). This inhibitor binds to the large S1’ binding pocket without apparent interactions with the catalytic zinc binding domain, justifying its micromolar potency (32). The weak binding may be favorable in our model in that MMP-13 activity is reduced, but not abolished, to levels seen in control animals (Fig. 6f). Also, DB04760, a pyrimidine dicarboxamide inhibitor, belongs to the class of non–zinc-chelating, S1’ pocket-binding compounds (33) and exhibited similar effects as CL-82198. Intriguingly, CL-82198 also has proven beneficial effects in decreasing cancer metastasis during which MMP-13 plays a role (53–55). MMP-13 has also been implicated in a variety of other conditions, including tendon injury and intestinal inflammatory diseases (42, 56, 57). Targeting this enzyme with these selective compounds could therefore provide multiple benefits. Intriguingly, recent data showed that paclitaxel also promotes metastasis (58), and thus, inhibitors targeting MMP-13 in neuropathy patients could provide additional benefits. Paradoxically, we found that MMP-13 inhibition of HEK001 cells promoted migration, suggesting that MMP-13 function under injury conditions may be different than in cancer. It is intriguing that in the wound setting, paclitaxel-induced cytосkeletal defects seem to be minor given that MMP-13 inhibition was able to rescue wound repair and promote HEK001 migration. Thus, paclitaxel concentrations used in our studies may primarily influence the ECM. Further studies are required to investigate the underlying basis.

Despite the fact that our findings strongly argue for epidermal influences on axons, it is possible that axons also directly uptake paclitaxel, as shown in mammalian cell culture studies (8). Although we did not detect tubulin tracker fluorescence in axons, we found it in some but not all RB cell bodies. One possibility is that only RB neurons that did not accumulate tubulin tracker innervated the caudal fin. Alternatively, tubulin tracker diffused into axons, but the concentrations were below detection limits, or rapid metabolic turnover of tubulin tracker within axons played a role. In this case, it is questionable whether such minute amounts could cause significant axon damage, a point that requires further investigation. In support of direct effects of paclitaxel is also the observation that growth cone dynamics were reduced. However, this phenotype and the lack of regenerative growth may also relate to perturbations in the ECM, leading to reduced substrate availability due to MMP-13–mediated collagen degradation. Overall, our findings argue for a primary role of epidermal damage in paclitaxel neurotoxicity, given that MMP-13 was specifically expressed in basal keratinocytes and because MMP-13 inhibition rescued short- and long-term paclitaxel neurotoxicity. The zebrafish larval skin resembles more closely the two-layered human fetal skin (59) and is innervated by axons of trigeminal and RB neurons, as opposed to adult skin that is innervated by DRG neurons, similar to mammals. Despite this difference, RB neurons are molecularly and functionally similar to DRG (60) and trigeminal neurons (61). However, we found a less robust larval phenotype following paclitaxel injections. This could be caused by the use of pulled glass needles instead of the Hamilton syringe that we used in adults. Glass needles cannot be precisely adjusted for the injection volume and thus may have increased injection variability. Consistently, we found that some tubulin tracker-injected animals showed weak fluorescence. It is further possible that the concentration used for larval injections (10 μM) was insufficient, as we found 22 μM to be optimal for incubation studies. Thus, a higher efficacy might be achieved when injecting 22 μM, which needs to be further investigated. Also, pharmacokinetic differences in paclitaxel metabolism could play a role, which may lead to more rapid paclitaxel turnover in larvae, as these are still actively growing. Because we used single daily injections, rapid turnover would cause a less robust phenotype than seen when larvae are incubated in the drug over prolonged time periods. This model is consistent with the rapid recovery of the touch response following the last injection. Despite these differences, reported overall similar phenotypes in larval and adult fish, also when compared with mammalian data suggesting that the zebrafish is a valid model to study paclitaxel neurotoxicity.

Materials and Methods

Animals were maintained and handled in strict accordance with good animal care practices as approved by the NIH Animal Care and Use Committee and MDI Biological Laboratory Institutional Assurance #A-3562-01 under protocol #14-09. Larval paclitaxel (22 μM) incubations were performed in Ringers solution and injections (10 μM) in PBS. Adults were injected with 0.09–0.113 mg/kg paclitaxel (87–97 μg/mL), DMSO served as control vehicle. CL-82198 (TOCRIS) and DB04760 (Santa Cruz Biotechnology) were administered at 10 μM, and DPI and Apocynin at 50 and 100 μM, respectively. For touch response, larvae were stimulated with a pipette tip at the distal tail fin until a response was observed. Adults were wrapped in plastic foil until calm, and the distal tail fin was stimulated with an insect pin until twitching of the fish was observed. For the mechanical stress assay, larvae were preincubated for injuries, and only unjured larvae were included. Five to six larvae were gently pipetted three times with a glass Pasteur pipette and analyzed for injuries.

Further for details, see SI Materials and Methods.

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