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Research Paper

Systemic administration of epothilone D improves functional recovery of walking after rat spinal cord contusion injury

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ABSTRACT

Central nervous system (CNS) injuries cause permanent impairments of sensorimotor functions as mature neurons fail to regenerate their severed axons. The poor intrinsic growth capacity of adult CNS neurons and the formation of an inhibitory lesion scar are key impediments to axon regeneration. Systemic administration of the microtubule stabilizing agent epothilone B promotes axon regeneration and recovery of motor function by activating the intrinsic axonal growth machinery and by reducing the inhibitory fibrotic lesion scar. Thus, epothilones hold clinical promise as potential therapeutics for spinal cord injury. Here we tested the efficacy of epothilone D, an epothilone B analog with a superior safety profile. By using liquid chromatography and mass spectrometry (LC/MS), we found adequate CNS penetration and distribution of epothilone D after systemic administration, confirming the suitability of the drug for non-invasive CNS treatment. Systemic administration of epothilone D reduced inhibitory fibrotic scarring, promoted regrowth of injured raphespinal fibers and improved walking function after mid-thoracic spinal cord contusion injury in adult rats. These results confirm that systemic administration of epothilones is a valuable therapeutic strategy for CNS regeneration and repair after injury and provides a further advance for potential clinical translation.

1. Introduction

Spinal cord injuries (SCIs) of traumatic or non-traumatic origin commonly disrupt spinal cord axons. Regeneration of these injured axons is prevented by a poor neuron-intrinsic regenerative potential (Blackmore et al., 2012; Canty et al., 2013; Liu et al., 2010; Moore et al., 2009; Park et al., 2008; Tedeschi and Bradke, 2017; Ylera et al., 2009) and by a variety of axon growth inhibitory molecules (Cregg et al., 2014; Filbin, 2003; Garcia-Alias and Fawcett, 2012; Hilton and Bradke, 2017; Schwab, 2004), which are expressed by oligodendrocytes, scar-forming glia and fibroblasts (Anderson et al., 2016; Hellal et al., 2011; Klapka et al., 2005; Pasterkamp et al., 1999; Ruschel et al., 2015). As a result, axons fail to re-innervate their former targets, leaving SCI patients with permanent impairments of sensorimotor functions and lifelong disabilities. To date, no clinically approved, disease modifying treatment for human SCI exists.

Recently, a number of therapeutic strategies to improve functional outcomes in SCI patients have entered clinical trials (Ahuja et al., 2017). However, many of these strategies including epidural stimulation (Harkema et al., 2011), the Rho inhibitor Cethrin (Fehlings et al., 2011), cell-based therapies (Manley et al., 2017) and wound healing matrices (Theodore et al., 2016) require surgical intervention to be efficacious. Thus, although these therapeutic interventions hold great promise to become a first line, clinical treatment for SCI, they have a limited availability for continuous, long-term treatment of human SCI.

Systemic delivery of pro-regenerative, blood-brain barrier permeable compounds have shown encouraging results in promoting spinal cord regeneration in experimental models of SCI (Lang et al., 2015; Ruschel et al., 2015) and have potential for treatment of SCI patients in sub-acute stages and during rehabilitation. Intraperitoneal (i.p.) administration of epothilone B improves functional recovery of walking in spinal cord injured rats by reactivating the intrinsic axon growth machinery and by reducing the inhibitory lesion scar (Ruschel et al., 2015). Epothilones have been tested in cancer clinical trials and have received FDA approval as cancer therapeutics (Goodin et al., 2004). Thus, repurposing epothilones for the treatment of SCI could provide a fast track toward clinical approval (Oprea et al., 2011).

Nevertheless, epothilone B showed significant adverse effects after daily dosing in immune deprived mice even when low doses were applied (Chou et al., 1998). This could have important safety implications for a potential clinical translation into SCI patients, which often exhibit impaired immune function (Popovich and McTigue, 2009). By contrast,

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daily-dosing of immune deprived mice with comparable doses of desoxy-epothilone B, also referred to as epothilone D, was well-tolerated, even though epothilone D exhibits a similar potency in vitro and in cultured cells (Chou et al., 1998). In addition, epothilone D showed CNS bioavailability and efficacy in rodent models of CNS disease (Brunden et al., 2010) and has been tested in clinical trials for CNS indications (Tsai and Boxer, 2014).

Here we report that systemic and post-injury administration of epothilone D improves functional recovery of hindlimb control in adult rats that underwent a mid-thoracic spinal cord contusion injury. Moreover, epothilone D reduced inhibitory fibrotic scar tissue at the lesion site and restored raphespinal innervation to the lumbar spinal cord. Together, these data suggest that epothilone D could be a promising therapeutic candidate for non-invasive treatment of CNS injuries with a translational perspective.

2. Material & methods

2.1. Animal studies ethical statement

All animal experiments in this study were performed in accordance with the German animal welfare law (TierSchG) and the EU Directive 2010/63/EU for animal experiments. The animals were housed and handled in accordance with good animal practice as defined by the Federation for Laboratory Animal Science Associations (FELASA).

2.2. Pharmacokinetic analysis

Adult, female Sprague-Dawley rats (200–250 g) were injected intraperitoneally (i.p.) with 0.75 mg per kg bodyweight (BW) of epothilone B (Selleck) or epothilone D (Abcam). Both drugs were diluted in 50% DMSO/Saline (1:1 mixture of DMSO and physiological saline at room temperature). Animals were euthanized at 6 h, 1 day, 7 days or 14 days after epothilone B/D injection and blood plasma and spinal cord tissue samples were collected. Spinal cord tissue was extracted and homogenized in PBS (1:1, w/v). Aliquots of homogenates and blood plasma were mixed with 1 volume acetonitrile containing 50 ng/ml diazepam as internal standard and the mixture was centrifuged at 12,000*g*. Aliquots of the resulting supernatants were diluted with deionized water (1:1, v/v) and transferred to autosampler vials for LC-MS analysis. Calibration standards in the range of 0.1–240 ng/ml and quality controls were prepared in the same way as the corresponding unknown samples. The HPLC system consisted of an autosampler and an HPLC pump (Surveyor, Thermo Scientific), connected to a triple quadrupole MS (TSQ Quantum Discovery Max, Thermo Scientific) equipped with an electrospray (ESI) interface (Thermo Fisher Scientific, USA) connected to a PC running the standard software Xcalibur 2.1. The HPLC pump flow rate was set to 600 µl/min and samples were separated on a Kinetex 2.6 µm Phenyl-Hexyl 100 50 × 2.1 mm analytical column with a Security Guard Ultra cartridge UHPLC Phenyl for 2.1 mm ID column (Phenomenex, Germany). Gradient elution was facilitated using acetonitrile/0.1% formic acid as organic phase (A) and 0.1% acetic acid (B): % A = 5 (0–0.1 min), 97 (0.4–1.7 min), 5 (1.8–2.5 min). Full scan mass spectra were acquired in the positive mode using syringe pump infusion to identify the protonated quasi-molecular ions $[M + H]^+$. Auto-tuning was carried out for maximizing ion abundance followed by the identification of characteristic fragment ions using a generic parameter set. Ions with the highest S/N ratio were used to quantify the item in the selected reaction monitoring mode (SRM) and as qualifier, respectively. The limit of quantification (LOQ) was defined as the lowest standard concentration taken for the corresponding calibration curve, i.e. 0.1 ng/ml.

2.3. Spinal cord injury studies

The spinal cord contusion injury was performed at thoracic spinal cord segment 9 using an Infinite Horizon impactor device (Precision Systems) as previously described (Scheff et al., 2003). Adult, female Sprague-Dawley rats (200–250 g) were deeply anesthetized by continuous inhalation of isoflurane (2% isoflurane/oxygen). Laminectomy of the thoracic vertebra 9 was performed to expose the spinal cord. The vertebral column was stabilized with 2 forceps, which fixed the lateral processes of thoracic vertebra 8 and 10. The exposed spinal cord was placed and aligned under the impactor according to the manufacturer's instructions. Finally, an impact of 150 kilodyne (kdyn) was applied to the spinal cord. The impact was controlled and monitored with the device-specific software. Inappropriate force impact curves led to exclusion of the animal from the study. A maximum variation of 10% from the set impact force of 150 kdyn was defined as acceptable range. At day 1 and day 15 after contusion injury, animals received a single i.p. injection of epothilone D (Abcam, 0.75 mg/kg BW, N = 15) or vehicle solution (50% DMSO/sterile saline, N = 17). Bodyweight of injected animals was monitored biweekly. One vehicle and three epothilone D injected animals were excluded from the study either because of irregular force impact curves or because no significant loss of hindlimb function was observed at 2 weeks after SCI (see horizontal ladder test), both of which indicating that the spinal cord impact may not have been appropriate. One vehicle and one epothilone D injected animal were excluded due to a recording error of the force-impact curves.

2.4. Horizontal ladder test

To analyze functional recovery of skilled walking, rats were tested on a foot misplacement device at 2, 4, 6 and 8 weeks after contusion injury. 13 vehicle treated animals and 14 epothilone D treated animals were recorded by video while walking over a 1 m long horizontal ladder, elevated 15 cm above the ground with a regular ladder-step distance of 2 cm. In cases where the animal stopped during the run, the trial was considered unquantifiable and repeated. The first two complete runs per animal were digitally examined frame by frame, the total number of major foot slips in between the ladder rungs were counted and normalized to the total number of steps. Foot slips were considered as ‘major’ when at least two third of the hind paw (complete metatarsus) slipped below the lower edge of the ladder rungs. One vehicle treated animal was excluded from subsequent quantification due to a recording error.

2.5. Immunohistochemistry

Immunohistochemistry was performed as previously described (Ruschel et al., 2015). For tissue fixation, animals were deeply anesthetized with an over dose of a ketamine xylazine mixture (1:2) and subsequently perfused transcardially with 4% paraformaldehyde (PFA) solution (in 0.1 M PBS). The spinal cord was dissected from the vertebral column, post-fixed in 4% PFA solution overnight and then placed in sterile 30% sucrose solution for 48 h. Subsequently, a 1 cm piece of spinal cord containing the lesion site and a caudally adjacent 0.5 cm piece of the lumbar spinal cord were embedded in embedding medium (Thermo Scientific) and frozen on dry ice. 25 µm sagittal sections of the lesion site and 20 µm coronal sections of the lumbar spinal cord were obtained using a cryostat (Leica) and mounted on Superfrost™ plus microscope slides (Thermo Scientific). Sections were quenched with 0.3 M glycine (in TBS) for 30 min and then permeabilized with TBS-TritonX-100 (0.5%) for 30 min at room temperature. Sections were transferred to custom made wet-chambers and blocked with 5% heat-inactivated goat serum (Invitrogen) in TBS-TritonX (0.5%) for 1 h. After blocking, sections were incubated at room temperature with primary antibodies diluted in TBS-TritonX (0.5%). Sagittal sections of the lesion site were incubated overnight with rabbit anti-laminin (1:50; Sigma, Cat# L9393) and monoclonal mouse anti-glial fibrillary acidic protein (GFAP; 1:200; Sigma, Cat# C9295) primary antibodies, while coronal sections of the lumbar spinal cord were incubated for 2 days with rabbit