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Tegaserod mimics the neurostimulatory glycan polysialic acid and promotes nervous system repair☆

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ABSTRACT

Glycans attached to the cell surface via proteins or lipids or exposed in the extracellular matrix affect many cellular processes, including neuritogenesis, cell survival and migration, as well as synaptic activity and plasticity. These functions make glycans attractive molecules for stimulating repair of the injured nervous system. Yet, glycans are often difficult to synthesize or isolate and have the disadvantage to be unstable in a complex tissue environment. To circumvent these issues, we have screened a library of small organic compounds to search for structural and functional mimetics of the neurostimulatory glycan polysialic acid (PSA) and identified the 5-HT_4 receptor agonist tegaserod as a PSA mimetic. The PSA mimicking activity of tegaserod was shown in cultures of central and peripheral nervous system cells of the mouse and found to be independent of its described function as a serotonin (5-HT_4) receptor agonist. In an *in vivo* model for peripheral nerve regeneration, mice receiving tegaserod at the site of injury showed enhanced recovery compared to control mice receiving vehicle control as evidenced by functional measurements and histology. These data indicate that tegaserod could be repurposed for treatment of nervous system injuries and underscores the potential of using small molecules as mimetics of neurostimulatory glycans.

1. Introduction

Polysialic acid (PSA) is a homopolymer of α-(2,8)-linked sialic acid residues numbering up to 200 in length, and is attached predominantly to the neural cell adhesion molecule (NCAM) (Finne et al., 1983; Muhlenhoff et al., 1996). PSA is expressed in the developing and adult nervous system of vertebrates, with expression localized to migrating cells, processes of neurons and glial cells, synapses and stem cells (Angata and Fukuda, 2010; Durbec and Cremer, 2001; Roche et al., 1997). PSA has been suggested to promote cell motility in the nervous system by primarily expanding the extracellular space due to its large hydration volume (Yang et al., 1994) and decreasing homophilic interactions of NCAM (Durbec and Cremer, 2001). PSA also mediates interactions of NCAM with heparan sulfate proteoglycans (Storms and Rutishauser, 1998), brain derived neurotrophic factor (Muller et al., 2000), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (Vaithianathan et al., 2004), N-methyl-D-aspartate receptors

Abbreviations: CDR, complementary determining region; cGN, cerebellar granule neuron; DRG, dorsal root ganglion; ELISA, enzyme-linked immunosorbent assay; FBA, foot base angle; GMP, good manufacturing processes; H, heavy chain; HRP, horse radish peroxidase; L, light chain; NCAM, neural cell adhesion molecule; NIH, National Institutes of Health; PBS, phosphate buffered saline, pH 7.3; PLL, poly-L-lysine; PLO, poly-L-ornithine; PLR, protraction limb ratio; PNS, peripheral nervous system; PSA, polysialic acid; RI, recovery index; SPR, surface plasmon resonance; 5-HT, serotonin (5-hydroxytryptamine).

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(Hammond et al., 2006; Senkov et al., 2006), histone H1 (Mishra et al., 2010) and myristoylated alanine-rich C kinase substrate (Theis et al., 2013).

The cell motility promoting effects of PSA are of interest for the treatment of nervous system injuries and disorders. Viral-induced expression of PSA enhances regeneration after spinal cord injury, promotes sensory neuron integration into the injured spinal cord, and increases Purkinje cell dendrite formation following injury (Zhang et al., 2007a, 2007b, 2007c). Overexpression of PSA on astrocytes improves axonal extension across spinal cord injuries (El Maarouf et al., 2006), and transplanted Schwann cells overexpressing PSA augment repair in both spinal cord (Luo et al., 2011; Papastefanaki et al., 2007) and peripheral nerve (Jungnickel et al., 2012). However, continuously expressed PSA slows the rate of myelination *in vivo* (El Maarouf et al., 2006; Franceschini et al., 2004), and high PSA expression correlates with invasiveness and malignancy of cancers (Petridis et al., 2009; Tanaka et al., 2001), suggesting that a dose- and time-constrained approach must be considered.

As PSA is rapidly degraded by sialidases in the complex tissue environment (Franz et al., 2005; Martini et al., 1994; Nagai et al., 1989), peptide mimetics of PSA were identified and shown to act as true functional counterparts of PSA (Torregrossa et al., 2004). Linear and cyclic PSA mimetic peptides have improved functional recovery following peripheral nerve and spinal cord injuries in mice (Marino et al., 2009; Mehanna et al., 2010, 2009). Small organic molecule mimetics of PSA offer further advantages for the development and regulatory approval for therapies. To build upon previous advances in identifying PSA mimetics and to increase the translational potential, we have screened a library of small compounds for mimetics of PSA and identified tegaserod. Tegaserod is a drug that received clinical approval for treatment of irritable bowel syndrome and constipation (Muller-Lissner et al., 2001) by stimulating 5-HT_4 receptors on enteric neurons (Liu et al., 2005, 2009). The present study shows that tegaserod has a second and distinct mechanism of action as a small organic mimetic molecule of PSA.

2. Materials and methods

2.1. Competition ELISA with small organic compounds

To identify small molecule PSA mimetics the NIH Clinical Collection 1 Library was screened using methods similar to those previously used by our group (Loers et al., 2013; Torregrossa et al., 2004). Briefly, the PSA mimicking peptide, NTHTDPYIYPIDC (Mehanna et al., 2009), coupled to catalase was immobilized on the surface of 384-well plates (3 μg/ml; 25 μl/well; overnight at 4 °C). Ten micromolar of molecules from the library were incubated with 0.1 μg/ml and 25 μl/well of the PSA-specific monoclonal antibody 735 (Frosch et al., 1985) for 1 h at room temperature and then added to the wells. An HRP-coupled secondary antibody (1:5000 in PBS; Jackson ImmunoResearch) and ortho-phenylenediamine (0.5 mg/ml, 5 min; Thermo Scientific) as HRP substrate were used to assess antibody binding at room temperature. Binding was quantified using an ELISA reader (490 nm; μQuant, Bio-TEK) and the software KCjunior (Bio-TEK). The PSA peptide mimetic was used as a positive control, and experiments were repeated three times to identify true hits.

Following the initial screen, a competition ELISA was performed with varying doses of tegaserod maleate (Sequoia Research Products Ltd.) and the negative control compound nitrendipine (Sequoia Research Products Ltd.). The PSA peptide mimetic coupled to catalase was immobilized, and wells were incubated with increasing concentrations of tegaserod and nitrendipine, pre-incubated with antibody 735 (0.1 μg/ml) for 1 h at room temperature.

2.2. Surface plasmon resonance (SPR)

Binding affinities of organic compounds to the antibody 735 were evaluated in a competition experiment by SPR measurements carried out on a BIAcore 3000 instrument (GE Healthcare Europe GmbH) with sensor chips maintained at 25 °C for all experimental steps (Schulze, 2000). The running buffer was phosphate buffered saline, pH 7.3 (PBS). PSA mimicking peptide coupled to catalase was covalently immobilized to CM5 sensor chips (carboxymethyl dextran; GE Healthcare Europe GmbH) via primary amino groups, using standard coupling protocols (Karlsson et al., 1991). In brief, the sensor surface was activated by a 7 min pulse of 0.2 M N-ethyl-N-(3-dimethylaminopropyl) carbodiimide and 50 mM N-hydroxysuccinimide. The PSA mimetic peptide solution (10 nM in 10 mM sodium acetate, pH 5.2) was then injected for 5–10 min. Ethanolamine (1 M, pH 8.5) was used to block remaining activated carboxyl groups (1 h). Ligand densities of 100–150 fmol/mm^2 were reached. Immobilized control peptide (10 nM PSA scrambled peptide mimetic coupled to catalase) was used as a reference surface. Regeneration of the sensor chip was achieved by injection of 10 mM glycine, pH 2.5, at 10 ml/min (two 30 s pulses). The data were analyzed using the BIA evaluation 3.0 software. All sensorgrams were corrected for background and bulk refractive index by subtraction of the reference.

To confirm that tegaserod binds specifically to antibody 735, a competition experiment was performed. First, the antibody (10 nM) was pre-incubated for 1 h at room temperature with different molar concentrations (1, 2, 5, 8, 15 and 30 μM) of tegaserod or the negative control compound nitrendipine. Then, the antibody/organic compound solutions were injected (1 ml/min) to the PSA peptide mimetic-coupled chip and binding was determined over 30 min. The surface of the sensor chip was subsequently regenerated with 10 mM glycine, pH 2.5, at 10 ml/min (three 30 s pulses).

2.3. Molecular modeling of tegaserod with the PSA-specific antibody 735

A model of PSA bound to the binding pocket of the antibody 735 was constructed using previous information (Evans et al., 1995). The 3-dimensional coordinates for antibody 735 were obtained from the Protein Data Bank (PDB id: 1PLG). A decamer of PSA was built using the program Discovery Studio (Accelrys Inc.) in helical conformation making approximately 1/2 of a turn within 17 Å ($n = 6$ residues per turn). Subsequently, we used the program PYMOL (Schrödinger Inc.) to produce a model of an eight-residue segment of PSA docked onto the antibody 735. The resulting model was manipulated in PYMOL to reproduce the specific intermolecular contacts between PSA and antibody 735 that had been identified (Evans et al., 1995). The PSA conformation and pose was then energy-minimized in complex with antibody 735 using Molecular Operating Environment (Chemical Computing Group) to construct the final model.

We carried out ligand docking of tegaserod with antibody 735. The crystal structure of antibody 735 was used for docking by isolating the immunoglobulin domains corresponding to the variable regions of the heavy and light chain of antibody 735, followed by hydrogen placement and energy minimization. We used Schrödinger's Glide ligand-docking software (Friesner et al., 2004) to manually construct a 12 Å cubic docking grid that included the entire complementary determining region (CDR) of antibody 735. We prepared tegaserod using Schrödinger's Ligprep software and carried out docking using Glide in standard precision mode. The top-ranked ligand pose was selected for further analysis.

2.4. In vitro analysis of neurite/process outgrowth

Primary cultures of cerebellar granule neurons (cGNs), dorsal root ganglion (DRG) neurons or Schwann cells were prepared from cerebella or dorsal root ganglia of 7-day-old (P7) C57BL/6J wild type mice as described (Kleene et al., 2001; Loers et al., 2005; Mehanna et al., 2009) and motoneurons were prepared from C57BL/6J wild type or $NCAM^{-/-}$ 14-day-old (E14) mouse embryos as described (Simova et al., 2006). In brief, 48-well plates were coated with 0.01% poly-L-lysine (PLL) or poly-L-ornithine (PLO) overnight at 4 °C. Schwann cells, cerebellar neurons, DRG neurons (PLL) or motoneurons (PLO) were seeded at a density of 1.25×10^4 (Schwann cells, DRG neurons and motoneurons) or 2.5×10^4 cells (cGNs) per well in 250 μl of corresponding serum-free culture medium and compounds were added 1 h after seeding. After maintenance for 24 h at 37 °C, cells were fixed with 2.5% glutaraldehyde and stained with 1% methylene blue/toluidine blue in 1% borax. Morphological quantification of neurite or process lengths was performed as described (Mehanna et al., 2009). Schwann cell processes and neurites of cGNs, DRG neurons and motoneurons with a length of at least one cell body diameter were counted and total neurite or process length per cell was determined by counting 50 cells in each of two wells per experiment using an AxioVision system 4.6 (Carl Zeiss). At least three independent experiments were performed for each culture condition.

2.5. Effects of tegaserod on femoral nerve regeneration

2.5.1. Mice

All experiments were conducted in accordance with the Rutgers Animal Care and Facilities Committee and the Institutional Animal Care and Use Committee (IACUC) and every effort was made to minimize animal suffering and the number of animals used in experiments. C57BL/6J wild type and $NCAM^{-/-}$ mice were used for all experiments and kept under standard laboratory conditions with food and water supply *ad libitum* and with an artificial 12 h light/dark cycle. Twelve-week-old C57BL/6J mice were subjected to femoral nerve injury as described (Mehanna et al., 2009), but with tegaserod substituting for the PSA peptide mimetic. Briefly, Puramatrix hydrogel (BD Biosciences, Franklin Lakes, NJ) contained within a polyethylene conduit was used as the delivery vehicle for tegaserod. Hydrogels with and without tegaserod were induced to gel using 2× PBS as the gelation stimulant within the conduits, which were then sutured into the injured site. Vehicle consisted of the same hydrogel contained within the conduit, but without tegaserod.