Experimental Eye Research 119 (2014) 97–105

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Ocular hypotensive efficacy of Src-family tyrosine kinase inhibitors via different cellular actions from Rock inhibitors

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ARTICLE INFO

Article history:
Received 17 May 2013
Accepted in revised form 27 November 2013
Available online 7 December 2013

Keywords:
intraocular pressure
trabecular meshwork
Src-family tyrosine kinase
PP2
Rock

ABSTRACT

We investigated the effects of Src-family tyrosine kinase (SFK) inhibitors on intraocular pressure (IOP) and trabecular meshwork (TM) cells. The SFK inhibitors, PP2, PP1, and damnacanthal, significantly lowered IOP from baseline following intracameral injection in ocular normotensive rabbits, and PP2 decreased trans-epithelial electrical resistance (TEER) of TM cell layers in a dose-dependent manner ranging from 0.1 μM to 100 μM. The maximal efficacy of PP2 on TEER was a reduction to 71.7% relative to the vehicle-treated group at 100 μM. PP2 decreased the adhesion of TM cells to culture surfaces either uncoated with specific ECM proteins dose-dependently or coated with extracellular matrix proteins such as laminin I, fibronectin, collagen type I and basement membrane extraction. Tyrosine phosphorylation of focal adhesion kinase and $p130^{cas}$ was decreased by PP2. On the other hand, major changes in actin staining of TM cells were not able to be detected after PP2 treatment, although quantitative analysis showed that PP2 induced some morphological changes which were in the different direction to those caused by Y-27632, a Rock inhibitor. Y-27632 at 10 μM increased the permeability of TM cell layers, but did not induce changes in the adhesion of TM cells. These results suggest that SFK inhibitors lower IOP, at least partly, by acting on TM cells in a manner that is distinct from Rock inhibitors.

1. Introduction

Glaucoma is a major ocular disorder that can result in blindness through an irreversible loss of visual function (Quigley, 1996, 2011; Weinreb, 2007). The main risk and prognostic factor supported by clinical evidence is elevated intraocular pressure (IOP) (The AGIS Investigators, 2000; Coleman and Miglior, 2008). Therefore, lowering IOP sufficiently to delay or prevent glaucomatous visual field loss is the most promising approach in current glaucoma treatment (Collaborative Normal-Tension Glaucoma Study Group, 1998; Goel et al., 2010; Quigley, 2011). However, given that approximately half of glaucoma patients are adjunctively treated with 2 or more medications for appropriate IOP control (Kass et al., 2010; Kaneko et al., 2012), and that current medications are not effective in all patients (Camras et al., 2003; Choplin et al., 2004), there remains a need for novel pharmacotherapies which can deliver stronger or longer-lasting ocular hypotensive efficacy for glaucoma treatment.

An increase in resistance through the conventional outflow route (i.e., a decrease in the outflow facility) is considered to contribute to the IOP elevation seen in primary open angle glaucoma (POAG) and during aging (Weinreb and Khaw, 2004; Gabelt and Kaufman, 2005; Acott and Kelley, 2008; Johnstone, 2009). An agent that can increase conventional outflow would thus be expected to display ocular hypotensive efficacy and to improve the impaired function of the conventional outflow pathway. Chemical agents that disrupt the cytoskeleton, including Rho-associated coiled coil-forming protein kinase (Rock) inhibitors and actin depolymerizers such as latrunculins, are candidates as new ocular hypotensive agents. Studies using these agents demonstrate that an increase in conventional outflow resulting in ocular hypotensive efficacy can be caused by changes in actin organization (Honjo et al., 2001; Rao et al., 2001; Okka et al., 2004; Gabelt and Kaufman, 2005; Tian and Kaufman, 2005; Lu et al., 2008; Williams et al., 2011). These changes result in alterations in cell morphology, contraction conditions, and adhesion to neighboring cells or the extracellular matrix (ECM).

Src-family tyrosine kinases (SFKs) are expressed in a wide variety of cells and play key roles in the regulation of proliferation,

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0014-4835/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.exer.2013.11.015

cytoskeletal alteration, differentiation, survival, adhesion, and migration (Thomas and Brugge, 1997; Elliott et al., 2011). This functional variety reflects their ability to interact with diverse classes of cellular receptors and distinct cellular targets. Moreover, a change in SFK activity is implied to cause pleiotropic reactions in cells. For example, Src-transformed cells show a loss of actin stress fibers as a result of suppression of Rho activity (Fincham et al., 1999), but conversely, there are reports that imply similar efficacy between inhibition of SFK- and Rho/Rock-mediated signaling pathways. PP2, a potent inhibitor of several SFKs (Hanke et al., 1996; Jones et al., 2002), inhibits the phosphorylation of myosin light chain (MLC) induced by ECM proteins such as fibronectin, laminin, and collagen type IV in cultured trabecular meshwork (TM) cells, as does a Rock inhibitor (Zhang et al., 2008). Such similarity has also been observed in other cell types, example being the prevention of transforming growth factor (TGF) β1-induced RhoA activation by Src inhibition in mesangial cells (Peng et al., 2008), and the disappearance of phospho-MLC from the cell periphery induced by PP2 and a Rock inhibitor in colon carcinoma cells (Avizienyte et al., 2004). Given the functions of SFKs, the efficacy of SFK inhibitors, and the regulation of conventional outflow, changes in SFK activity are likely to play some roles in conventional outflow. However, it is not known how SFKs function in ocular tissues, or whether SFK inhibitors display ocular hypotensive efficacy.

The purpose of this study was to assess the potential of SFK inhibitors as prospective new ocular hypotensive agents. To this end, we first evaluated their effect on IOP in ocular normotensive rabbits. Having found that they were effective, we explored their potential mechanism by evaluating effects of PP2, a selected representative of the SFK inhibitors evaluated in this study, on the permeability of TM cell layers, morphology, adhesion, and tyrosine phosphorylation of focal adhesion kinase (FAK) and $p130^{cas}$ in TM cells, and by comparing them with the corresponding effects of a Rock inhibitor.

2. Materials and methods

2.1. Chemicals and drug preparation

PP2 and PP1, SFK inhibitors, and PP3, a negative control of PP2 that lacks kinase inhibitory activity against SFKs, were purchased from Tocris (Ellisville, MO). Y-27632, a Rock inhibitor, and damnacanthal, an SFK inhibitor, were purchased from Sigma–Aldrich (St Louis, MO) and Merck Millipore (Darmstadt, Germany), respectively. For the IOP study, the drugs were dissolved in dimethyl sulfoxide (DMSO; Wako, Osaka, Japan) and diluted in saline to 1 mM, with a final DMSO concentration of 0.5%. For *in vitro* studies, the drugs were dissolved in DMSO and then diluted using 'experiment medium' [low glucose Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Kyoto, Japan) supplemented with 3% fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mM L-glutamine, 25 μg/ml gentamicin, and 2.5 μg/ml amphotericin B], to yield 0.1–100 μM drug solutions with the final DMSO concentration being 0.2%. In all treatments, 'vehicle' means the solvent at the same concentration as that in the drug solution.

Mouse monoclonal antibodies to human FAK and $p130^{cas}$ were purchased from Millipore and BD Biosciences (San Jose, CA), respectively. Rabbit polyclonal antibodies to phospho-FAK(Tyr^{576}) and phospho-$p130^{cas}$(Tyr^{410}) were purchased from Life Technologies (Carlsbad, CA) and Cell Signaling Technology (Danvers, MA), respectively. Donkey horseradish peroxidase (HRP)-conjugated anti-mouse IgG and anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

2.2. Animals

Japanese white rabbits (Kitayama Labes, Nagano, Japan) weighing 3.0–4.0 kg were used. All experiments were conducted in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and the internal ethics code for animal study of Santen Pharmaceutical Co., Ltd. Animals were housed under a 12-h light–dark cycle (light phase: 7 am–7 pm; dark phase: 7 pm–7 am).

2.3. IOP measurement, anesthesia, and drug administration

A calibrated pneumotonometer (Model 30 Classic; Reichert, Depaw, NY) was used to measure IOP. IOP measurement was performed on conscious animals under local anesthesia induced by topical administration of 0.4% oxybuprocaine hydrochloride solution (Benoxil ophthalmic solution 0.4%, Santen Pharmaceutical, Osaka, Japan).

IOP was measured before and at 2, 4, 6, and 8 h after drug administration. Intracameral injections were made using a microsyringe (Hamilton, Reno, NV) fitted with a 30-gauge needle, the volume administered being 20 μl. Drug concentration in the anterior chamber was estimated at 100 μM for the reason that the administered drug (20 μl) was diluted 10-fold by aqueous humor (200 μl). All drugs and vehicles were administered unilaterally, with the fellow eye remaining untreated. Administration was performed at around 11 am in all experiments. Drug-induced IOP changes were firstly compared with baseline in IOP, and then compared with the vehicle-treated group. Baseline was measured 2 days before the treatment day at each scheduled time point on the drug treatment day without drug administration.

2.4. Cell culture

Primary human TM cells were purchased from ScienCell Research Laboratories (Carlsbad, CA) and maintained in 'maintenance medium' (low glucose DMEM supplemented with 10% FBS, 2 mM L-glutamine, 25 μg/ml gentamicin, and 2.5 μg/ml amphotericin B) at 37 °C, under 5% CO_2. Cells that had undergone subculture passage less than 12 times from thawed cells were used for the present work.

2.5. Trans-epithelial electrical resistance (TEER) measurement

TEER measurement in TM cell layers was selected as an *in vitro* model to evaluate changes in cell morphology and/or cell adhesion which can suggest changes in outflow resistance of the TM, on the basis of previous reports (Li et al., 2004; Russ et al., 2010). Changes in permeability of cell layers were detected as changes in TEER. TM cells were seeded onto the membrane insert (0.4-μm pore; upper chamber) of a Transwell (Coster; Corning Life Sciences, Acton, MA) in maintenance medium except in wells for blank. The TEER of cell layers was measured using a voltohmmeter (EVOMX and ENDOHM; World Precision Instruments, Sarasota, FL), as described previously (Li et al., 2004; Burke et al., 2004). After the presence of sable TEER had been confirmed for several days, vehicle or drugs (PP2, 0.1–100 μM; PP3, 100 μM; Y-27632, 10 μM) in experiment medium were applied individually to both the upper and the lower chambers. The basis of the drug concentration was: PP2, kinase inhibitory activity (IC_{50}) and *in vivo* IOP-lowering efficacy; PP3, equivalency to the highest concentration of PP2; Y-27632, the previous report regarding its efficacy on cell permeability (Rao et al., 2001) and almost equivalent IOP-lowering efficacy to PP2 at 100 μM in a