Journal of Controlled Release 264 (2017) 34–44

Contents lists available at ScienceDirect

Journal of Controlled Release

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

Increased delivery of chemotherapy to the vitreous by inhibition of the blood-retinal barrier

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ARTICLE INFO

Keywords:
Blood-retinal barrier
Retinoblastoma
Topotecan
Pantoprazole
Microdialysis
Rabbit
ABC transporters
BCRP
ABCG2/BCRP
P-gp
ABCB1/P-gp
Pediatric cancer
Vitreous
Retina
Distribution
Delivery
Xenograft

ABSTRACT

Treatment of retinoblastoma -a pediatric cancer of the developing retina- might benefit from strategies to inhibit the blood-retinal barrier (BRB). The potent anticancer agent topotecan is a substrate of efflux transporters BCRP and P-gp, which are expressed at the BRB to restrict vitreous and retinal distribution of xenobiotics. In this work we have studied vitreous and retinal distribution, tumor accumulation and antitumor activity of topotecan, using pantoprazole as inhibitor of BCRP and P-gp. We used rabbit and mouse eyes as BRB models and patient-derived xenografts as retinoblastoma models. To validate the rabbit BRB model we stained BCRP and P-gp in the retinal vessels. Using intravitreous microdialysis we showed that the penetration of the rabbit vitreous by lactone topotecan increased significantly upon concomitant administration of pantoprazole ($P = 0.0285$). Pantoprazole also increased topotecan penetration of the mouse vitreous, measured as the vitreous-to-plasma topotecan concentration ratio at the steady state ($P = 0.0246$). Pantoprazole increased topotecan antitumor efficacy and intracellular penetration in retinoblastoma *in vitro*, but did not enhance intratumor drug distribution and survival in mice bearing the intraocular human tumor HSJD-RBT-2. Anatomical differences with the clinical setting likely limited our *in vivo* study, since xenografts were poorly vascularized masses that loaded most of the vitreous compartment. We conclude that pharmacological modulation of the BRB is feasible, enhances anticancer drug distribution into the vitreous and might have clinical implications in retinoblastoma.

Chemical compounds included in this manuscript: Topotecan (PubChem CID: 60700)
Pantoprazole sodium (PubChem CID: 15008962)

1. Introduction

Retinoblastoma is the most frequent ocular cancer in children [1]. Patients with advanced intraocular retinoblastoma typically present with massive retinal tumors, frequently disseminated as diffuse small tumors (seeding) in subretinal or vitreous location [2,3]. Treatment of free-floating vitreous seeding with systemic and intra-arterial chemotherapy remains an unmet medical need because the activity of the inner blood-retinal barrier (BRB) restricts the penetration of xenobiotic agents to the avascular vitreous compartment [4–6]. Recently, drug delivery to the vitreous has been improved by new local techniques of administration, including intravitreal injection, which is now used as standard of care for retinoblastoma vitreous seeding in several ocular oncology centers [7,8]. However, intravitreal injection is not sufficiently efficient in cases with concomitant presence of active subretinal tumors, and it is technically not feasible when diffuse seeding among the four quadrants of the eye compromise the safety of the procedure [9]. These patients might benefit from new strategies to inhibit the BRB

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http://dx.doi.org/10.1016/j.jconrel.2017.08.018
Received 31 May 2017; Received in revised form 17 August 2017; Accepted 18 August 2017
Available online 19 August 2017

during intravenous or intra-arterial administration of chemotherapy.

Although the function of the BRB is still poorly understood, it may involve the activity of drug transporters located at the retinal vessels such as the ATP-binding cassete (ABC) family (*e.g.*, BCRP/ABCG2, P-gp/ABCB1 and MRPs/ABCCs) [5,10,11] and the solute carrier (SLC) family [12,13]. The question whether the inhibition of these drug transporters at the BRB increases vitreous and retinal distribution of substrate anticancer agents has not been adequately addressed.

Among the clinically relevant anticancer drugs in retinoblastoma, topotecan is a semisynthetic camptothecin showing potent preclinical activity against several pediatric solid tumors [14–16]. Topotecan is given as intravenous low pH formulation to stabilize the active lactone form [17]. In the body fluids, due to neutral pH, the lactone is reversibly hydrolyzed to carboxylate topotecan (inactive), following a first order kinetic process, until reaching the equilibrium at a lactone:carboxylate ratio of approximately 1:9 [18]. The maximum concentration of lactone topotecan achieved in vitreous samples (aspirated with needle) upon the administration of high dose (0.5 mg/kg) in rabbits is 10 ng/mL (20 nM), and the vitreous-to-plasma area under the concentration-time curve (*AUC*) ratio is 0.2, suggesting active efflux from the vitreous compartment [19]. In fact, lactone topotecan is a substrate of the efflux pumps BCRP and P-gp, and inhibition of such pumps at the blood-brain barrier (BBB) leads to increased topotecan penetration of the central nervous system [20]. Because BCRP and P-gp are also located in the inner BRB (retinal vessels) [5,10], we hypothesized that their inhibition with pantoprazole (a clinically available drug used to reduce acid production of the stomach) could increase topotecan distribution in the vitreous compartment. In combination with chemotherapy agents, pantoprazole inhibits BCRP and P-gp [21,22]. Pantoprazole has also been shown to enhance the distribution of anticancer agents in solid tumors [23] and it has proven safe at high dose (240 mg; intravenous, i.v.) in adult patients with cancer [24].

In this work we used rabbits and mice as *in vivo* BRB models to study the ocular distribution of topotecan upon coadministration of pantoprazole. We additionally studied the effect of pantoprazole on topotecan tumor distribution and efficacy in retinoblastoma cells and intraocular xenografts in mice.

2. Materials and methods

2.1. Drugs and reagents

Topotecan (4 mg vials for i.v. injection) was from GSK (Brentford, Middlesex, UK). Pantoprazole sodium was from Baliarda (Buenos Aires, Argentina) or from Sigma-Aldrich (Tres Cantos, Madrid, Spain). Reagents for HPLC were from Merck (Darmstadt, Germany). Reagents for cultures were from Life Technologies (Grand Island, NY, USA).

2.2. Immunohistochemistry (IHC)

Four micron sections of formalin-fixed, paraffin-embedded (FFPE) tissues were used. Human enucleated eyes with retinoblastoma were obtained under an IRB-approved protocol and informed consent. Primary antibodies anti-BCRP/ABCG2 (clone BXP-21, ab3380; 1:50; Abcam, Cambridge, UK), anti-P-glycoprotein (EPR10363, ab170903; 1:250; Abcam), anti-CD31 (ab28364; 1:100; Abcam) and anti-human nuclei (MAB4383, 1:200; Merck Millipore, Watford, UK) were used to stain BCRP (rabbit and human), P-gp (rabbit and human), endothelial cells (mouse and human), and human nuclei, respectively.

2.3. Vitreous and plasma protein binding in rabbits

The unbound fraction of drugs determines the distribution processes through the active transport mechanisms involved in the BBB, the BRB and the tumor cells [25]. Thus, we calculated the unbound fraction of lactone and carboxylate topotecan in vitreous and plasma. We used the ultrafiltration method (Centrifree Ultrafiltration Device with Ultracel YM-T membrane, Merck Millipore, Billerica, MA). Briefly, topotecan at clinically relevant concentrations 20 and 100 ng/mL (50:50 mixture of carboxylate and lactone forms) was incubated 10 min in rabbit vitreous and plasma, respectively, at 37 °C, and then ultrafiltrated in triplicate. Vitreous and plasma protein binding were calculated as previously described [26].

2.4. Effect of pantoprazole on topotecan distribution in rabbit vitreous dialysates

A previously described ocular microdialysis sampling method was used to study the concentrations of free (protein-unbound) lactone and carboxylate topotecan in the vitreous of New Zealand albino rabbit eyes [27]. This animal experiment was approved by the local animal experimentation committee (CICUAL 5947817). Each animal (5 rabbits, 10 eyes; purchased from Izaguirre, Buenos Aires, Argentina) received both treatments, *i.e.*, topotecan 0.25 mg/kg i.v. bolus, or topotecan 0.25 mg/kg i.v. bolus preceded by pantoprazole 3.5 mg/kg bolus, 15 min before topotecan. Treatments were separated by 7 days and assigned randomly the first day. The first day of the experiments a central catheter was placed in the right jugular vein of each rabbit to infuse intravenous drugs and to withdraw blood samples under general anesthesia with ketamine-xylazine (37.5 mg/kg and 5 mg/kg). An intravitreal microdialysis probe was sutured to the sclera of the right eye and vitreous dialysates were obtained under general anesthesia [27]. After a 40 min period of equilibration of the probe, the animal received the treatments i.v., vitreous dialysates were obtained every 30 min for 3 h and both topotecan forms (lactone and carboxylate) were quantified immediately by HPLC as previously described [19]. A retrodialysis experiment infusing 500 ng/mL topotecan was performed at the end of the experiment to calibrate the probe, and the animals were recovered [27]. Upon profuse rinsing of the central catheter, blood samples were obtained at 0.05, 0.25, 1.5 and 3 h after the administration of topotecan, plasma was precipitated in cold methanol and supernatants were analyzed immediately by HPLC [19]. Seven days later (day 8) a similar experiment was performed by cannulating the left jugular vein and inserting a microdialysis probe in the left eye before the administration of the second treatment. Thus, after the completion of both experiments, each animal produced paired vitreous and plasma topotecan pharmacokinetic data with and without pantoprazole. Vitreous dialysate and plasma samples were obtained at time zero during the second experiment, to ensure complete wash out of topotecan received in the previous (day 1) experiment. After the end of the second microdialysis experiment animals were euthanized under general anesthesia with a rapid intravenous bolus injection of sodium thiopental (100 mg).

Topotecan *AUC*s were calculated by the trapezoid method to quantify exposure to unbound topotecan (lactone and total) in plasma ($AUC_{u,plasma}$) and vitreous ($AUC_{u,vitreous}$) of each individual experiment. The maximum concentration achieved in plasma (C_{max}) was calculated by extrapolation at time 0 of the initial fast exponential decay curve. The extent of penetration of vitreous by unbound topotecan ($P_{vitreous}$) was calculated as the vitreous-to-plasma *AUC* ratio (Eq. (1)).

$$P_{vitreous} = \frac{AUC_{u,vitreous}}{AUC_{u,plasma}} \quad (1)$$

2.5. Retinoblastoma cultures and xenografts

Seven patient-derived retinoblastoma tumor models (HSJD-RBT-1, HSJD-RBVS-1, HSJD-RBT-2, HSJD-RBVS-3, HSJD-RBT-5, HSJD-RBT-7 and HSJD-RBT-8) were cultured as tumorspheres in neural stem cell medium (serum-free) [28]. Retinoblastoma cell line Y79 was obtained from Sigma-Aldrich and cultured as previously described [28]. Intraocular xenografts were established in immunodeficient mice