International Journal of Pharmaceutics 506 (2016) 371–381

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Expression of glutathione transferases in corneal cell lines, corneal tissues and a human cornea construct

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ARTICLE INFO

Article history:
Received 22 January 2016
Received in revised form 18 April 2016
Accepted 19 April 2016
Available online 22 April 2016

Keywords:
Cornea
Cell culture *in vitro* model
Metabolism
Glutathione transferase
GSTP1-1
GSTO1-1

ABSTRACT

Glutathione transferase (GST) expression and activity were examined in a three-dimensional human cornea construct and were compared to those of excised animal corneas. The objective of this study was to characterize phase II enzyme expression in the cornea construct with respect to its utility as an alternative to animal cornea models. The expression of the GSTO1-1 and GSTP1-1 enzymes was investigated using immunofluorescence staining and western blotting. The level of total glutathione transferase activity was determined using 1-chloro-2,4- dinitrobenzene as the substrate. Furthermore, the levels of GSTO1-1 and GSTP1-1 activity were examined using S-(4-nitrophenacyl)glutathione and ethacrynic acid, respectively, as the specific substrates. The expression and activity levels of these enzymes were examined in the epithelium, stroma and endothelium, the three main cellular layers of the cornea. In summary, the investigated enzymes were detected at both the protein and functional levels in the cornea construct and the excised animal corneas. However, the enzymatic activity levels of the human cornea construct were lower than those of the animal corneas.

1. Introduction

The approval of new active pharmaceutical ingredients (APIs) requires investigation of their efficacy, safety and quality. In general, drug efficacy and safety is preclinically tested using animal models, which have several disadvantages over *in vitro* models and raises ethical concerns. Animal husbandry is time-consuming and cost-intensive and cannot be neglected. Furthermore, most experimental animals must be sacrificed for evaluation of the test results. In addition, poor standardization leads to high variability in the animal-based data. A major concern is to what extent the results of animal experiments are transferable to humans (Reichl et al., 2004; Reichl et al., 2005). Several studies have shown that the corneas of the commonly utilized animal models exhibit a morphology that is slightly different from that of the human cornea. For instance, the thickness of the epithelia and the tightness of their junctions in rabbit and porcine corneas are quite different, resulting in different permeation coefficients of APIs (Hahne and Reichl, 2011). Because of these disadvantages, several cell culture-based *in vitro* models have been developed with the aim of reducing and/or replacing animal testing.

Based on the expected permeation route of APIs, *in vitro* models were created using appropriate cells. In the case of topically applied ophthalmic drugs, the human cornea provides the main permeation barrier (Burstein and Anderson, 1985) and should therefore be reproduced in tissue-engineering labs. Several cell culture models of the cornea have been developed (Pepić et al., 2014; Reichl et al., 2011). However, only a few of these models were tested for the permeation of ophthalmic drugs (Becker et al., 2008; Hahne and Reichl, 2011; Reichl et al., 2004; Reichl and Müller-Goymann, 2001; Tak et al., 2001; Tegtmeyer et al., 2004; Toropainen et al., 2001).

To allow its widespread use as an alternative to animal models, a cornea model should be a three-dimensional (3D) construct produced using immortalized human cells that is highly similar to the human cornea and should be characterized as thoroughly as possible (Kölln and Reichl, 2015; Reichl et al., 2011). The most promising *in vitro* cornea model appears to be the Hemicornea construct, which was established and pre-validated by Hahne et al. (Hahne et al., 2012; Hahne and Reichl, 2011). This model, which contains immortalized human corneal cells (epithelial cells and keratocytes), has been evaluated with respect to the permeation coefficients of selected APIs

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http://dx.doi.org/10.1016/j.ijpharm.2016.04.053
0378-5173/

(Hahne et al., 2012; Hahne and Reichl, 2011) and the expression of drug transporter proteins (Verstraelen and Reichl, 2013, 2014).

However, the intraocular concentration of topically applied ophthalmic drugs does not depend only on the permeation barrier properties of the cornea and its levels of transporter proteins but may also be affected by its content of metabolic enzymes and the metabolic degradation of APIs during trancorneal passage. Although the Hemicornea construct contains cells of human origin, the Simian virus 40 (SV40)-immortalization process used to create them may have modified their protein expression patterns (Greco et al., 2010). Therefore, in a preliminary study, the Hemicornea construct was further characterized by investigating the expression of several phase I and phase II enzymes on the mRNA level relative to those of the human corneal epithelium (Kölln and Reichl, 2012). Briefly, 13 phase I and phase II enzymes were found to be expressed on the messenger RNA (mRNA) level in the Hemicornea construct with an expression pattern that was very similar to that of the *in vivo* human corneal epithelium. To characterize the metabolic properties of the Hemicornea construct, we decided to focus on the phase II enzyme family in the present study. These detoxification enzymes are subdivided into more than four groups but mainly consist of UDP-glucuronosyltransferases, sulfotransferases, N-acetyltransferases and glutathione transferases, with the latter representing one of the major phase II enzyme families (Jancova et al., 2010).

Glutathione transferases (GST) have been discovered in many tissues including the cornea of humans and several other mammals (Awasthi et al., 1980; Bilgihan et al., 2003; Gondhowiardjo and van Haeringen, 1993; Saneto et al., 1982; Sastry et al., 1995; Singh et al., 1985; Watkins et al., 1991). To date, three mammalian GST superfamilies have been identified, consisting of cytosolic proteins, mitochondrial proteins and membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEGs). The cytosolic GSTs form the most highly differentiated superfamily. Cytosolic Alpha (α), Zeta (ζ), Theta (θ), Mu (μ), Pi (π), Sigma (σ) and Omega (ω) classes have been identified in humans. Those seven classes of soluble proteins comprise 17 different enzymes (Hayes et al., 2005). Based on the GST nomenclature proposed by Mannervik et al., GST classes are abbreviated in Roman capital letters and the specific enzymes are differentiated using Arabic numbers (Mannervik et al., 2005). GSTs exist as dimeric proteins. Generally, these enzymes form homodimeric structures, which are indicated using the same Arabic number for each subunit (Mannervik et al., 2005). For example, the homodimeric Pi glutathione transferase, which is composed of two 1 subunits, is abbreviated as GSTP1-1. However, heterodimeric GST structures are known to form, particularly by members of the Alpha and Mu classes (Hayes and Pulford, 1995).

However, to our knowledge, this is the first study focusing on the differences in phase II enzyme expression in the corneal layers. This topic is important because the corneal layers exhibit different barrier properties against APIs. The epithelium is the rate-limiting layer for all substances except small lipophilic compounds (Prausnitz and Noonan, 1998). In contrast, the stroma forms a barrier against lipophilic drugs (Prausnitz and Noonan, 1998). Because of these different barrier properties, the transit times of permeating APIs vary in the different corneal layers. Therefore, a closer investigation of the enzymatic activity in the corneal layers will increase the knowledge concerning possible metabolic transformations of drugs occurring during the transcorneal transport process in both *ex vivo* and *in vitro* models.

Because the glutathione transferases GSTO1-1 and GSTP1-1 were detected at the mRNA level in the Hemicornea construct and human corneal epithelium (Kölln and Reichl, 2012), further investigation of the expression of those enzymes is warranted. Therefore, one aim of the present study was to examine whether GSTO1-1 and GSTP1-1 were also expressed on the protein level in the Hemicornea construct and in the commonly used animal models, the porcine and rabbit cornea. Additionally, the functional activity of glutathione transferases in general and the selected enzymes GSTO1-1 and GSTP1-1 in particular was investigated in these models.

2. Materials and methods

2.1. Materials

Potassium chloride and magnesium sulfate heptahydrate were obtained from Acros Organics (Geel, Belgium). Dulbecco's Modified Eagles Medium (DMEM), epidermal growth factor (EGF), fetal bovine serum (FBS), L-glutamine, Ham's F12, 10× Minimum Essential Medium (MEM), non-essential amino acids (NEAA) and phosphate buffered saline (PBS) were obtained from Biochrom (Berlin, Germany). Transwell® inserts (polycarbonate membrane, 1.12 cm^2, 3.0-μm pore size) were purchased from Corning Costar (Acton, MA, USA). Triton X-100 was obtained from ICN Biomedicals (Aurora, OH, USA). Soybean trypsin inhibitor was acquired from Invitrogen (Karlsruhe, Germany). Keratinocyte Growth Medium (KGM), composed of Keratinocyte Basal Medium (KBM) and SingleQuots supplements containing amphotericin B, bovine pituitary extract (BPE), epidermal growth factor (EGF), gentamicin sulfate, hydrocortisone and insulin, were purchased from Lonza (Rockland, ME, USA). Hydriodic acid and sodium dihydrogen phosphate monohydrate were obtained from Merck (Darmstadt, Germany). Polyvinylidene fluoride (PVDF) blotting membrane (0.45-μm pore size) was purchased from Millipore (Schwalbach, Germany). Amphotericin B, penicillin G sodium salt, streptomycin sulfate and trypsin-EDTA were purchased from PAA (Linz, Austria). The PCR Mycoplasma I/C Test kit was obtained from PromoCell (Heidelberg, Germany). The Lumi-LightPlus Western Blotting kit was purchased from Roche (Mannheim, Germany). Acetic acid, D-glucose monohydrate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium bicarbonate, sodium chloride, sodium dodecyl sulfate (SDS) and sodium hydroxide were obtained from Roth (Karlsruhe, Germany). Tissue culture flasks were obtained from Sarstedt (Nümbrecht, Germany). Acrylamide/bis-acrylamide solution, 2-Bromo-4′-nitroacetophenone, 1-chloro-2,4-dinitrobenzene (CDNB), calcium chloride dihydrate, cholera toxin, dimethyl sulfoxide (DMSO), dithiothreitol (DTT), ethacrynic acid, ethanol, ethylenediaminetetraacetic acid (EDTA), L-glutathione (reduced) (GSH), glycine, 2× Laemmli sample buffer, 2-mercaptoethanol, protease inhibitor cocktail, rat tail collagen, skim milk powder, sucrose, tetramethylethylenediamine (TEMED), Tris(hydroxymethyl)aminomethane (Tris) base, Tris-HCl and Tween 20 were purchased from Sigma (Deisenhofen, Germany). Four-well coverglass chambers, a multicolor broad range protein ladder, Neg 50 frozen sectioning medium and polylysine-coated slides were obtained from Thermo Scientific (St. Leon-Rot, Germany).

The primary monoclonal anti-actin, anti-GSTO1 and anti-GSTP1 antibodies were obtained from Sigma (Deisenhofen, Germany). All of the primary antibodies were produced in mice. The peroxidase-conjugated IgG and fluorescein isothiocyanate (FITC)-conjugated IgG (H + L) secondary antibodies were obtained from Millipore (Schwalbach, Germany). These antibodies were produced in goats. The FITC-conjugated rabbit IgG antibody was purchased from Sigma (Deisenhofen, Germany). All of the secondary antibodies were directed against mouse antigens.

Krebs-Ringer buffer (KRB) contained the following substances per 1000 mL of double-distilled water: 6.8 g sodium chloride, 0.4 g potassium chloride, 0.14 g sodium dihydrogen phosphate monohydrate, 2.1 g sodium bicarbonate, 3.575 g HEPES, 1.1 g D-glucose