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Cytochrome P450 Activity in *Ex Vivo* Cornea Models and a Human Cornea Construct

Christian Kölln, Stephan Reichl*

Institut für Pharmazeutische Technologie, Technische Universität Braunschweig, Braunschweig, Germany

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ABSTRACT

The pharmacokinetic behaviors of novel ophthalmic drugs are often preliminarily investigated in preclinical studies using *ex vivo* animal cornea or corneal cell culture models. During transcorneal passage, topically applied drugs may be affected by drug metabolizing enzymes. The knowledge regarding the functional expression of metabolic enzymes in corneal tissue is marginal; thus, the aim of this study was to investigate cytochrome P450 activity in an organotypic three-dimensional human cornea construct and to compare it with porcine and rabbit corneas, which are commonly used *ex vivo* cornea models. The total cytochrome P450 activity was determined by measuring the transformation of 7-ethoxycoumarin. Furthermore, the expression of the cytochrome P450 enzyme 2D6 (CYP2D6) was investigated at the protein level using immunohistochemistry and western blotting. CYP2D6 activity measurements were performed using a D-luciferin-based assay. In summary, similar levels of the total cytochrome P450 activity were identified in all 3 cornea models. The protein expression of CYP2D6 was confirmed in the human cornea construct and porcine cornea, whereas the signals in the rabbit cornea were weak. The analysis of the CYP2D6 activity indicated similar values for the human cornea construct and porcine cornea; however, a distinctly lower activity was observed in the rabbit cornea.

Introduction

During the development of novel active pharmaceutical ingredients (APIs) or the approval of new drugs, substantial information concerning drug efficacy and safety is required. Furthermore, pharmacokinetic data, such as the drug absorption rate and bioavailability, are also necessary. This information concerns APIs that act systemically, as well as topically applied drugs, such as ophthalmics. In general, preclinical studies are performed using animal models, which exhibit various disadvantages. For example, interindividual differences result in poor standardization, which leads to high variability in the data. Most importantly, the transferability to humans is questionable.[1,2] For example, the expressions of histological characteristics, such as the corneal thickness or epithelial barrier properties of rabbit and porcine corneas, which comprise commonly utilized animal models, are different, which results in distinct permeation behavior of the API.[3] In addition, the collection of experimental data raises ethical problems because most animals must be sacrificed. To reduce or replace animal testing, the development of alternative cell culture models is required. In the case of ophthalmic drugs, the majority of APIs are available as eye drop formulations, whereas the cornea is known to provide the main barrier for topically applied drugs.[4] Over the past 2 decades, several groups have developed various cell culture equivalents of the cornea.[3,5] However, a limited number of these constructs have been tested with regard to the barrier properties against ophthalmic drugs.[1,3,6-10]

The unique corneal structure, which comprises 3 main layers, is one reason for the remarkable barrier properties of the cornea. The outer multilayered epithelium has a hydrophobic character and exhibits a high expression of tight junctions, which results in a strong permeation barrier, especially for hydrophilic compounds and macromolecules.[11] The hydrophilic stroma represents approximately 90% of the corneal thickness and consists of approximately 75% water forming a permeation rate limiting layer for lipophilic substances. The single-layered corneal endothelium is more hydrophobic and only marginally contributes to the drug permeation barrier.[12] Thus, to reproduce the permeation barrier of the human cornea, the cultivation of at least the epithelium and the stromal layer appears to be essential.[13] These multilayered constructs, which are also referred to as three-dimensional (3D) constructs, initially

* *Correspondence to:* Stephan Reichl (Telephone: +49-531-391-5651; Fax: +49-531-391-8108).
E-mail address: s.reichl@tu-braunschweig.de (S. Reichl).

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consisted of primary animal cells and subsequently consisted of immortalized cells of human origin.[14] The latter constructs promise improved standardization combined with increased similarities to humans.

An organotypic 3D cell culture construct of the human cornea that complies with these requirements is the hemicornea construct (HCC), which was established by our group in 2011.[3] For the widespread use of these tissue models in the drug development process, additional characterization must be performed to demonstrate the comparability with the human cornea or the commonly utilized animal models, as well as the usefulness for drug absorption studies. This specifically applies if cell lines are used instead of primary cells because the immortalization procedure may lead to a modified protein expression pattern.[15] The permeation barrier equivalence of the HCC with *ex vivo* cornea models has been extensively demonstrated in drug absorption studies.[3,16] Moreover, similar expression patterns of several efflux drug transporter proteins have been demonstrated for the HCC compared with the human cornea.[17,18] However, in addition to passive diffusion and active drug transport, other factors, such as drug metabolism, may affect APIs during transcorneal passage. For example, since decades corneal esterases are used for the prodrug approach of many APIs, such as latanoprost or dipivefrine, to improve their ocular bioavailability. These phase I enzymes convert the prodrug to its active form[19] and have previously been proven in a precursor model of the HCC.[20]

However, in view of hepatic metabolism, other phase I enzymes are primarily involved in drug metabolism. In the human liver, approximately 75% of drug metabolism is attributed to cytochrome P450 enzymes (CYP),[21] whereas 5 main CYP enzymes (1A2, 2C9, 2C19, 2D6, and 3A4) are actively involved.[22,23] The CYPs represent a group of hemoproteins that are embedded in the endoplasmic reticulum.[24] The reaction with a suitable substrate proceeds in several steps and is initiated with the reduction of Fe^{3+} to Fe^{2+} in the heme moiety by the NADPH-P450 reductase, which is also located in the endoplasmic reticulum.[24] Briefly, several subsequent reaction steps lead to the formation of FeO^{3+}, which is a reactive intermediate that oxidizes the substrate.[23] The cytochrome P450 nomenclature is based on the sequence identity. The different enzymes are grouped into families (Arabic numbers), subfamilies (roman capitals), and individual enzymes (Arabic numbers).[23] By date, approximately 53 different human CYP enzymes have been identified in humans.[23] CYPs exhibit various functions in mammals.[23] For example, they are responsible for the metabolization of benzo[a]pyrene as a component of city smog or cigarette smoke, as well as halogenated hydrocarbons and aromatic amines.[25] Furthermore, they catalyze the metabolization of many drugs, such as antibiotics, chemotherapeutic agents, and other common APIs.[25]

CYPs exhibit a widespread distribution in the human body, including the ocular tissues. To date, phase I activity has been identified inter alia in the porcine lens,[26] as well as in bovine, porcine, and rabbit retinas.[26-29] Furthermore, phase I activity has been demonstrated in the bovine and porcine iris,[26,27] rabbit conjunctiva,[29] and bovine and porcine ciliary bodies.[26-28] Similarly, phase I enzyme activity has been identified in bovine, porcine, rabbit, and human corneas.[26-31] In the cornea, CYPs have been demonstrated as a third mechanism to metabolize arachidonic acid in addition to cyclooxygenase and lipoxygenase.[32] The 2 major metabolites, 12(R)-hydroxy-5,8,10,14-eicosatetraenoic acid and 12(R)-hydroxy-5,8,14-eicosatrienoic acid, inhibit Na-K-ATPase, increase corneal thickness, and induce intraocular pressure.[33] Moreover, aryl hydrocarbon hydroxylase activity, which is associated with CYP1A1, was detectable in the bovine cornea.[27] ω hydroxylase activity, which is associated with CYP4A activity,[34] has been reported in the porcine cornea.[26] A CYP4B1 isoform that is involved in the metabolization of arachidonic acid may be present in the rabbit cornea.[35] In addition, 7-ethoxycoumarin *O*-deethylase activity, which is associated with several P450 enzymes,[36] was detectable in bovine[28] and rabbit corneas.[29]

However, little is known regarding the corneal expression of CYPs involved in drug metabolization. Reliable data are primarily available regarding the messenger RNA (mRNA) level for the expression of cytochromes, including 2A6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5, in the human cornea.[37,38] To date, information regarding their functional expression is lacking. In addition, investigations regarding CYP expression in the HCC are critical for further characterization of this cornea model. Thus, in a preliminary study, we examined the CYP expression at the mRNA level as an initial step.[38] Briefly, with the exception of CYP2C19, all previously identified cytochrome P450 enzymes of the human cornea were recovered in the HCC.

Based on these promising findings, the aim of this study was to investigate the functional expression of CYPs in the HCC. Because of the poor availability of freshly isolated human donor corneas, the obtained results were compared with porcine and rabbit corneas. The cytochrome P450 activity was monitored via the measurement of 7-ethoxycoumarin *O*-deethylase activity using a fluorescence method. 7-Ethoxycoumarin is metabolized by the CYPs 1A2, 2A6, 2B6, 2C8, 2C9, 2D6, 2E1, 3A3, 3A4, 3A5, and 4B1.[36] This compound is a substrate for a substantial number of CYPs; thus, the measured activity hereinafter is referred to as the total cytochrome P450 activity. In our previous mRNA study, the highest expression level in the corneal cell lines was identified for CYP2D6. Furthermore, timolol maleate, which is routinely used in the treatment of glaucoma disease, has been described as a substrate for CYP2D6.[39] Thus, knowledge regarding the metabolic activity of CYP2D6 in the HCC compared with the commonly utilized animal models was of particular interest. The presence of CYP2D6 in the corneal tissues and construct was investigated using immunohistochemistry and western blot techniques. The CYP2D6 activity was subsequently analyzed with a commercially available kit based on a luminescence measurement. Moreover, the CYP expression was investigated in each single layer (epithelium, stroma, and endothelium) of the *in vitro* and *ex vivo* cornea models.

Materials and Methods

Materials

Potassium chloride and magnesium sulfate heptahydrate were obtained from Acros Organics (Geel, Belgium). Dulbecco's modified eagle's medium, epidermal growth factor (EGF), fetal bovine serum (FBS), L-glutamine, Ham's F12, 10× minimum essential medium (MEM), non-essential amino acids, and phosphate buffered saline (PBS) were obtained from Biochrom (Berlin, Germany). Transwell® inserts (polycarbonate membrane 1.12 cm^2, pore size 3.0 μm) were purchased from Corning Costar (Acton, MA). Triton X-100 was obtained from ICN Biomedicals (Aurora, OH). Soybean trypsin inhibitor was acquired from Invitrogen (Karlsruhe, Germany). Keratinocyte growth medium (KGM), which was composed of keratinocyte basal medium and SingeQuots supplements that contained amphotericin B, bovine pituitary extract, EGF, gentamicin sulfate, hydrocortisone, and insulin, was purchased from Lonza (Rockland, ME). Sodium borate and sodium dihydrogen phosphate monohydrate were obtained from Merck (Darmstadt, Germany). A polyvinylidene fluoride blotting membrane (0.45 μm pore size) was purchased from Millipore (Schwalbach, Germany). Amphotericin B, penicillin G sodium salt, streptomycin sulfate, and trypsin-EDTA (ethylenediaminetetraacetic acid) were obtained from PAA (Linz, Austria). A Lumi-Light^{Plus} Western Blotting Kit was