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A primary fish gill cell culture model to assess pharmaceutical uptake and efflux: Evidence for passive and facilitated transport

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ABSTRACT

The gill is the principle site of xenobiotic transfer to and from the aqueous environment. To replace, refine or reduce (3Rs) the large numbers of fish used in *in vivo* uptake studies an effective *in vitro* screen is required that mimics the function of the teleost gill. This study uses a rainbow trout (*Oncorhynchus mykiss*) primary gill cell culture system grown on permeable inserts, which tolerates apical freshwater thus mimicking the intact organ, to assess the uptake and efflux of pharmaceuticals across the gill. Bidirectional transport studies in media of seven pharmaceuticals (propranolol, metoprolol, atenolol, formoterol, terbutaline, ranitidine and imipramine) showed they were transported transcellularly across the epithelium. However, studies conducted in water showed enhanced uptake of propranolol, ranitidine and imipramine. Concentration-equilibrated conditions without a concentration gradient suggested that a proportion of the uptake of propranolol and imipramine is *via* a carrier-mediated process. Further study using propranolol showed that its transport is pH-dependent and at very low environmentally relevant concentrations (ng L^{-1}), transport deviated from linearity. At higher concentrations, passive uptake dominated. Known inhibitors of drug transport proteins; cimetidine, MK571, cyclosporine A and quinidine inhibited propranolol uptake, whilst amantadine and verapamil were without effect. Together this suggests the involvement of specific members of SLC and ABC drug transporter families in pharmaceutical transport.

1. Introduction

There are currently over 140,000 compounds that are being reassessed for their bioconcentrative properties as a part of the EU Registration, Evaluation, Authorization & Restriction of Chemicals (REACH) initiative (REACH, 2009). Conventionally, the main determinant used for assessing bioconcentration of a compound is the octanol–water partitioning coefficient (K_{ow}), a measure of hydrophobicity that drives sorption and accumulation, and a main input parameter in quantitative structure–activity relationships (QSARs) (Hansch, 1969). However, this may not be fully applicable to pharmaceuticals, many of which are polar and ionizable (Hermens et al., 2013). In this case, the pH-corrected octanol–water partitioning coefficient, D_{ow}, may be used, but this fails to take into account other major interactions such as hydrogen bonding and van der Waals forces, as well as uptake *via* carrier-mediated processes (Dobson and Kell, 2008; Sugano et al., 2010).

In vivo ecotoxicology testing produces bioconcentration factor (BCF) values that indicate the potential of a compound to bioconcentrate within an organism ($OECD_{305}$, 2012). Fish are exposed to highly lipophilic compounds *via* the diet, whilst to others *via* the water; the principle being to use the uptake and depuration rates to calculate the propensity of a compound to bioconcentrate. Typically, each test can use up to 108 fish per compound and many thousands of fish are used for this test every year (Scholz et al., 2013). There is currently a desire to develop alternative methods to replace these standardized whole fish studies to recognize and classify environmental hazards (Creton et al., 2013; Wolf et al., 2007). This requires the identification and validation of appropriate *in vitro*

Abbreviations: 3Rs, reduce, refine, replace; A, apical; ABC, ATP-binding cassette; B, basal; BCF, bioconcentration factor; BTA, bi-directional transport assay; CETA, concentration equilibrated transport assay; Ci, Curie; Dpm, disintegrations per minute; K_{ow}, octanol–water partitioning coefficient; MRP, multidrug resistance protein; OCT, organic cation transporter; OECD, Organisation for Economic Co-operation and Development; P_{app}, apparent permeability coefficient; pK_a, dissociation constant; QSARs, quantitative structure–activity relationships; REACH, Registration, Evaluation, Authorization & Restriction of Chemicals; SLC, solute carrier; TER, transepithelial resistance; TEP, transepithelial potential; TR, transport ratio.

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systems that could replace such studies (Baron et al., 2012; Uchea et al., 2013). To find alternatives to the $OECD_{305}$ water exposure it is necessary to identify a suitable fish gill model that mimics the intact organ because the gill, being constantly and continuously exposed to substances in water, is the principle site of xenobiotic uptake (Bury et al., 2014).

Fletcher et al. (2000) developed a double-seeding technique that enables primary gill cells to be cultured on permeable membrane inserts in a two-compartment model. This cultured epithelium comprises the different cell types (mitochondrial rich cells, respiratory cells and mucus cells) found in the gill and produces high transepithelial resistance (reviewed by Bury et al., 2014). Importantly, the system is able to tolerate apical freshwater and produces a negative transepithelial potential, further simulating the *in vivo* scenario. This is crucial when investigating the transport of ionizable compounds such as pharmaceuticals that may behave differently in culture medium and water. Furthermore, the gill cells from two fish can be used to create up to 72 individual gill epithelial inserts for assaying, thus potentially reducing numbers of fish in *in vivo* testing.

The present study thus aims to use the *in vitro* gill to investigate the uptake and efflux of seven pharmaceuticals representing a range of classes. We hypothesize that both passive transcellular and carrier-mediated transport of xenobiotics across the gill are likely principle drivers in determining the rate of uptake of waterborne compounds (Mckim and Erickson, 1991). Passive transcellular transport depends on the pH of the solution, acid–base constants (pK_a) and the lipophilicity of the compound, whereas facilitated transport may be *via* members of the solute carrier (SLC) and ATP-binding casette (ABC) transporter families (Dobson and Kell, 2008). Therefore, to investigate carrier-mediated transport for some of these pharmaceuticals, concentration-equilibrated, pH-dependent, and concentration-dependent assays, as well as membrane channel inhibitor studies were conducted. In the context of this work, paracellular transport refers to the movement of compounds over membranes between cells and passive transport refers to concentration-dependent transcellular processes, whereas facilitated transport indicates concentration-independent carrier-mediated transport *via* membrane channel proteins. In addition, the uptake of propranolol across the *in vitro* gill model was compared to *in silico* and *in vivo* data (Owen et al., 2009), to demonstrate the use of this model as a predictive tool for pharmaceutical uptake.

2. Materials and methods

2.1. Animal husbandry

Gill cells for the use in primary cultures were obtained from juvenile diploid rainbow trout weighing 50–120 g purchased from a trout farm (Hampshire, UK). Fish were acclimatized in three 1000 L fiberglass aquaria at King's College, London, and maintained at 13–14 °C in recirculating aerated city of London tap water ($[Na^+]$ = 0.53 mM, $[Ca^{2+}]$ = 0.92 mM, $[Mg^{2+}]$ = 0.14 mM, $[K^+]$ = 0.066 mM and $[NH_4^+]$ = 0.027 mM), which was passed through carbon, mechanical and biological filters. Photoperiod was maintained at a constant 14 h light/10 h dark cycle and fish were fed a daily 1% (w/w) ration of fish chow.

2.2. Gill cell culture

Sterile techniques were used throughout all cell culture procedures. Equipment, containers and solutions were autoclaved or sterile filtered (0.2 μm, Corning). The gill cell isolation procedure was based on methods previously documented (Fletcher et al., 2000) and the cell culture double-seeded insert (DSI) technique as described by Walker et al. (2008) and Wood et al. (2002). Briefly, primary gill cells are isolated, washed and resuspended in L-15 medium (Invitrogen) supplemented with FBS (5% (v/v)) (Sigma) and seeded onto a permeable polyethylene terephthalate (PET) membrane inserts with 0.4 μm pores with an area of 0.9 cm^2 and maintained at 18 °C. This Transwell system (Corning) has an apical compartment above and a basal compartment below.

The development of an intact and electrically tight gill epithelium was monitored daily through 'blank'-corrected measurements of transepithelial resistance (TER) using a custom-modified epithelial tissue voltohmeter (EVOMX; World Precision Instruments) fitted with chopstick electrodes (STX-2). The same device was used to measure transepithelial potential (TEP) before and after freshwater application. DSI epithelia that reached a TER of ≥5 kΩ cm^2 were considered developed and electrically 'tight' for experimental procedures. In this instance, DSI preparations were washed twice with PBS (to remove any media supplemented with FBS) and exposed to radiolabeled pharmaceuticals apically in either L-15 medium (without FBS) or freshwater (2.0 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.8 mM $NaHCO_3$, 77.1 μM KCl at pH 7.7), or basally, always in L-15 medium. L-15 medium has an osmolarity of 300–320 mOsm kg^{-1} (Invitrogen) and that of freshwater is around 15 mOsm kg^{-1}. All experiments and exposures are based on individual inserts (n) derived from at least one biological replicate. Due to the seeding procedure over two days, one biological replicate is derived from two fish.

2.3. Membrane permeability

Paracellular permeability was measured using the paracellular marker ^{14}C-mannitol (20 Ci $mmol^{-1}$, Amersham Biosciences, CAS no. 88404-24-4). Thirty-seven DSI epithelia with TER values ranging from 0 to 14 kΩ cm^2 were exposed to 0.013 μCi (2.2×10^5 dpm) ^{14}C-mannitol in 1.5 mL sterile freshwater in the apical compartment, with 2.0 mL L-15 medium in the basal (Hubatsch et al., 2007). From this, a TER value at which paracellular transport is at its most minimal can be deduced as a threshold for when epithelia are ready for transport assays (≥5 kΩ cm^2). Aliquots of 100 μL were taken from the apical and basal compartments at time 0 and 24 h, and placed in 2 mL liquid scintillation fluid (Ecolume) and radioactivity measured by beta counting (Tri Carb 460CD liquid scintillation system; Packard). Mannitol flux after 24 h was calculated using Eq. (1):

$$\text{Permeability}\,(\text{cm s}^{-1}) = \frac{[\Delta M]_{BL} \times \text{volume}}{M_{AP} \times \text{time} \times 3600 \times \text{area}} \quad (1)$$

where $[\Delta M]_{BL}$ is the change in radioactivity in the basal compartment, M_{AP} is the radioactivity at the start, time is 24 h and area is 0.9 cm^2 (Fletcher et al., 2000).

2.4. Radiolabeled pharmaceuticals

All drugs used in transport assays were at a concentration of 1 μg L^{-1} to represent the levels detected in the environment whilst remaining within detectable limits (Table 1). These were purchased radiolabeled and re-suspended in ethanol or methanol with a final solvent concentration in assay conditions of <0.0003%, and chosen to demonstrate a range of different classes (β_1-, β_2- and non-specific β-receptor agonists, a H_2-receptor agonist and a tricyclic anti-depressant) with mid-range log K_{ow} values (see Table 1). This method of using labeled compounds allows for the recovery of label during cell-free conditions to calculate the amount that sticks to plastic ware. Furthermore, the label may be detected as either the parent compound or biotransformed products. ^{3}H-propranolol hydrochloride (29.0 Ci $mmol^{-1}$,