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Biorelevant media resistant co-culture model mimicking permeability of human intestine

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ABSTRACT

Cell culture models are currently used to predict absorption pattern of new compounds and formulations in the human gastro-intestinal tract (GIT). One major drawback is the lack of relevant apical incubation fluids allowing mimicking luminal conditions in the GIT. Here, we suggest a culture model compatible with biorelevant media, namely Fasted State Simulated Intestinal Fluid (FaSSIF) and Fed State Simulated Intestinal Fluid (FeSSIF). Co-culture was set up from Caco-2 and mucus-secreting HT29-MTX cells using an original seeding procedure. Viability and cytotoxicity assays were performed following incubation of FeSSIF and FaSSIF with co-culture. Influence of biorelevant fluids on paracellular permeability or transporter proteins were also evaluated. Results were compared with Caco-2 and HT29-MTX monocultures. While Caco-2 viability was strongly affected with FeSSIF, no toxic effect was detected for the co-cultures in terms of viability and lactate dehydrogenase release. The addition of FeSSIF to the basolateral compartment of the co-culture induced cytotoxic effects which suggested the apical mucus barrier being cell protective. In contrast to FeSSIF, FaSSIF induced a slight increase of the paracellular transport and both tested media inhibited partially the P-gp-mediated efflux in the co-culture. Additionally, the absorptive transport of propranolol hydrochloride, a lipophilic β-blocker, was strongly affected by biorelevant fluids. This study demonstrated the compatibility of the Caco-2/HT29-MTX model with some of the current biorelevant media. Combining biorelevant intestinal fluids with features such as mucus secretion, adjustable paracellular and P-gp mediated transports, is a step forward to more realistic *in-vitro* models of the human intestine.

1. Introduction

Intestinal barrier models have been extensively developed over the last 20 years to predict drug absorption. Such cell line based models exhibit morphological and functional properties similar to cells from the human intestine. For instance, the Caco-2 cell line, one of the major representatives of these cell lines, develop microvilli and are inter-linked by tight junctions and express numerous transporters such as the efflux pump P-glycoprotein (P-gp). Associations of different cell types in one cell lines were suggested to turn these models more realistic and come closer to the barrier properties of the human small intestine (Antunes et al., 2013; Lasa-Saracíbar et al., 2014). The combination of Caco-2 and HT29-MTX mucin-secreting cells in one co-culture model has been reported, elucidating that the confluent cells were covered by a mucin layer and exhibited tailor-made barrier properties regarding the P-gp expression and paracellular permeability (Béduneau et al., 2014; Calatayud et al., 2012; Chen et al., 2010).

The choice of transport buffer is also critical to predict accurately the *in-vivo* fate of formulations, excipients as well as

Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; DPBS, Dulbecco's phosphate-buffered saline; FaSSIF, Fasted State Simulated Intestinal Fluid; FCS, fetal calf serum; HBSS, Hank's balanced salt solution; HCl, hydrochloride; FeSSIF, Fed State Simulated Intestinal Fluid; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LDH, lactate dehydrogenase; LY, Lucifer yellow; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide); MTX, methotrexate; NACC, (*N*-acetyl cysteine); P_{app}, permeability coefficient; P-gp, P-glycoprotein; TB, transport buffer; TEER, transepithelial electrical resistance.

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drugs. Bioavailability studies are usually performed in saline solution like Hanks' balanced salt solutions (HBSS) supplemented with D-glucose and HEPES. However, the physicochemical and biochemical properties of HBSS-like buffers are very different from the intestinal fluid. Biorelevant media simulating the fluid in the upper small intestine under preprandial and postprandial conditions were suggested and named "Fasted State Simulated Intestinal Fluid" (FaSSIF) and "Fed State Simulated Intestinal Fluid" (FeSSIF) accordingly (Dressman et al., 1998). To mimic the human intestinal fluids, various parameters were then adjusted in FeSSIF and FaSSIF, including pH, osmolality and concentrations of sodium taurocholate (NaTC) and lecithin. Biorelevant media were first used to predict *in-vitro* dissolutions of oral dosage forms. However, a study in healthy human volunteers emphasized significant differences between FaSSIF and FeSSIF compared with the *in-vivo* conditions (Porter et al., 2007). In consequence, their compositions were then revised (FaSSIF-V2 and FeSSIF-V2) (Jantratid et al., 2008). Maleate buffer added to FaSSIF-V2 was used to adjust pH at 6.5 and can delay the oxidation of lipophilic components while the addition of pancreatin in FaSSIF-V2 was considered as optional (Klein, 2010). Nevertheless, pancreatic lipases are involved in the digestion of glycerides and lipid-based excipients and consequently, they may affect the drug release from formulations.

The association of biorelevant fluids with cellular models of intestinal barrier would represent a promising strategy to predict more accurately the bioavailability of drugs, especially the influences from new excipients and smart dosage forms. Such a strategy is currently limited by the cytotoxicity of the proposed media on the cell culture models

Essentially all the components, acidic pH, presence of surfactants in high concentrations and enzymes for the FeSSIF-V2 may alter the viability and the functionality of cells. Ingels et al. (2002) showed that FaSSIF did not affect the integrity of Caco-2 monolayers whereas a toxic effect of FeSSIF on Caco-2 cells was reported.

This present work evaluates the possibility to combine biorelevant media with a mucus producing *in-vitro* co-culture model of the intestinal barrier. HT29-MTX/Caco-2 co-cultures were exposed to FaSSIF and FeSSIF close to the updated V2 version during 2 h and the influence on cell viability was recorded. Then, integrity barrier was assessed by TEER measurements and paracellular permeability. Effects of simulated intestinal media on the P-gp-mediated transport and on the absorption of a lipophilic compound were studied in comparison to Caco-2 and HT29-MTX cells in monoculture. Finally, stability of the co-culture during repeated exposures to FeSSIF was assessed by transport studies and TEER measurements.

2. Materials and methods

2.1. Cells and reagents

Caco-2 cells were obtained from American Type Culture Collection (ATCC) at passage 47. The HT29-MTX cell line was kindly provided by Dr. Thécla Lesuffleur from INSERM UMR S 938 in Paris, France, at passage 13. All the cell culture reagents were purchased from Fisher Scientific (Illkirch, France). Sodium taurocholate, Lucifer yellow ((6-amino-2,3-dihydro-1,3-dioxo-2-hydrazinocarbonylamino-1H-benz(d,e) isoquinoline-5,8-disulfonic acid dilithium salt), verapamil HCl (5-(*N*-(3,4-dimethoxyphenylethyl) methylamino)-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile hydrochloride), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), D-glucose, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), NACC (*N*-acetyl cysteine), pancreatin, PFA (paraformaldehyde), Pierce LDH Cytotoxicity Assay kit, rhodamine 123, alcian blue and sodium hydroxide were provided by Sigma-–Aldrich (Saint-Quentin-Fallavier, France). Propranolol hydrochloride and digoxin were purchased from Tokyo Chemical Industry (Tokyo, Japan). Mouse UIC2 anti-P-gp antibody, Alexa fluor® – conjugated goat anti-mouse secondary antibody and mouse control IgG2a isotype were provided by Abcam (Paris, France).

2.2. Cell culture conditions

Culture media was composed of Dulbecco's Modified Eagle Media Gluta-Max™ containing 15% of inactivated fetal bovine serum, 1% non-essential amino acids and 1% antibiotics (100 μg/ml streptomycin and 100 UI/ml penicillin). Caco-2 and HT29-MTX were seeded at densities of 5000 and 10,000 cells per well, respectively. For the co-culture monolayer, Caco-2 and HT29-MTX were seeded at days 0 and 3, respectively. Monolayers were used for transport experiments between day 21 and 31 post Caco-2 seeding.

A transport buffer (TB) was used for all permeability experiments. TB was composed of 25 mM D-(+)-glucose and 10 mM HEPES in Hank's balanced salt solution, with Ca^{2+} and Mg^{2+}, adjusted at pH 7.4 with 1 M sodium hydroxide solution.

2.3. Preparation of FaSSIF and FeSSIF

Formulations of FaSSIF and FeSSIF were performed from the updated compositions of FaSSIF-V2 and FeSSIF-V2 except for NaTC and pancreatin (Jantratid et al., 2008). The composition is reported in Table 1. Preparation of biorelevant media was mainly established according the procedures developed by Jantratid et al. (2008) and Klein (2010). Briefly, blank FaSSIF was prepared with maleic acid, sodium hydroxide, sodium chloride in deionized water. After incorporation of NaTC in blank FaSSIF, phosphatidylcholine was added. The formulation was gently stirred during 4 h to obtain a clear micellar solution. Blank FeSSIF was first prepared by adding maleic acid, sodium hydroxide and calcium chloride in deionized water. Solution at 50 U lipase/ml was obtained after centrifugation of a suspension of pancreatin in blank FeSSIF at 15,000 g and at 4 °C.

NaTC was added to FeSSIF solution followed by phosphatidylcholine previously dissolved in dichloromethane at an initial concentration of 100 mg/ml. Glycerol monooleate in dichloromethane and sodium oleate in water were finally added. Mixture was then stirred for at least 1 h under a hood in order to evaporate dichloromethane. Vial containing FeSSIF was weighted before and after evaporation to confirm the complete elimination of organic

Table 1
Compositions of transport buffer, FaSSIF and FeSSIF.

	Transport buffer	FaSSIF	FeSSIF
Hank's balance salt solution	500 ml	–	–
D-(+)-glucose	25 mM	–	–
HEPES	10 mM	–	–
Sodium chloride	–	68.6 mM	125.5 mM
Calcium chloride	–	–	5 mM
Maleic acid	–	19.1 mM	55.05 mM
Sodium hydroxide	–	34.8 mM	81.7 mM
Sodium oleate	–	–	0.8 mM
Glycerol monooleate	–	–	5 mM
Sodium taurocholate	–	3 mM	5 mM
Phosphatidylcholine	–	0.2 mM	2 mM
Pancreatin	–	–	50 U/ml (lipase)
Sodium hydroxide 1N	q.s. ad pH 7.4	q.s. ad pH 6.5	q.s. ad pH 5.8