International Journal of Pharmaceutics 370 (2009) 68–74

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

# HO-1-u-1 model for screening sublingual drug delivery—Influence of pH, osmolarity and permeation enhancer

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ARTICLE INFO

*Article history:*
Received 3 July 2008
Received in revised form
14 November 2008
Accepted 15 November 2008
Available online 24 November 2008

*Keywords:*
HO-1-u-1
Sublingual delivery
β-Blocker
pH
Osmolarity
Enhancer

ABSTRACT

HO-1-u-1 (Ueda-1) is a human tumor cell line established from human sublingual squamous cell carcinoma. In previous study, HO-1-u-1 cell line was grown on cell culture inserts and utilized as in vitro model for screening sublingual drug delivery. The aim of current study was to further investigate the effects of pH, osmolarity and permeation enhancer, sodium glycodeoxycholate (GDC) on the permeability of three β-blockers with different lipophilicities. The cytotoxicity was evaluated by MTS/PES assay. The permeability studies were carried out using the cell culture model and compared with that obtained from fresh porcine sublingual mucosa. The results showed the enhancement effects caused by pH, osmolarity and GDC were highly lipophilicity-dependent and in the order atenolol > metoprolol > propranolol. The apparent permeability coefficients ($P_{app}$) of all the three β-blockers were significantly increased by increasing pH. However, less enhancing effects were observed by non-physiological osmolarity or the presence of GDC in permeability study using both cell culture and porcine sublingual mucosa. The present results suggested that the HO-1-u-1 cell culture model maybe a useful and effective in vitro model for evaluating the enhancement effects and mechanism in sublingual drug delivery.



## 1. Introduction

Sublingual mucosa offers an attractive route of administration for systemic drug delivery. In comparison to oral drug administration, drug can be directly absorbed via sublingual mucosa and then delivered into systemic circulation, thus the gastrointestinal degradation and first-pass metabolism in liver will be bypassed (Squier and Hall, 1985). However, due to the small absorption area and diluting effect by saliva, the absorption is comparatively slow for the compounds with high molecular weight or low lipophilicity. To enhance sublingual absorption and improve bioavailability, permeability enhancing methods could be evaluated and applied in the sublingual dosage forms. The most commonly used enhancing methods include altering pH and osmolarity, or adding permeation enhancers (Nielsen and Rassing, 1999). To evaluate sublingual absorption as well as these enhancing approaches, in vivo perfusion models using animals with large sublingual area (i.e. dogs, pigs and sheep) have been developed (Rathbone, 1996). These approaches, however, are complicated, time-consuming, and with high costs. Thus simple in vitro models from animal sublingual epithelium, or cell culture should be developed for evaluation of sublingual drug delivery.

HO-1-u-1 (Ueda-1) is a human tumor cell line established from human sublingual squamous cell carcinoma (Miyauchi et al., 1985). In our recent study, this cell line grown on cell culture insets has been validated as an in vitro model for sublingual drug delivery screening (Wang et al., 2007). The preliminary validation works revealed that HO-1-u-1 cells grown on suitable cell culture inserts differentiated into stratified epithelial-like morphology with histological feathers resembling the human sublingual epithelium. The transport studies further showed the cell layers may provide a permeability barrier to both hydrophilic and lipophilic markers and β-blockers.

To further validate HO-1-u-1 cell culture model for screening sublingual drug delivery, the enhancing methods described above will be evaluated by measuring the permeability of three β-blockers with similar molecular weight (250–300) and p$K_a$ (9.2–9.7), and great variance in lipophilicity. In vitro permeability studies using porcine sublingual mucosa will be also conducted and compared with cell culture model.

## 2. Materials and methods

### 2.1. Materials

HO-1-u-1 cell line derived from the squamous cell carcinoma of the floor of the mouth was purchased from Health Science Research Bank (Tokyo, Japan). Dulbecco's Modified Eagle Medium

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doi:10.1016/j.ijpharm.2008.11.010

(high glucose, with L-glutamine, 110 mg/ml sodium pyruvate and pyridoxine hydrochloride), Ham' F12 Nutrient Mixture (with L-glutamine), Hank's Balanced Salt Solution (HBSS), Trypsin-EDTA (0.25%, 1 mM EDTA), Penicillin-Streptomycin (100 IU/ml), Gentamicin (10 mg/ml), Fetal Bovine Serum (USA, qualified) and other cell culture mediums were purchased from Invitrogen Life Sciences (Hong Kong). BD Falcon cell culture inserts (polyethylene terephthalate, 0.45 μm pore size) and BD Falcon six-well culture plates (TC-treated, notched) were obtained from Becton Dickinson Labware (NJ, USA). Tissue culture flasks (75 $cm^2$, TC-treated) and other cell culture consumables were supplied by IWAKI (Tokyo, JP). CellTiter96® $AQ_{ueous}$ One Solution Cell Proliferation Assay was provided by Promega Co. (Madison, WI, USA). Atenolol, (±)-metoprolol tartrate salt, (±)-propranolol hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO, USA). [$^3$H]-water, [$^3$H]-testosterone (250 μCi) and [$^{14}$C]-mannitol (50 μCi) were ordered from Amersham Biosciences (Buckinghamshire, UK). OptiPhase HiSafe 3 scintillation cocktail were supplied by PerkinElmer Life Sciences (Turku, Finland).

### 2.2. Determination of distribution coefficient (log D) of selected β-blockers

1-Octanol and deionized water were pre-equilibrated at room temperature (20 °C) for 24 h. After separation of the two phases, exactly 3 ml of octanol was transferred into a screw capped glass tube, and then mixed with the 3 ml drug solutions (200 μg/ml in 0.05 M phosphate buffer) with various pH levels at room temperature. The two phases were allowed to continuously equilibrate using a tube rotator. After 24 h, the two phases were left to separate and the concentration of drug in buffer and octanol were measured using the HPLC methods described by Wang et al. (2007). Each experiment was performed in four replicates. The distribution coefficient was determined according to the following equation:

$$\log D = \log \frac{C_w V_w}{C_{oct} V_{oct}} \quad (1)$$

where $C_w$ and $C_{oct}$ are drug concentrations in aqueous phase and octanol phase after partition experiment. $V_w$ and $V_{oct}$ are the volume of aqueous phase and octanol phase, respectively.

### 2.3. Cell cultures

HO-1-u-1 cells were maintained in 75 $cm^2$ T-flasks and incubated at 37 °C in 90% relative humidity atmosphere of 5% $CO_2$ and 95% air. The culture medium consisted of Dulbecco's Modified Eagle's Medium - F12 Medium (1:1 mixed) with 10% fetal bovine serum, 50 μg/ml gentamicin, 100 IU/ml penicillin and 100 μg/ml streptomycin. When 70% cell confluence was reached, the cells were dissociated by 0.25% trypsin-EDTA and then seeded in the BD-Falcon filter inserts at a density of $1.5 \times 10^5$ cells/well. Cells were cultured in the inserts at 37 °C with 2 ml cell culture medium in the apical chamber and 2.5 ml in the basolateral chamber, and incubated for 21–23 days. The culture medium was changed 3–4 times a week. Cells with passage number from 1 to 11 were used.

### 2.4. Cytotoxic effect of pH, osmolarity and sodium glycodeoxycholate on HO-1-u-1 cell

To investigate cytotoxic effects from non-physiological pH, isotonic phosphate buffers with pH ranging from 2 to 11 were prepared using 85% phosphoric acid or 20% sodium hydroxide. Sodium chloride solutions (pH 7.4) with osmolarity ranging between 0 and 550 mOsm were also prepared. Sodium glycodeoxycholate was dissolved in isotonic phosphate buffers (pH 7.4) with final concentration between 0 and 5 mM for the cytotoxic assay.

HO-1-u-1cells were seeded at the density of $1 \times 10^4$ cells/well ($n = 4$) in a 96-well culture plate. The plate was incubated in the cell culture incubator for 48 h. After removing the cell culture medium, 100 μl testing solutions with various pH, osmolarity or GDC were added to each well. The plate was then incubated for 5 h in the incubator. The testing solution was removed, and rinsed with 100 μl HBSS. The cells received 20 μl MTS/PES reagent, then diluted by 100 μl HBSS. The period of incubation was 4 h, the optical density (OD) at 490 nm was recorded and the relative cell activity was calculated according to the following equation (Wang et al., 2007):

$$\text{Relative cellular activity } (\%) = \frac{OD_{test} - OD_{blank}}{OD_{control} - OD_{blank}} \times 100 \quad (2)$$

where $OD_{test}$ is the OD of wells with cells, testing solution and MTS/PES reagent; $OD_{control}$ is the OD of wells with cells, HBSS and MTS/PES reagent; $OD_{blank}$ is the OD of wells with HBSS and MTS/PES reagent and in absence of cells.

### 2.5. pH effect on the integrity of HO-1-u-1 cell culture model

The pH effect on the integrity of HO-1-u-1 cell culture model was performed by measuring the apparent permeability coefficients ($P_{app}$) of hydrophilic marker [$^{14}$C]-mannitol and lipophilic marker [$^3$H]-testosterone across cell layers. These markers represent paracellular and transcellular transports, respectively. [$^{14}$C]-mannitol and [$^3$H]-testosterone were dissolved in HBSS with a final concentration of 200 and 500 nCi/ml, respectively. Initially, the cell culture medium in the apical and basolateral compartments was removed, and the compartments were rinsed twice by HBSS and equilibrated at 37 °C in HBSS for 20 min. Equilibrating HBSS was then removed followed by adding 1.5 ml pre-warmed (37 °C) test solution to the apical compartment. The samples (200 μl) were taken from the basolateral compartment every 15 min for 2 h, and the volume of HBSS was replaced after each sampling.

Each sample was mixed with a 2 ml scintillation cocktail and the radioactivity was determined by a TRI-CARB 2900TR Liquid Scintillation Analyzer. All experiments were performed in triplicate. The apparent permeability coefficients ($P_{app}$) of the two markers were expressed as

$$P_{app} = \frac{dQ/dt}{A C_0} \quad (3)$$

where $dQ/dt$ represents the rate at which markers appear in basolateral compartment on steady state; $A$ is the surface area of the cell layers and $C_0$ is the initial concentration in apical chamber.

Transepithelial electrical resistance (TEER) was also monitored with an epithelial voltohmmeter (World Precision Instruments, Sarsota, FL, USA) before and after the permeability study, the resistance of cell-free filter was monitored as the background.

### 2.6. Permeability study of selected β-blockers using HO-1-u-1 cell culture model

Atenolol, metoprolol and propranolol were dissolved into the previously prepared solution with various pHs, osmolarities, and GDC levels, to a final the concentration of 200 μg/ml. Permeability studies were then carried out according to the method described in Section 2.5. Sample concentrations were determined using the HPLC methods described by Wang et al. (2007). The apparent permeability coefficients ($P_{app}$) were calculated using Eq. (3).

### 2.7. Permeability study of selected β-blockers using porcine sublingual mucosa

Porcine sublingual mucosa was selected since its permeability of tritiated water and other markers are very similar to that from

human's mucosa (Lesch et al., 1989). Briefly, the sublingual tissue were excised from white domestic pigs (male, 50–100 kg) in local slaughterhouse and stored in 0.15 M isotonic phosphate buffer at 4 °C. All tissues were used within 2 h of slaughter. The epithelium layer was mechanically separated from the underlying connective tissue using a surgical scissor. The separated epithelial layer was then mounted between the donor chamber and receiver chamber of the diffusion cells (the surface of mucosa was placed facing the donor chamber).

The permeation experiments were conducted using a diffusion chamber system (PermeGear Co., PA, USA). The Side-Bi-Side horizontal diffusion cells with a diffusion area of 0.196 $cm^2$ and a volume of 4 ml for each chamber were used. The temperature was maintained at 37 °C. Solution in each chamber was stirred with Teflon coated magnetic bar. After the sublingual mucosa were equilibrated with isotonic phosphate buffer in both chambers at 37 °C for 30 min, the receiver chamber and donor chamber were filled with 4 ml prewarmed isotonic phosphate buffer (pH 7.4) and testing solutions prepared in 2.6, respectively. Samples of 200 μl were withdrawn from the receiver chamber at predetermined time intervals with refilling the same volume of buffer. Samples were analyzed by scintillation counter or HPLC. Triplicate experiments were conducted for each testing compound. $P_{app}$ was calculated using Eq. (3).

### 2.8. Permeability coefficients of ionized species and unionized species

The individual permeability coefficients of ionized species and unionized species were calculated according to the following equation

$$P_{app} = P_i X_i + P_u X_u \quad (4)$$

where $P_{app}$ is the apparent permeability coefficient of total drug penetrating across the membrane, and $P_i$ and $P_u$ are the permeability coefficients of the ionized species and unionized species, respectively. $X_i$ and $X_u$ represent the fractions of the ionized species and unionized species, and can be obtained using the following equations

$$X_i = \frac{1}{1 + 10^{(pH - pK_a)}} \quad (5)$$

$$X_u = \frac{1}{1 + 10^{(pK_a - pH)}} \quad (6)$$

The p$K_a$ for atenolol, metoprolol, and propranolol were reported to be 9.32, 9.23, and 9.23 (Schoenwald and Huang, 1983). At pH 5.4, more than 99.5% species are ionized for all the three compounds, thus, $P_i$ is approximately equal to the $P_{app}$ at pH 5.4. Then $P_u$ can be obtained from the $P_{app}$ at pH 9.0 using Eqs. (4)–(6).

### 2.9. Statistical analysis

All data were reported as mean ± S.D. Unpaired Student's *t*-test was applied to analyze the significant differences between two groups. Statistical significant difference among three or more groups was also calculated by one-way ANOVA using Tukey's test. A $p < 0.05$ is considered to be statistically significant for all tests.

## 3. Results

### 3.1. log D of selected β-blockers

log *D* of the three β-blockers at pH 5.4–10 is shown in Table 1. As weakly basic compounds, the ionization and lipophilicity are greatly affected by the solution pH. The log *D* increased quickly at higher pH as expected.

**Table 1**
Distribution coefficients (log *D*) of the three β-blockers at various pHs ($n = 4$).

| pH | log *D* | | |
|---|---|---|---|
| | Atenolol | Metoprolol | Propranolol |
| 5.4 | −2.26 | −1.61 | 0.84 |
| 7.4 | −1.92 | 0.01 | 1.51 |
| 9.0 | −1.22 | 0.58 | 2.13 |
| 10.0 | −0.37 | 1.20 | 2.87 |

### 3.2. Cytotoxic effect of pH, osmolarity and sodium glycodeoxycholate

The relative activities of HO-1-u-1 cells exposed to buffer with pH ranging from 2 to 11 were presented in Fig. 1(a). The results indicated that HO-1-u-1 cells were more sensitive to acidic buffer than to basic buffer. When the pH was controlled between 7.0 and 9.0, the relative activity of cells could be higher than 80%.

Fig. 1(b) revealed that the relative activities of HO-1-u-1 cells were unaffected by solution osmolarity in the range of 50–500 mOsm. When the osmolarity was less than 50 mOsm, or higher than 500 mOsm, however, the declining trends on cell relative activities appeared.

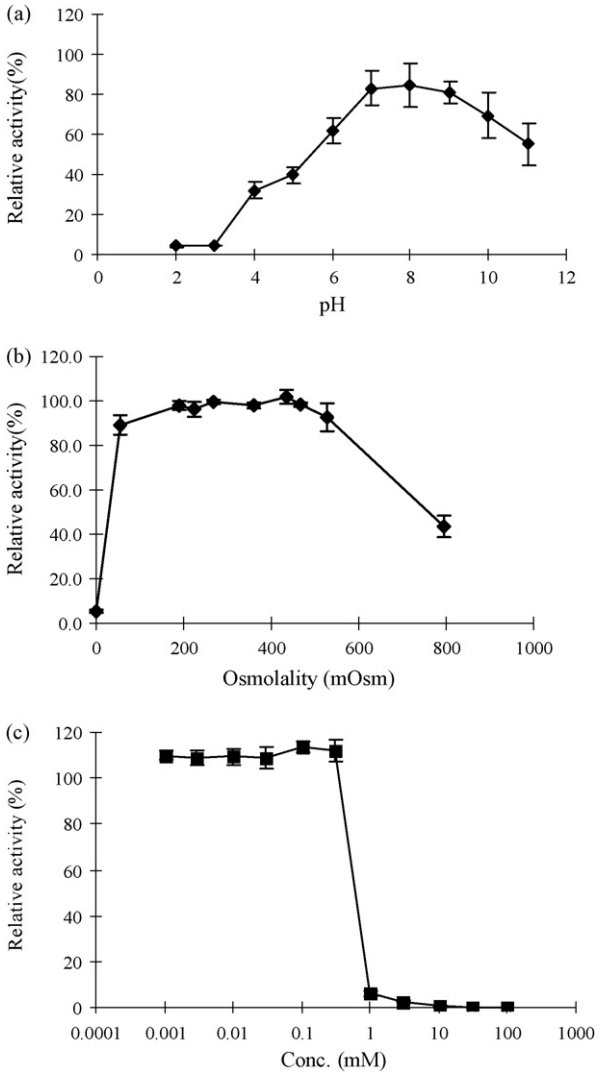


**Fig. 1.** The activity of HO-1-u-1 cells (a) at different pH levels; (b) in the presence of PBS solutions (pH 7.4) different osmolarity; (c) towards different concentrations of GDC by MTS/PES assay. Each point is expressed as mean ± S.D., $n = 4$.

**Table 2**
PH effect on the $P_{app}$ of [$^{14}$C]-mannitol through HO-1-u-1 cell culture model ($n = 3$).

| pH | TEER (Ohm) | | $P_{app} \pm$ S.D. ($\times 10^{-6}$ cm/s) | $p^*$ |
|---|---|---|---|---|
| | Pre-study | Post-study | | |
| 3.5 | 175.4 ± 2.5 | 158.3 ± 7.8 | 1.92 ± 0.18 | 0.076 |
| 5.0 | 169.9 ± 7.4 | 156.5 ± 5.7 | 2.05 ± 0.33 | 0.118 |
| 7.5 | 171.1 ± 5.6 | 169.3 ± 7.3 | 1.52 ± 0.27 | – |
| 8.5 | 177.2 ± 2.7 | 162.8 ± 2.4 | 1.49 ± 0.15 | 0.694 |
| 10.0 | 167.5 ± 9.2 | 163.0 ± 1.9 | 1.63 ± 0.14 | 0.290 |

* Compared with the $P_{app}$ at pH 7.4 by unpaired Student's $t$-test.

As shown in Fig. 1(c), when the HO-1-u-1 cells were exposed to sodium GDC at the concentration below 0.2 mM, the cell activities remained at about 100%. However, the activities quickly declined to 5% when the GDC concentration increased to 1 mM. These results suggest that GDC could generate cytotoxic effect to HO-1-u-1 cells at concentrations greater than 0.2 mM.

### 3.3. pH effect on the integrity of HO-1-u-1 cell culture model

The permeability of two neutral markers, [$^{14}$C]-mannitol and [$^{3}$H]-testosterone were measured at non-physiological pH levels and then compared with that obtained at pH 7.4 by unpaired Student's $t$-test (Tables 2 and 3). The results indicated the $P_{app}$ values of two markers were independent of solution pH. The TEER values did not significantly alter at all pH levels before and after the permeability studies, which suggested that the integrity of cell layers were not affected by non-physiological pH.

### 3.4. Permeabilities of β-blockers at various pHs in HO-1-u-1 model and porcine sublingual mucosa

The $P_{app}$ values of the three compounds at non-physiological pH levels were compared with that obtained at pH 7.4 by unpaired Student's $t$-test (Fig. 2) in both HO-1-u-1 model and porcine sublingual mucosa model. In HO-1-u-1 model, the $P_{app}$ of hydrophilic atenolol effectively increased at pH 10.0. For more lipophilic metoprolol and propranolol, however, the $P_{app}$ values were more significantly enhanced by pH. In porcine sublingual mucosa model, the permeabilities of theses compounds were also found to be highly dependent on pH. The $P_{app}$ values of three compounds all increased at pH > 9.0. The enhancement effects were dependent on not only the pH level but also the drug lipophilicity. The highest enhancement ratio obtained among all the test compounds was found to be 12.31 for hydrophilic atenolol at pH 10.0 when compared with that at pH 7.4. Additionally, the permeability were analyzed using one-way ANOVA followed by Tukey's test. For atenolol and propranolol across sublingual mucosa, the $P_{app}$ at pH 10.0 were statistically significantly different from those at pH 5.4 and pH 7.0 ($p < 0.05$). For metoprolol across sublingual mucosa, the $P_{app}$ at pH 9.0 and pH 10.0 are significantly increased from those at pH 5.4 and pH 7.0 ($p < 0.05$).

**Table 3**
PH effect on the $P_{app}$ of [$^{3}$H]-testosterone through HO-1-u-1 cell culture model ($n = 3$).

| pH | TEER(Ohm) | | $P_{app} \pm$ S.D. ($\times 10^{-6}$ cm/s) | $p^*$ |
|---|---|---|---|---|
| | Pre-study | Post-study | | |
| 3.5 | 167.5 ± 7.6 | 153.9 ± 4.7 | 6.74 ± 0.71 | 0.119 |
| 5.0 | 178.2 ± 6.9 | 154.2 ± 5.0 | 6.16 ± 0.52 | 0.188 |
| 7.5 | 176.1 ± 3.4. | 164.3 ± 7.0 | 5.38 ± 0.23 | – |
| 8.5 | 181.6 ± 9.2 | 167.8 ± 2.1 | 5.33 ± 0.62 | 0.886 |
| 10.0 | 175.5 ± 4.1 | 156.8 ± 6.4 | 5.60 ± 0.34 | 0.126 |

* Compared with the $P_{app}$ at pH 7.4 by unpaired Student's $t$-test.

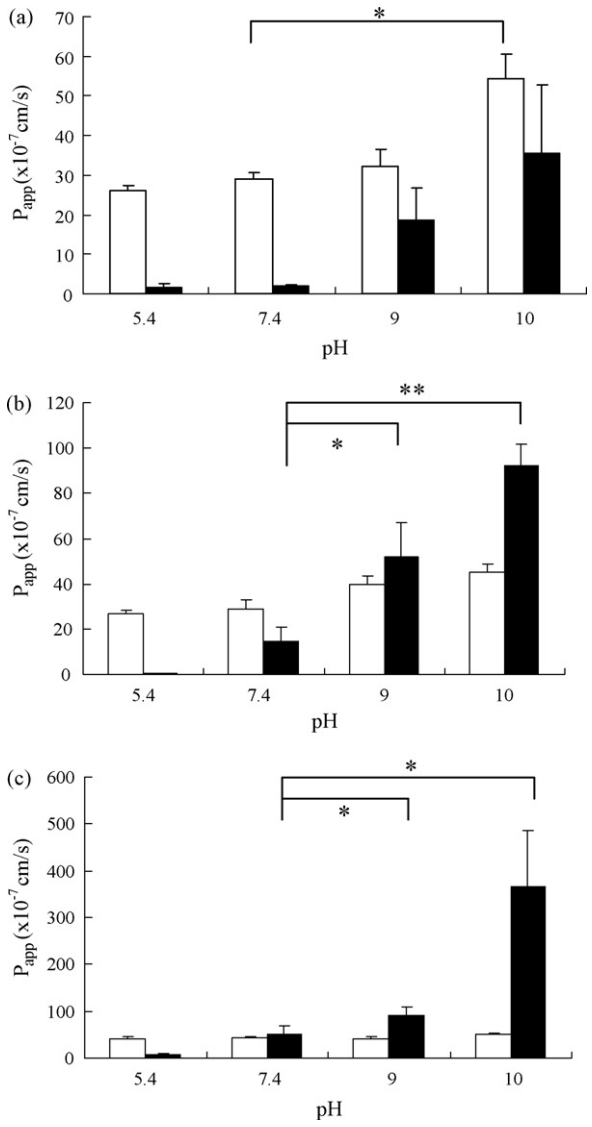


**Fig. 2.** The pH effect on the $P_{app}$ of (a) atenolol; (b) metoprolol; (c) propranolol across HO-1-u-1 cell culture model (□) and porcine sublingual mucosa (■). Data are expressed as mean ± S.D., $n = 3$. *$p < 0.05$ when compared with the $P_{app}$ at pH 7.4 by unpaired Student's $t$-test.

### 3.5. Permeabilities of selected β-blockers at various osmolarity

In Fig. 3, the $P_{app}$ values of testing compounds at non-physiological osmolarity are presented and compared with that obtained at physiological level (about 290 mOsm) by Student's $t$-test. The results indicted that the non-physiological osmolarity only had slight effect on the permeabilities of these compounds across either HO-1-u-1 cell layers or porcine sublingual mucosa, except that the $P_{app}$ of atenolol significantly increased at extreme low and high osmolarity. No significant enhancing effect of osmolarity was found on the lipophilic drugs, propranolol ($p = 0.079$, one-way ANOVA) and metoprolol ($p = 0.055$, one-way ANOVA). However, the $P_{app}$ of the hydrophilic β-blocker, atenolol, was significantly improved ($p = 0.000$, one-way ANOVA), especially at 0 and 550 mOsm.

### 3.6. Effect of sodium glycodeoxycholate (GDC) on the permeabilities of selected β-blockers

The enhancement effects for three testing compounds were in the order of atenolol > metoprolol > propranolol, which appeared to

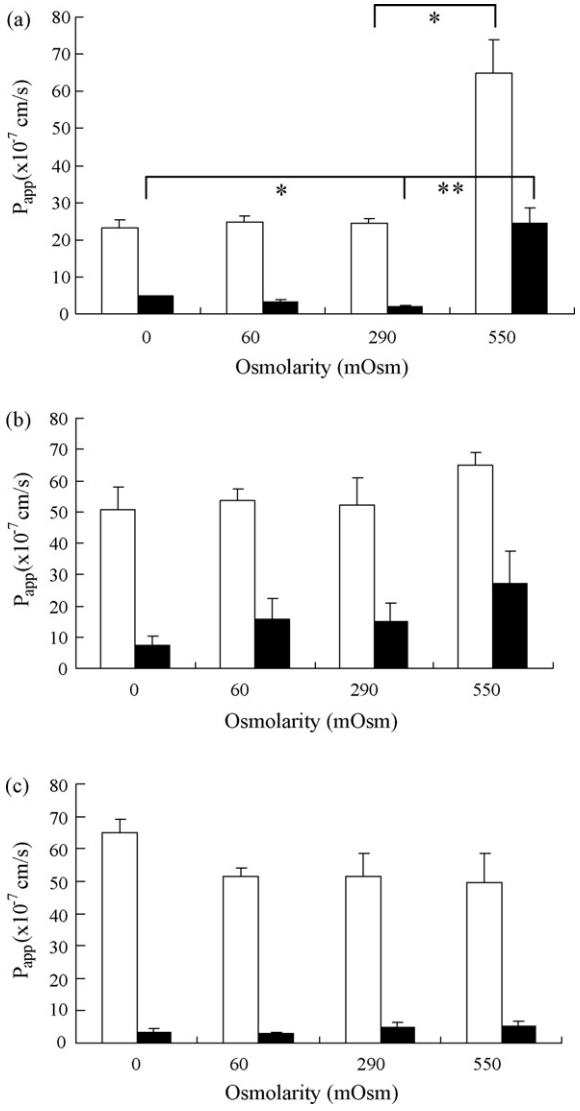


**Fig. 3.** $P_{app}$ of (a) atenolol; (b) metoprolol; (c) propranolol across HO-1-u-1 cell culture model (□) and porcine sublingual mucosa (■) with various osmolarity. Data are expressed as mean ± S.D., $n = 3$. $^{*}p < 0.01$; $^{**}p < 0.001$ when compared with the $P_{app}$ at 290 mOsm by unpaired Student's $t$-test.

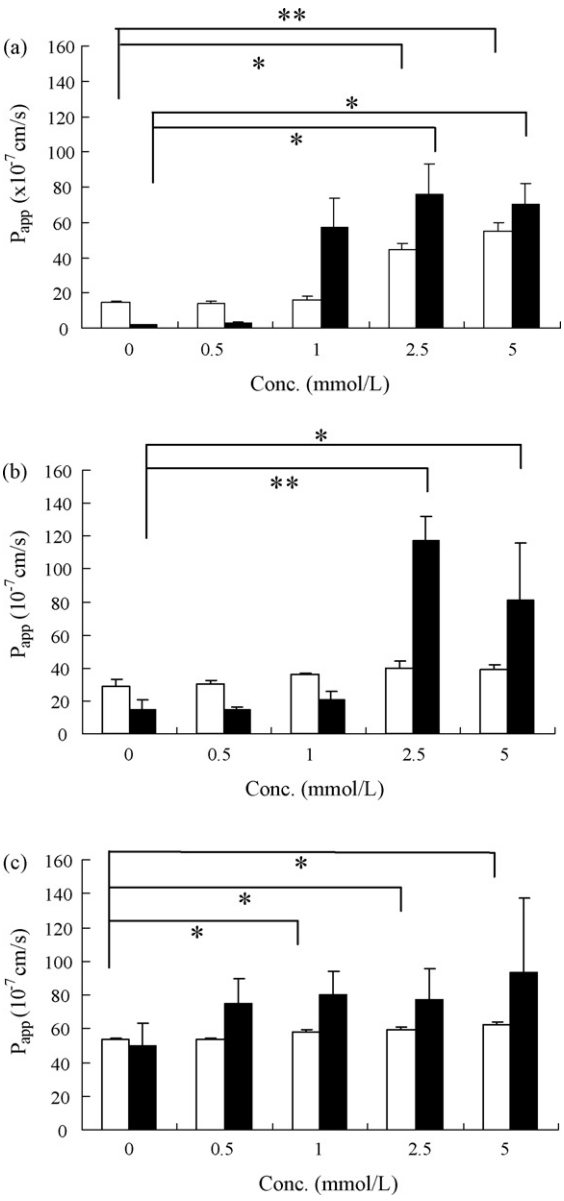


**Fig. 4.** $P_{app}$ of (a) atenolol; (b) metoprolol; (c) propranolol across HO-1-u-1 cell culture model (□) and porcine sublingual mucosa (■) in the presence of various concentrations of sodium glycodeoxycholate (GDC). Data are expressed as mean ± S.D., $n = 3$. $^{*}p < 0.05$, $^{**}p < 0.001$ when compared with the $P_{app}$ without GDC by unpaired Student's $t$-test.

be dependent on their lipophilicity (Fig. 4). Comparing the $P_{app}$ values obtained in the presence and absence of GDC for all three β-blockers, only the permeability of hydrophilic atenolol was significantly enhanced by GDC at a concentration greater than 2.5 mM in HO-1-u-1 cell culture model.

In permeability study using sublingual mucosa, the trend of enhancement on $P_{app}$ was similar to that in HO-1-u-1 cell culture model. The $P_{app}$ of atenolol increased nearly 30-fold when the GDC concentration reached 1.0 mM or higher comparing to that obtained at GDC concentration of 0.5 mM or below ($p = 0.001$, one-way ANOVA followed by Tukey's test). The $P_{app}$ of metoprolol was significantly improved by GDC with a concentration of 2.5 mM in comparison to 1 mM ($p = 0.000$, one-way ANOVA followed by Tukey's test). No further enhancement effect was observed at higher concentrations. In fact, at higher GDC concentrations (2.5–5 mM), a decreased trend was found for the $P_{app}$ of metoprolol and atenolol. For propranolol, however, no significant enhancement effect was observed in the presence of GDC at concentrations from 0 to 5 mM ($p > 0.05$, one-way ANOVA).

### 3.7. Permeability coefficients of ionized species and unionized species

The individual permeability coefficients of ionized and unionized species across cell culture as well as sublingual mucosa were calculated from the $P_{app}$ of the three drugs at pH 5.4 and 9.0 (Tables 4 and 5). Similar trend was shown between cell culture and sublingual mucosa. The permeability coefficients for both species

**Table 4**
Permeability coefficients of ionized species ($P_i$) across HO-1-u-1 cell culture model and porcine sublingual mucosa ($n = 3$).

| | $P_i$ (×$10^{-7}$ cm/s) | | |
|---|---|---|---|
| | Atenolol | Metoprolol | Propranolol |
| HO-1-u-1 | 25.97 ± 1.53 | 26.73 ± 1.66 | 39.90 ± 6.69 |
| SL mucosa | 1.73 ± 0.91 | 2.37 ± 0.12 | 7.53 ± 2.58 |

**Table 5**
Permeability coefficients of unionized species ($P_u$) across HO-1-u-1 cell culture model and porcine sublingual mucosa ($n = 3$).

| | $P_u$ ($\times 10^{-7}$ cm/s) | | |
|---|---|---|---|
| | Atenolol | Metoprolol | Propranolol |
| HO-1-u-1 | 45.25 ± 16.33 | 62.69 ± 10.61 | 41.38 ± 8.12 |
| SL mucosa | 53.66 ± 23.34 | 137.87 ± 42.02 | 237.11 ± 42.18 |

were also lipophilicity-dependent across cell culture and sublingual mucosa.

## 4. Discussion

Extreme low or high pH may influence the integrity of cell layer. To identify if the enhancing effects were caused by the changes from cell layer integrity, or the ionization state of the three β-blockers, integrity of cell layer should be screened before the permeability study on β-blockers. [$^{14}$C]-mannitol and [$^{3}$H]-testosterone were selected because they are neutral compounds and their ionization state is not affected by solution pH. The permeability study using two markers with various lipophilicity showed that cell layer integrity was not significantly altered by altering solution pH, despite that the cytotoxicity was found at extreme pHs.

The permeabilities of all the three β-blockers with different lipophilicities were effectively enhanced by increasing the solution pH. Since β-blockers belong to weak base with the p$K_a$ ranging from 8.8 to 9.6 (Schoenwald and Huang, 1983), the alteration of solution pH changes the degree of ionization, and then affect the log $D$ and permeability across cell culture or porcine sublingual mucosa.

The permeability enhancement effects by pH were highly dependent on the lipophilicity of compounds. Since high pH may effectively increase the lipid solubility of hydrophilic β-blockers (i.e. atenolol), more percentage of drug molecules transported through the transcellular route. For lipophilic β-blockers (i.e. propranolol), increasing pH showed lower enhancing effect in comparison to hydrophilic compounds.

The mechanisms for the permeability enhancement by osmolarity include changing barrier function of cell layers or ionic strength of testing solution (Nielsen and Rassing, 1999; Sugawara et al., 2002). In this study, The $P_{app}$ of hydrophilic atenolol increased over 20 times when the solution osmolarity increased to 1095 mmol/l. However, the $P_{app}$ of more lipophilic metoprolol and propranolol were not significantly improved, which indicates that the permeability of transcellular route may not be altered by non-physiological osmolarity.

There are several mechanisms proposed to explain the permeability enhancement effect by bile salts including GDC. Firstly, bile salts may involve in the formation of micelles which interact with the membrane components, thereby increasing membrane fluidity and permeability (Tengamnuay and Mitra, 1990; Shao and Mitra, 1992). The results showed that the enhancement effect of GDC on the three β-blockers across porcine sublingual mucosa was also highly affected by drug lipophilicity. The $P_{app}$ of hydrophilic atenolol was significantly increased when GDC concentration reached its CMC. Secondly, the bile salts can increase the paracellular transport by disruption of the hemidesmosomes (Hoogstraate et al., 1996). This may explain the effective enhancement of hydrophilic atenolol in our study. Thirdly, as a surfactant, GDC enhances dissolution and solubility of the studied compounds (Gibaldi and Kanig, 1965). GDC could effectively improve the permeability of the compounds (i.e. hydrophilic compounds) through paracellular route. This is also consistent with the study by Deneer et al. (2002) who investigated the enhancement effects of buccal transport of flecainide and sotalol by bile salts. Their results showed that the bile salts enhanced the paracellular transport of more polar compounds such as flecainide than sotalol.

In this study, when the GDC concentrations reached about 2.5 mM, further increases in its concentration could result in an declining trend on permeability. The reason for this observation is possibly related to the critical micelle concentration (CMC). As some drug is incorporated into the GDC micelles when the GDC concentration is higher than CMC, the free drug concentrations in the solution decreases and thus the apparent permeability coefficient declines. The CMC of GDC is about 1.5–3 mM (Roda et al., 1983; Nielsen and Rassing, 1999).

The objective for calculating the individual permeability coefficients of ionized and unionized species was to further investigate the different barrier characteristics between two models, especially paracellular and transcellular routes for different species. The results showed the $P_{app}$ increased with drug's lipophilicity in both models. The cell culture models, however, showed a narrow dynamic range in comparison to porcine sublingual model, which may be caused by the loose connection between cells and the less cell layers compared with sublingual epithelium. The $P_u$ for propranolol across cell culture model was only $4.14 \pm 0.81 \times 10^{-6}$ cm/s, compared with $6.27 \pm 1.06 \times 10^{-6}$ cm/s for metoprolol (Table 5). Such declining trend can be explained by the high fraction of unionized propranolol binding on cell layer (Wang et al., 2007). For the ionized species, however, no declining trend was found for the $P_i$ since the binding fraction is very low for ionized species.

## 5. Conclusion

In the permeability studies using both HO-1-u-1 cell culture model and porcine sublingual mucosa, the permeabilities of selected β-blockers under various pHs, osmolarity and different concentrations of sodium glycodeoxycholate suggested that the enhancement effects were highly dependent on drug lipophilicity. For hydrophilic compound atenolol, significant enhancing effects were observed. However, less enhancing effects were shown to moderate lipophilic compound metoprolol and high lipophilic compound propranolol. Among the three enhancement methods investigated, change of pH appears to be an effective approach to enhance β-blockers permeation.

## Acknowledgements

This work was supported by Direct Grant (No. CUHK 2041010) from The Chinese University of Hong Kong and ITF DDC Gant (No. ITS/174/00) from Innovation & Technology Commission of Hong Kong, SAR.

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