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An approach for rapid development of nasal delivery of analgesics—Identification of relevant features, *in vitro* screening and *in vivo* verification

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ABSTRACT

Drug delivery via the nasal route is gaining increasing interest over the last two decades as an alternative to oral or parenteral drug administration. In the current study an approach for rapid identification of relevant features, screening and *in vivo* verification of potential therapeutic agents for nasal delivery was carried out using "analgesic agents" as an example. Four such drug candidates (rizatriptan, meloxicam, lornoxicam and nebivolol) were initially identified as potentially viable agents based on their therapeutic use and physicochemical characteristics. An *in vitro* screening was then carried out using the Calu-3 cell line model. Based on the *in vitro* screening results and the reported pharmacokinetic and the stability data, meloxicam was predicted to be the most promising drug candidate and was subsequently verified using an *in vivo* animal model. The *in vivo* results showed that nasal administration of meloxicam was comparable to its intravenous administration, with respect to plasma drug concentration and AUC_{0-2h}. In addition, nasal absorption of meloxicam was much more rapid with higher plasma drug concentration and AUC_{0-2h} than that of oral administration. The current approach appears to be capable of developing "analgesic agents" suitable for nasal delivery. Further studies are needed to prove the clinical advantage of the specific selected agent, meloxicam, by nasal administration in patients.

1. Introduction

The most desirable and common route of drug administration is the oral route due to its convenience and compatibility. However, drugs which possess certain properties are not suitable for oral administration (Davis and Illum, 2003), for example: (1) drugs which are extensively degraded by the gut enzymes or metabolized by the liver that can result in very low bioavailability, (2) drugs which can cause gastrointestinal intolerance, and (3) drugs which are only slowly absorbed when rapid action is desired.

Although parenteral routes of administration (such as the intravenous, intramuscular and subcutaneous injections) can be employed to overcome the above-mentioned drawbacks, the disadvantages of the injections are obvious: they are invasive, inconvenient and more expensive. Other routes of administration such as buccal, ocular, sublingual, rectal, vaginal, pulmonary and nasal drug are potential alternatives. Among these, nasal drug delivery can offer a convenient and rapid effect since mucosa is relatively highly permeable (Corbo et al., 1990; Rojanasakul et al., 1992). An ideal nasal drug candidate should possess the following attributes (Behl et al., 1998): (1) appropriate aqueous solubility to provide the desired dose in a 25–150 μl volume of formulation administered per nostril; (2) appropriate nasal absorption properties; (3) no nasal irritation from the drug; (4) a suitable clinical rationale for nasal drug delivery, e.g. need for rapid onset of action; (5) low dose, e.g. below 25 mg per dose; (6) no toxic nasal metabolites; (7) no offensive odors/aroma associated with the drug; and (8) suitable stability characteristics.

In consideration of above criteria we believe that potential candidates for nasal delivery can be identified relatively rapidly, especially for those drug candidates that are already on the market, by the following steps: (a) determine a therapeutic category that can benefit from nasal delivery (rapid action), (b) review/identify relevant physicochemical properties, (c) *in vitro* screening, and (d) *in vivo* confirmation. To illustrate the application of the above concept, four small molecule drug candidates with "analgesic" property for various types of "pain" relief, namely rizatriptan, meloxicam, lornoxicam and nebivolol, were selected for evaluation. Since these drugs are already on the market, key information on their physicochemical properties most relevant to nasal delivery can be obtained via data mining and their relevant parameters further calculated. The values of certain relevant parameters for these four drugs as calculated from ACD/Labs Software (Advanced Chemistry Development Inc., Canada) are summarized in Table 1. Also as marketed drugs, they are known to be stable at room temperature for the approved dosage form and they do not possess offensive

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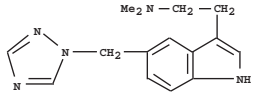
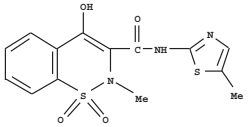
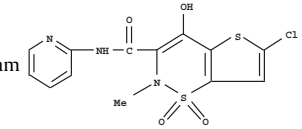
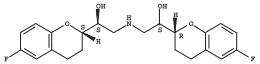
Table 1
Physicochemical properties of the four drug candidates.

Drugs	Molecular weight	pK_a		Log *P*	Log D				Aqueous solubility and stability[d]
		Most acidic	Most basic		pH 6	pH 7	pH 8	pH 9	
Rizatriptan 	269.34	16.98 ± 0.30	9.49 ± 0.28	0.956 ± 0.569	−2.00	−1.44	−0.54	0.35	Very good
Meloxicam 	351.40	4.50 ± 0.00/4.18[a]	3.20 ± 0.50/1.09[a]	2.709 ± 0.000	1.20/1.01[a]	0.25	−0.49	−0.75	Good
Lornoxicam 	371.82	4.50 ± 0.00/4.70[b]	3.71 ± 0.19	2.546 ± 0.064	1.03	0.08	−0.65	−0.91	Good
Nebivolol 	405.43	14.29 ± 0.20	8.65 ± 0.29/8.22[c]	3.671 ± 0.812/3.23[c]	1.15	2.03	2.94	3.51	Good

Note: All data are calculated values (ACD/Labs Software V8.19, Advanced Chemistry Development Inc., Canada), unless specified.

[a] Data were obtained from the experimental values from Luger et al. (1996).

[b] Data were obtained from the experimental values from Riad and Moussa (2008).

[c] Data were obtained from the experimental values from Cheymol et al. (1997).

[d] Based on approved solid dosage form or injectable solution.

odor. Additional relevant information for each drug is summarized below:

Rizatriptan is an active serotonin 5-HT1 receptor agonist useful for the treatment of acute migraine attacks. At present, rizatriptan is commercially available as oral tablet. The recommended initial dose for rizatriptan is 5 or 10 mg.

Meloxicam is a nonsteroidal anti-inflammatory drug (NSAID). Its main mechanism of action is decreasing prostaglandin synthesis by inhibiting cyclo-oxygenase (COX). Meloxicam exhibits analgesic, antipyretic and anti-inflammatory properties. The usual recommended oral dose is 7.5–15 mg/day; a dose as high as 30 mg/day may also be employed if needed (Euller-Ziegler et al., 2001). A parenteral formulation of meloxicam (15 mg/1.5 ml) has been developed for situations requiring rapid analgesia, such as acute mechanical lower back pain, sciatica and acute flares of osteoarthritis (Davies and Skjodt, 1999).

Lornoxicam is also a NSAID with potent analgesic and anti-inflammatory activity. Additionally, activation of the opioid neuropeptides system may be also part of its mechanism of analgesic action. Because of its potent analgesic effect, lornoxicam is used as injection to treat acute moderate post-operative pain after surgery and pain associated with acute lumbo-sciatica, in addition to its usual indications as an oral NSAID for inflammatory arthropathies, mechanical soft tissue pain and migraine. Lornoxicam is commercially available as 4 mg or 8 mg tablet. The powder for injection contains 8 mg lornoxicam per vial.

Nebivolol is a novel beta 1-blocker that combines beta-adrenergic blocking activity with a vasodilating effect. In addition to its use as antihypertensive agent, nebivolol is useful for the treatment of angina cordis and congestive heart failure. At present, the drug is available as 5 mg oral tablet.

Based on information from data mining, the above four drug candidates all meet 6 out of the 8 attributes (criteria) for nasal delivery (Behl et al., 1998) with exception of criteria 3 and 6 that relate to nasal irritation and toxic nasal metabolites, which requires separate testing after appropriate nasal formulation is developed. While these candidates appear to also meet criteria 2 (appropriate nasal absorption properties, i.e. good solubility and reasonable Log *P* value or high Log *P* with low dose needed for 150 μl per nostril administration) their nasal permeation property needs to be confirmed. To further demonstrate the proof-of-concept of developing nasal delivery for the drug candidates, the initial screening of permeability (step c) of the above four drugs was carried out *in vitro* using the Calu-3 cell line model followed by *in vivo* study in rats (step d). The Calu-3 cells are derived from human bronchial adenocarcinoma and have been reported to form "tight" polarized, mucus producing cell monolayers with apical microvilli (Foster et al., 2000; Grainger et al., 2006; Li et al., 2006; Mathias et al., 2002). At present, this cell line has been used as a model for both pulmonary epithelium (Florea et al., 2006; Foster et al., 2000; Mathias et al., 2002) and nasal epithelium (Li et al., 2006; Seki et al., 2007; Witschi and Mrsny, 1999). Based on the *in vitro* screening results (step c), the most promising drug candidate will further undergo *in vivo* pharmacokinetics verifications (i.e. oral, i.v. and nasal absorption studies) in rats (step d).

2. Materials and methods

2.1. Material

2.1.1. Chemicals

Rizatriptan benzoate, lornoxicam and meloxicam were obtained from Iffect Chemphar Co., Ltd. (China). Nebivolol hydrochloride was provided by Hwasun Biotechnology Co., Ltd. (China).

Marker compounds used for the cell line model validation included [^{3}H]-dexamethasone (250 μCi) from GE Healthcare Bio-Sciences Corp. (USA), [^{14}C]-mannitol (50 μCi) from Sigma–Aldrich Co. (USA), [^{3}H]-testosterone (250 μCi) and [^{3}H]-water from Amersham Biosciences (UK), and [^{14}C]-PEG 4000 (0.5 mCi/g) from PerkinElmer Life Sciences (USA). OptiPhase HiSafe 3 scintillation cocktail used for the radioactivity counting was supplied by PerkinElmer Life Sciences (Finland). Propranolol hydrochloride, the non-radioactive marker used for the validation, was purchased from Chang Zhou Guangming Biochemical Laboratories (BP2000/USP24, China). CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS/PES assay) used for the cytotoxicity measurement was obtained from Promega Corporation (USA). Phosphate buffered saline tablet was supplied by Sigma–Aldrich Co. (USA). All other chemicals were at least of analytical grade and all solvents were HPLC grade.

2.1.2. Cells and cell culture

Calu-3 cell line (passage 18) was obtained from the American Type Culture Collection (USA). Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (Ham, 1:1, with L-glutamine, 2.438 g/L sodium bicarbonate), fetal bovine serum (certified), non-essential amino acids (×100), penicillin–streptomycin (10,000 units/ml penicillin G sodium and 10,000 μg/ml streptomycin sulfate), trypsin–EDTA (0.25%) and Hank's Balanced Salt Solution (HBSS) were supplied by Invitrogen (USA). Collagen type I (from rat tail) was provided by Sigma–Aldrich Co. (USA). Pipettes (5 ml and 10 ml) and 12 well Transwell® plates (0.4 μm pore size, 1.12 cm^2, polycarbonate membrane insert) were purchased from Corning Costar Co. (USA). Tissue culture flasks (75 cm^2), centrifuge tubes (15 ml and 50 ml), 96-well plates and other cell culture consumables were supplied by IWAKI (Japan).

2.2. Validation of Calu-3 cell line model

2.2.1. Cell culture

Calu-3 cells from the American Type Culture Collection (ATCC) were cultured in 75 cm^2 flasks using 15 ml medium and incubated at 37 °C in 90% relative humidity atmosphere of 5% CO_2–95% air. The medium was prepared with 500 ml Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (1:1), 50 ml fetal bovine serum, 5 ml nonessential amino acid solution and 5 ml penicillin/streptomycin solution (10,000 units/ml penicillin G sodium and 10,000 μg/ml streptomycin sulfate). The medium was changed every other day. When reached 70%–80% confluence, the cells were dissociated with 5 ml 0.25% trypsin–EDTA solution, then subcultured at a split ratio of 1:3 or seeded in the Transwell® inserts. Before seeding the cells, the Transwell® inserts were coated with a collagen layer. Cells were seeded on the apical surface of Transwell® inserts at a density of 2×10^5 cells/well in 0.2 ml medium, followed by addition of 0.8 ml medium to the basolateral chamber. Two days after seeding, the medium in the apical chamber was withdrawn to create an air-interfaced culture condition and the medium in the basolateral chamber was replaced every two days. The integrity of the confluent cell monolayers was evaluated by measuring transepithelial electrical resistance (TEER) using the epithelial voltohmmeter from World Precision Instruments (USA). Only the monolayers with culturing days of 15–21 and TEER greater than 500 Ω × cm^2 were used in the experiments. The passages used for the experimentation were between 21 and 25.

2.2.2. Permeabilities of marker compounds

The permeabilities of markers were examined by measuring their apparent permeability coefficients (P_{app}). [^{14}C]-mannitol, [^{14}C]-PEG 4000, [^{3}H]-dexamethasone, [^{3}H]-testosterone and [^{3}H]-water were diluted or dissolved in HBSS with a final concentration

of 10^3, 10^3, 10^3, 500, and 200 nCi/ml, respectively. Propranolol was dissolved in HBSS to reach a concentration of 10^{-4} M.

On the day of experiment, the medium in the basolateral chamber of the Transwell® plate was removed. After washing with 37 °C HBSS, the cell monolayers were equilibrated in HBSS (0.2 ml in apical chamber, 0.8 ml in basolateral chamber) at 37 °C for 10 min. Then, the HBSS in the apical chamber was aspirated and 0.2 ml of pre-warmed test solutions (37 °C) were added to the apical chamber. The Transwell® plate was maintained at 37 °C during the experiment. Samples (100 μl) were taken from the basolateral chamber at various time intervals, and the same amount of 37 °C HBSS was supplemented immediately after every sampling to maintain the constant volume of the fluid in the receiver chamber. At the end of the experiment, 100 μl samples were taken from both the apical and basolateral chambers to calculate the recovery of the tested compounds according to Eq. (1) in Section 2.5.

For the radioactive samples, they were mixed with 1.5 ml scintillation cocktail and their radioactivity was determined in a TRI-CARB 2900TR Liquid Scintillation Analyzer from PerkinElmer (USA). The concentrations of propranolol in the samples were determined by an HPLC system consisting of a Waters 2695 solvent delivery module, a Waters 996 photodiode array (PDA) detector and a BDS C_{18} reversed phase column (4.6 mm I.D. × 250 mm, 5 μm, Thermo Hyersil). The mobile phase, containing 35% acetonitrile and 65% 25 mM phosphate buffer (pH 4.0) with 0.5% triethylamine, was run using the isocratic mode. The flow rate was set at 1.0 ml/min and the UV measurement was conducted at 213 nm.

The apparent permeability coefficient was calculated according to Eq. (2) in Section 2.5.

2.3. Initial screening of the drug candidates in Calu-3 cell line model

2.3.1. Effect of concentration on the permeabilities of drug candidates across Calu-3 cell line model

Each of the four drugs was dissolved in HBSS to prepare three concentrations for evaluation: for rizatriptan, 5×10^{-4}, 1×10^{-3} and 5×10^{-3} M were prepared for meloxicam and lornoxicam, 5×10^{-5}, 1×10^{-4} and 2×10^{-4} M were prepared for nebivolol, 1×10^{-5}, 2×10^{-5} and 5×10^{-5} M were prepared in HBSS containing 1% DMSO.

The permeation studies were conducted in the same way as that described in permeabilities of marker compounds. The concentrations of the tested drugs were determined by HPLC (Table 2). The recovery and the apparent permeability coefficient of the tested compounds were calculated according to Eqs. (1) and (2), respectively.

2.3.2. Effect of pH on the permeabilities of drug candidates across Calu-3 cell line model

Rizatriptan, meloxicam and lornoxicam were dissolved in HBSS and concentrations of 5×10^{-4}, 1×10^{-4} and 1×10^{-4} M were respectively prepared. The pHs of the drug loading solutions were adjusted to 6, 7, 8 and 9 using phosphoric acid or sodium hydroxide before they were loaded to the apical side of the Calu-3 monolayers. The permeation studies, sample analysis, and the calculations were carried out as described above.

2.4. In vivo absorption studies of meloxicam in rat model

2.4.1. Animals

Male Sprague–Dawley rats weighing 220–240 g were supplied by the Laboratory Animal Services Center at the Chinese University of Hong Kong. The rats were housed in an air-conditioned room under a 12/12 h light/dark cycle. The experiment was conducted after approval by Animal Ethics Committee of the Chinese University of Hong Kong.

Based on the *in vitro* results, meloxicam was chosen for the *in vivo* study. Three groups of rats were assigned to receive different routes of administration of meloxicam with 7–8 rats in each group. For oral and intravenous administrations, a light surgery of the rat was performed the day before the experiment. The rat was anesthetized with an intraperitoneal injection of a mixture containing 60 mg/kg ketamine and 6 mg/kg xylazine. For oral administration, the right jugular vein of rat was exposed and cannulated with a polyethylene tube (0.5 mm ID, 1.0 mm OD, Portex Ltd., UK). For intravenous administration, the right jugular vein of rat was exposed and cannulated with two polyethylene tubes (0.4 mm ID, 0.8 mm OD, Portex Ltd., UK), for drug administration and blood sampling, respectively. The polyethylene tubes were then guided subcutaneously to place at the back of the rat. After the surgery, the rat was allowed to recover over night under fasting condition with free access to water.

For nasal administration, the rat was first anesthetized with an intraperitoneal injection of a mixture containing 60 mg/kg ketamine and 6 mg/kg xylazine on the day of experiment. The rat was then placed in supine position and its body temperature was maintained by a heating lamp. The trachea of the rat was cannulated from an incision made in the neck with a polyethylene tube inserted to aid breathing. Also, a sealed polyethylene tube was inserted through the esophagus into the posterior part of the nasal cavity to prevent drainage of the drug solution from the nasal cavity. Subsequently, the right jugular vein of the rat was exposed and cannulated with a polyethylene tube (0.5 mm ID, 1.0 mm OD, Portex Ltd., UK).

2.4.2. Drug administrations and sample collections

For nasal delivery, meloxicam was dissolved in phosphate buffered saline (pH 7.4) containing 3% Tween 80 to reach a final concentration of 3.2 mg/ml. A dose of 1 mg/kg was administered to the nasal cavity of rat via a polyethylene tube (0.4 mm ID, 0.8 mm OD, Portex Ltd., England). Blood samples were withdrawn from the jugular vein before and at 2, 4, 6, 10, 20, 40, 60, 90 and 120 min post-dosing, respectively.

For oral delivery, the drug solution, which was diluted 10 times with phosphate buffered saline (pH 7.4) from that prepared for nasal delivery, was given to rat at a dose of 1 mg/kg by gastric gavages. Blood samples were withdrawn from the jugular vein before and at 10, 20, 30, 45, 60, 90 and 120 min post-dosing, respectively.

For intravenous delivery, the same drug solution as that prepared for oral delivery was administered to rat as bolus injection at the dose of 1 mg/kg via one of the two cannulated polyethylene tubes. To avoid contamination, blood samples were withdrawn from the other cannulated tube before and at 2, 4, 6, 10, 20, 40, 60, 90 and 120 min post-dosing, respectively.

After each sampling, an equal volume of heparinized normal saline solution (20 IU/ml) was immediately injected via jugular vein catheter. The collected blood samples were centrifuged at 13,000 rpm for 5 min and the plasma was collected and stored at −80 °C until analysis.

2.4.3. HPLC assay

In 100 μl of plasma sample, 50 μl of internal standard (lornoxicam) was added, followed by 100 μl of 10% H_3PO_4 to acidify the sample. The acidified mixture was then extracted with 1 ml ethyl acetate by vortexing for 2 min. After centrifuging at 3000 rpm for 10 min, the organic layer was collected and evaporated to dryness in a vacuum concentrator. The residue was reconstituted with 100 μl of 40% acetonitrile in 20 mM NaH_2PO_4 buffer solution (pH 5.2). An aliquot of 20 μl was injected into the HPLC system for analysis.

Table 2
Summary of HPLC assay conditions for the four drug candidates.

Compounds	Inject volume (μl)	Isocratic mobile phase	Wavelength of UV detector (nm)
Rizatriptan	20	14% acetonitrile in 20 mM NaH_2PO_4 buffer solution (0.5%TEA, pH 5.2)	225
Meloxicam	20	35% acetonitrile in 20 mM NaH_2PO_4 buffer solution (pH 5.2)	364
Lornoxicam	20	35% acetonitrile in 50 mM NaAc buffer solution (pH 5.8)	380
Nebivolol	50	50% acetonitrile in 20 mM NaH_2PO_4 buffer solution (0.5%TEA, pH 3.0)	281

The HPLC system comprised a Waters 2695 solvent delivery module, a Waters 996 photodiode array (PDA) detector and a BDS C_{18} reversed phase column (4.6 mm I.D. × 250 mm, 5 μm, Thermo Hyersil). The mobile phase consisted of 25% acetonitrile and 75% 20 mM NaH_2PO4 buffer solution (pH 5.2). The flow rate was 1.0 ml/min, and the total running time was 15 min. The autosampler was set at 10 °C. The wave-length of the UV detector was set at 364 nm for meloxicam and lornoxicam, the internal standard (IS).

In order to avoid undue bias to the low concentrations of the standard curve by the high concentrations, a weight of $1/x^2$ was applied to the calibration curves (GraphPad Prism 3.0, GraphPad Software Inc., USA). The calibration curve was linear over the concentration range of 0.1757–35.14 μg/ml with regression coefficients $R^2 \geq 0.9993$. The RSD was not more than 9.46% in both intra-day and inter-day precision studies. The accuracy calculated as the percentage difference of the determined concentration from the nominal value was within the range of −3.27% to 5.73%.

2.5. Data analysis

In Sections 2.2.2 and 2.3.1, the recovery of each tested compounds was calculated according to the following equation:

$$\text{Recovery } (\%) = \frac{A_{\text{apical}} + A_{\text{basolateral}}}{A_0} \times 100 \quad (1)$$

where A_0 is the initial amount of the compound, A_{apical} is the amount of the compound in the apical chamber at the end of the permeation studies, and $A_{\text{basolateral}}$ is the cumulative amount of the compound permeated to the basolateral chamber at the end of the permeation studies.

In Sections 2.2.2 and 2.3.1, the apparent permeability coefficient was calculated according to Eq. (2):

$$P_{\text{app}} = \frac{dQ/dt}{A} \times C_0 \quad (2)$$

where dQ/dt (mol/s) is the steady-state rate of change in cumulative mass permeated, A (cm^2) is the surface area for permeation, and C_0 (mol/ml) is the initial dosing concentration in the donor chamber.

The plasma drug concentrations versus time profiles were analyzed with WinNonlin Standard 2.1 (Pharsight Corporation, USA). Non-compartmental model was used to estimate the area under the plasma concentration – time curve from 0 to 2 h ($AUC_{0-2\,h}$). Maximum plasma concentration (C_{max}) and the time to reach the maximum plasma concentration (T_{max}) were read directly from the plasma concentration–time profile.

All data were reported as mean ± SD. Statistical significant difference among more than two groups was evaluated by one-way ANOVA. A $p < 0.05$ was considered statistically significant for all tests.

3. Results and discussions

3.1. Validation of Calu-3 cell line model

The apparent permeability coefficients (P_{app}) and recoveries of the six marker compounds across the Calu-3 cell line model are shown in Table 3. [^{14}C]-mannitol and [^{14}C]-PEG 4000 were commonly used as paracellular markers (Pezron et al., 2002; Wadell et al., 2003), while [^{3}H]-dexamethasone, [^{3}H]-testosterone and propranolol were usually used as transcellular markers (Mathias et al., 2002; Nielsen and Rassing, 2000; Wang et al., 2007). Tritiated water was also used as a marker, and was expected to transport across the cell monolayer freely (Nielsen and Rassing, 2000). Based on their lipophilities and transport mechanisms, the permeabilities of various marker compounds were expected to be in the following order: water > transcellular markers > paracellular markers. Our experimental results of the rank order for permeabilities were consistent with the above prediction. In addition, the recoveries of the transcellular markers were lower than those of the paracellular markers, probably due to the higher cellular uptake of the transcellular markers than that of the paracellular markers by Calu-3 cell monolayers.

Table 3
Apparent permeability coefficients (P_{app}) and recoveries of the six marker compounds across Calu-3 cell line model ($n = 3$ or 4).

Marker compounds	P_{app} ($\times 10^{-7}$ cm/s)	Recovery (%)
Mannitol	1.14 ± 0.11	92 ± 3
PEG 4000	2.57 ± 0.21	94 ± 2
Dexamethasone	64.81 ± 2.68	86 ± 4
Testosterone	205.01 ± 9.41	73 ± 3
Propranolol	227.27 ± 5.50	91 ± 2
H_2O	398.07 ± 57.62	93 ± 3

The P_{app} values of mannitol and dexamethasone obtained in the current study were compared with those reported in previous literature (Mathias et al., 2002). The published and current experimental P_{app} values of mannitol were $1.35 \pm 0.3 \times 10^{-7}$ cm/s and $1.14 \pm 0.11 \times 10^{-7}$ cm/s, respectively. The published and current experimental P_{app} values of dexamethasone were $74.2 \pm 4.94 \times 10^{-7}$ cm/s and $64.81 \pm 2.68 \times 10^{-7}$ cm/s, respectively.

In summary, the permeabilities of various marker compounds obtained from current study are comparable to those published previously, indicating that the currently developed Calu-3 cell line model is similar to those developed in other labs and thus suitable for screening of the candidate drugs.

3.2. Initial screening of the drug candidates in Calu-3 cell line model

3.2.1. Effect of concentration on the permeabilities of drug candidates across Calu-3 cell line model

To determine whether the four drug candidates permeate the Calu-3 cell monolayers through passive diffusion or active transport, the permeabilities of the drug candidates at three different loading concentrations were investigated (data shown in Table 4). Although the permeability of rizatriptan seemed to increase at the higher concentration, the P_{app} values of rizatriptan were in the range of 10^{-7} cm/s, which is comparable to most of the paracellular markers (Mathias et al., 2002; Pezron et al., 2002; Wadell et al., 2003) and much less than that of meloxicam and lornoxicam ($P_{\text{app}} > 10^{-5}$ cm/s). Therefore, it may be concluded that rizatriptan permeates the Calu-3 cell monolayers mainly via paracellular passive diffusion.

Table 4
Permeabilities (P_{app}) and recoveries of the four drug candidates across Calu-3 cell line model at 3 different loading concentrations (n = 3 or 4).

Compounds	Loading conc. (μM)	P_{app} ($\times 10^{-7}$ cm/s)	Recovery (%)	p (compare P_{app}) (one-way ANOVA)	
Rizatriptan	500	6.86 ± 0.13	92 ± 1	500 vs. 1000	0.998
	1000	6.84 ± 0.29	91 ± 3	500 vs. 5000	<0.0005
	5000	9.95 ± 0.49	88 ± 3	1000 vs. 5000	<0.0005
Meloxicam	50	176.14 ± 15.04	84 ± 5	50 vs. 100	0.488
	100	188.66 ± 16.72	88 ± 6	50 vs. 200	0.182
	200	196.60 ± 12.61	96 ± 5	100 vs. 200	0.739
Lornoxicam	50	146.85 ± 5.32	73 ± 2	50 vs. 100	0.379
	100	140.42 ± 6.12	77 ± 4	50 vs. 200	0.883
	200	149.05 ± 7.75	83 ± 2	100 vs. 200	0.199
Nebivolol	10	ND[a]	6 ± 2	NA[b]	NA[b]
	20	ND[a]	6 ± 2	NA[b]	NA[b]
	50	6.26 ± 0.24	11 ± 1	NA[b]	NA[b]

[a] Not detectable.
[b] Not applicable.

For meloxicam and lornoxicam, their P_{app} values were comparable among the 3 different loading concentrations. Most likely, the passive diffusion is the main transport pathway for meloxicam and lornoxicam to permeate across the Calu-3 cell line model.

Comparing with the other three candidates, nebivolol is most lipophilic with a calculated Log *P* of 3.7 (ACD/Labs Software V8.19, Advanced Chemistry Development Inc., Canada), which led to a very low solubility (<8 μg/ml) and an extraordinarily high cellular uptake in the Calu-3 cell line model (recovery <15%). Such high cellular uptake of nebivolol resulted in a high cellular toxicity (evaluated by MTS/PES assay, data not shown) and low permeability in the Calu-3 cell line model. Within the non-toxic concentration range, the P_{app} of nebivolol could not be determined at the two lower loading concentrations, and the P_{app} obtained at the higher concentration was very low ($6.26 \pm 0.24 \times 10^{-7}$ cm/s). In view of these results, no further investigation of nebivolol was carried out in the Calu-3 cell line model.

For further *in vivo* studies, drugs with such low solubility as nebivolol should employ very high concentrations of co-solvents to increase its solubility to the extent that it can be delivered in a therapeutically relevant dose in a 25–150 μl volume of formulation administered per nostril. However, high concentrations of solubility enhancers tend to have high risks of nasal mucosal irritation. Therefore, nebivolol may not be a good candidate for nasal drug delivery.

3.2.2. Effect of pH on the permeabilities of drug candidates across Calu-3 cell line model

The permeabilities of rizatriptan, meloxicam and lornoxicam across Calu-3 cell line model at different pH levels are presented in Table 5. As expected, with the increase of pH, the P_{app} of rizatriptan increased, whereas the P_{app} of meloxicam and lornoxicam decreased.

These experimental results were consistent with the theoretical predictions based on the ionization of the candidate drugs. Since these three drugs are all weak organic acids or bases, they exist in aqueous solutions in both ionized and unionized forms. Unionized species of a drug are more lipophilic than ionized species, resulting in a better absorption. The pH of a drug solution can affect the percentage of unionized species of a drug and thus affects its permeability. Rizatriptan is a weak base with a calculated pK_a of 9.49 (ACD/Labs Software V8.19, Advanced Chemistry Development Inc., Canada), so theoretically its permeability should increase when pH increases. Meloxicam and lornoxicam are weak acids, both with calculated pK_a of 4.50 (ACD/Labs Software V8.19, Advanced Chemistry Development Inc., Canada), so theoretically their permeabilities should decrease when pH increases. The present results are consistent with these theoretical considerations (see Table 5).

3.2.3. Selection of drug candidate for further in vivo studies

Based on the results of the *in vitro* permeation studies, rizatriptan was excluded for further *in vivo* studies due to its low permeability, and nebivolol was excluded due to both low permeability and solubility. The selection between the two remaining drugs was conducted based on their pharmacokinetic features and stability.

The T_{max} of meloxicam was reported to be 6–11 h after oral administration (Davies and Skjodt, 1999; Türck et al., 1996), while that of lornoxicam was 0.5–3 h (Hitzenberger et al., 1990; Olkkola

Table 5
Percentages of unionized species, permeabilities (P_{app}) and recoveries of rizatriptan, meloxicam and lornoxicam across Calu-3 cell line model at different pH levels (n = 3 or 4).

Compounds	pH	% Unionized[a]	P_{app} ($\times 10^{-7}$ cm/s)	Recovery (%)	p (comparison of P_{app}) (one-way ANOVA)	
Rizatriptan	6	0.032	3.84 ± 0.46	94 ± 2	6 vs. 7/6 vs. 8/6 vs. 9	0.014/0.001/<0.0005
	7	0.323	6.09 ± 0.72	93 ± 4		
	8	3.135	7.21 ± 0.89	90 ± 7	7 vs. 8/7 vs. 9/8 vs. 9	0.302/0.001/0.029
	9	24.448	9.21 ± 1.20	92 ± 5		
Meloxicam	6	3.065	341.15 ± 31.98	92 ± 2	6 vs. 7/6 vs. 8/6 vs. 9	<0.0005/<0.0005/<0.0005
	7	0.315	232.17 ± 7.30	87 ± 7		
	8	0.032	175.87 ± 9.90	85 ± 5	7 vs. 8/7 vs. 9/8 vs. 9	0.017/0.002/0.781
	9	0.003	161.11 ± 10.61	94 ± 2		
Lornoxicam	6	3.065	256.72 ± 14.02	73 ± 5	6 vs. 7/6 vs. 8/6 vs. 9	<0.0005/<0.0005/<0.0005
	7	0.315	178.99 ± 15.09	75 ± 3		
	8	0.032	164.36 ± 11.14	78 ± 6	7 vs. 8/7 vs. 9/8 vs. 9	0.440/0.046/0.486
	9	0.003	150.55 ± 12.76	77 ± 5		

[a] Calculated based on Henderson–Hasselbalch equation.

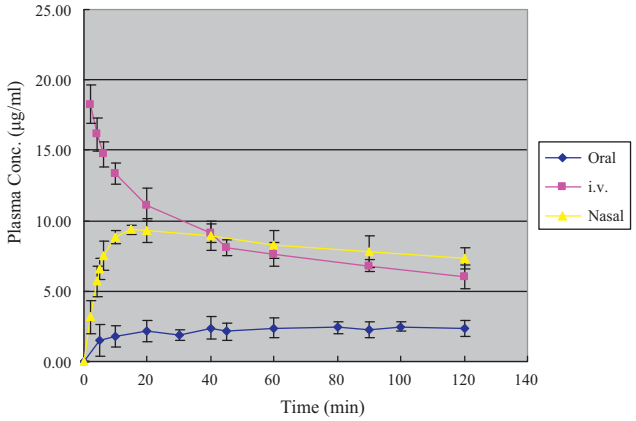


Fig. 1. Plasma drug concentrations vs. time profile in rats after nasal, oral and intravenous administration of 1 mg/kg meloxicam. The data are expressed as mean ± SD, $n = 7$ or 8.

et al., 1994). Hence, the room for improvement exists for meloxicam, but not lornoxicam.

In terms of stability, lornoxicam was reported to be unstable in solution and would form a large number of degradation products within weeks on standing at room temperature (Penkler and Whittaker, 1996). As no published report on the stability of meloxicam in solution was available, we performed some preliminary experiment and found that meloxicam was stable in phosphate buffered saline (pH 7.4) after 24 h at room temperature (remaining percentage of 100.44 ± 1.79%, $n = 3$), whereas the remaining percentage of lornoxicam under the same condition was 87.90 ± 2.65 ($n = 3$). Therefore, it can be concluded that meloxicam is more stable than lornoxicam in solution. Based on above data, meloxicam was selected to be the most promising of the four drug candidates for further *in vivo* confirmation study for nasal delivery.

3.3. *In vivo absorption studies of meloxicam through the nasal, intravenous and oral routes in rat model*

The plasma drug concentration vs. time profile in rats after nasal, intravenous and oral administration of 1 mg/kg meloxicam is shown in Fig. 1. The results demonstrated that meloxicam was quickly absorbed after nasal administration (T_{max} = 22.86 ± 12.54 min) with high plasma concentrations (C_{max} = 9.51 ± 0.73 μg/ml), which were comparable with those obtained after intravenous administration. On the contrary, meloxicam given via the oral route was absorbed slowly, with a plasma concentration of 2.38 ± 0.57 μg/ml achieved at 2 h. Our finding is consistent with the literature (Busch et al., 1998), where C_{max} of meloxicam following an oral administration in rat (1 mg/kg) was not reached until 4.4–6.8 h. In addition, the plasma concentrations of meloxicam obtained after oral administration were much lower than that obtained after nasal and intravenous administration. The $AUC_{0-2\,h}$ after nasal administration of 1 mg/kg meloxicam was 971.13 ± 90.46 μg × min/ml, which is comparable ($p = 0.144$) to that from intravenous administration (1055.11 ± 72.68 μg × min/ml) and much higher than that from oral administration (262.59 ± 63.71 μg × min/ml) ($p < 0.0005$). These data confirmed the initial Calu-3 cell line permeation results, which indicated that meloxicam may be well absorbed via nasal route of administration. The higher $AUC_{0-2\,h}$ after nasal compared to oral administration could be due to the bypass of first pass metabolism of meloxicam in the liver following nasal administration.

The *in vivo* pharmacokinetics investigation lasted only 2 h in the current study, which is a limitation of the rat model. Since the trachea of such rat model was cannulated, it should be kept under anaesthetized condition during the experimental period. Therefore, the *in vivo* pharmacokinetics studies could not last too long under such a setting. As the objective of the current study was to verify rapid onset of action from nasal route compared to oral administration, the early time points after drug administration would be the relevant data to obtain.

The current *in vivo* study did not provide complete pharmacokinetic profile due to sampling limitations. Since the $t_{1/2}$ of meloxicam in rat has been reported to be very long (~20 h) (Busch et al., 1998; Han and Choi, 2007), further studies are needed to confirm the influence of nasal administration on the $t_{1/2}$ of meloxicam.

4. Conclusions

Our present work demonstrated that relatively rapid identification of potential therapeutic class of pharmacologic agents suitable for nasal delivery by a stepwise approach is feasible. Following such an approach, nasal delivery of meloxicam was found to be comparable to its intravenous administration, with respect to plasma drug concentration and $AUC_{0-2\,h}$. In addition, nasal delivery of meloxicam resulted in a much more rapid absorption with higher plasma drug concentration and $AUC_{0-2\,h}$ than that of oral administration. Further studies are needed to prove the clinical advantage of the specific selected agent, meloxicam, by nasal administration in patients.

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