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Evaluation of Cell Permeation of a Potent 5α-Reductase Inhibitor Using MALDI-TOF MS

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_N_-(Dicyclohexyl)acetyl-piperidine-4-benzylidene-4-carboxylic acid (1), although a very potent *in vitro* 5α-steroid reductase (5αR) type 2 inhibitor, showed only marginal *in vivo* activity in rats. Since this could be due to hindered cellular uptake of the carboxylic acid, acid (1) and its corresponding methyl ester (1a) were compared with respect to their permeation properties. In the parallel artificial membrane permeation assay (PAMPA), 1a showed a higher %flux of 55 versus 6 for 1. Considering the high potency of 1 and better permeation of 1a, the use of 1a as a prodrug for 1 was explored using the human prostate carcinoma cell line DU145. Esterase activity, a prerequisite for this prodrug concept was detected employing 4-nitrophenyl acetate (4-NPA) as a substrate. After incubation of DU145 cells with 1 and 1a, respectively, permeated 1a and its hydrolysis to 1 were unequivocally observed by MALDI-TOF MS analyses, whereas 1 could not be detected inside the cells above the detection limit. Regarding biological activity, 1a showed a stronger inhibition of 5αR in intact DU145 cells than 1 (IC_{50} values, 4 μM and >10 μM for 1a and 1, respectively). These results suggest that the *in vivo* activity of 1 might be increased by the use of its methyl ester prodrug 1a.

Keywords: Cellular uptake; Prodrug; 5α-Reductase inhibitors; Matrix assisted laser desorption ionization mass spectrometry (MALDI-MS); Parallel artificial membrane permeation assay (PAMPA); Benign prostatic hyperplasia (BPH)

INTRODUCTION

Benign Prostatic Hyperplasia (BPH), characterized by prostatic enlargement and lower-tract urinary obstruction, is the most common benign tumor affecting over 50% of men above the age of 60.[1] Based on the pathophysiology of the disease, one of the therapeutic strategies is the inhibition of steroid-5α-reductase (5αR) that catalyzes the reduction of testosterone (T) to dihydrotestosterone (DHT), the most potent androgen in men.

We[2–5] and others[6–8] have focused on the development of several classes of non-steroidal inhibitors of 5αR, designed as mimics of the steroidal substrate. We recently synthesized and evaluated several *N*-acyl-4-benzylidenepiperidine-4′-carboxylic acids, the most active ones displaying inhibitory activities in the low nanomolar range.[9,10] These inhibitors are amongst the most potent non-steroidal 5αR type 2 inhibitors reported so far.

The lead compound **1** (Figure 1) of this series, reported by Picard *et al.*,[9] is a highly potent inhibitor of human 5αR type 2 exhibiting an IC_{50} of 60 nM. The reference compound finasteride was found to be even more active (IC_{50}: 3 nM). Considering its high activity *in vitro* towards human and rat enzymes, it was subjected to *in vivo* studies. It exhibited only moderate activity in a rat prostate weight test (49% inhibition after subcutaneous application and 36% after peroral application). Similar results were obtained with other carboxylic acids of this class, whereas finasteride (Figure 1) showed 76% inhibition at a 10-fold lower dose.[9]

This suggests that pharmacokinetic factors like absorption, partitioning or biodegradation are playing a role in the decreased *in vivo* potency of **1**. One important aspect of drug absorption is the ability of compounds to cross the cell membrane barrier. In order to investigate cell permeability of these carboxylic acids, the acid **1** was evaluated as a model compound. The methyl ester of **1**, **1a** (Figure 1) was used for the comparison of cell permeation.

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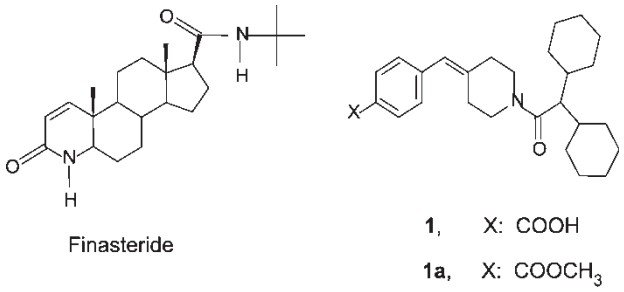

FIGURE 1 Structures of finasteride, **1** and **1a**.

Since esterases are often detected in many tissues[11–13] we hypothesize that in their presence this compound might serve as a prodrug, releasing the active compound **1** after cellular uptake and hydrolysis. To check the cell permeability of **1** and **1a**, a prostate cancer cell line, DU145 (expressing 5αR type 1), was employed.[14–16]

In the current paper, we present permeability studies with **1** and **1a** using an artificial membrane assay (PAMPA).[17] Esterase activity in DU145 cells was monitored with 4-nitrophenyl acetate (4-NPA) as substrate. In order to compare cell permeation of **1** and **1a** including intracellular hydrolysis of **1a**, MALDI-TOF MS was used. Finally, the inhibitory activities of **1** and **1a** in intact DU145 cells were determined.

MATERIALS AND METHODS

Materials

Compounds **1** and **1a** have been previously synthesized.[9] 4-NPA was purchased from Lancaster Synthesis GmbH (Frankfurt am Main, Germany). Sinapinic acid and trypsin were obtained from Fluka-Chemie GmbH (Taufkirchen, Germany). RPMI (Roswell Park Memorial Institute 1440 Medium) was obtained from CC Pro GmbH (Neustadt, Germany). Fetal calf serum (FCS) was purchased from Invitrogen GmbH (Karlsruhe, Germany). The DU145 cell line was supplied by DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkulturen), (Braunschweig, Germany) and human prostatic tissue (BPH patients) was provided by the Department of Urology, Saarland University (Homburg, Germany). All solvents used were of HPLC grade.

Artificial Membrane Permeation and Physicochemical Parameters

The PAMPA assay was performed according to the method described by Kansy *et al.*[17] The stock solutions of the compounds were prepared in ethanol (5 mM), which were then diluted further using tris buffer (pH 7.4) leading to a final concentration of 500 μM for the assay. The assays were performed in triplicates and the optical densities of the donor and the acceptor wells were measured spectrophotometrically.

The pKa of compound **1** was calculated using ACD labs website.[18] For the determination of the $\log P_{Oct}$ value of **1**, titration was carried out pH-metrically using octanol-water as described by Avdeef *et al.*[19–21] The experiments were performed on a PCA 200 Titrator (Sirius Analytical Instruments Ltd., Forest Row, East Sussex, UK) and the results were processed with the help of Refine 200 V1.0 software. The samples were run in triplicate with octanol-water ratios from 1:100 to 5:100. The GOF ("goodness–of–fit" = reduced chi–square) values were calculated as described in the application guide provided by Sirius Analytical Instruments Ltd[22] and were found to be in the range of 1.00 to 1.42. The assays were commenced at basic pH and terminated at acidic pH. The logP of **1a** was calculated using Hansch/Fujita's π-system as described by Rekker and Mannhold.[23] The π values were taken from the literature.[24,25]

Esterase Activity Determination in DU145 Cells

The esterase activity in DU145 cells was determined by monitoring the conversion of 4-nitrophenyl acetate (1 mM, 500 μM, 50 μM) to its coloured product *p*-nitrophenol during an incubation period of 3 h at a wavelength of 405 nm using a Multilabel Plate Reader (Wallac1420 Victor2, PerkinElmer Life Sciences). A day before the experiment, DU145 cells were seeded in a 24 multi-well plate at a density of 170,000 cells/well and allowed to become adherent overnight. The medium was removed and the cells were washed with PBS buffer (pH 7.4) five times. 4-NPA dissolved in tris-HCl buffer (0.1 M, pH 7.4) was added to the cells in a final volume of 1 mL. A control (without cells) containing 4-NPA in the respective concentrations was also incubated to determine the weak formation of *p*-nitrophenol that spontaneously occurs. The control values were subtracted from the sample values at different timepoints. The samples for each concentration were run in triplicate. The esterase activity was determined using a calibration curve for *p*-nitrophenol.

Assay for MALDI-MS Analyses

DU145 cells (1.7 million) were incubated in a petri dish and were allowed to become adherent overnight. The stock solution of compound **1** in methanol (20 μL) was added to the cells in a final volume of 5 ml (final concentration: 10 μM). After 6 h of incubation in an atmosphere containing 5% CO_2 at

37°C, the medium was removed and the cells were washed (3 mL, 4 times) with the washing buffer (PBS buffer, pH 7.4, containing 1% bovine serum albumin). After a first round of washing, trypsin was added and the detached cells were collected again with 3 mL of washing buffer. For the second round of washing, the cell suspension was centrifuged at 480 × g for 2 min at RT. The supernatant obtained was collected and the cell pellet was resuspended in 3 mL of fresh washing buffer. This step was repeated four times and all the washings were collected. Finally, the cells were homogenized in PBS buffer (3 mL) using an ultrasonic rod at 0°C (5 times for 10 s). The homogenate and the washings were then extracted each using liquid-liquid extraction (LLE). Diethyl ether (6 mL) was added to the samples under acidic conditions (pH 2). After vortex-mix (2 min) and centrifugation (480 × g, 5 min), the aqueous phase was frozen using dry ice and the ether layer was decanted. The extraction was repeated twice using 3 mL of ether. The ether layers were collected, dried over sodium sulphate and evaporated to dryness under N_2. The residue was reconstituted with 50 μL of methanol. The resulting samples were then analyzed by MALDI-MS. The assay was performed in duplicate including a control (without inhibitor) and a blank (without cells). The incubation with **1a** followed by LLE was performed in the same way.

Determination of MALDI-detection Limit in the Cell Homogenate

DU145 cells were homogenized as described above. A total of 30 μL of **1** or **1a** (10 μM, 5 μM, 2.5 μM, 1.25 μM, 0.75 μM and 0.312 μM in methanol) were added to the homogenate that gave a final volume of 3 mL. The resulting suspensions were incubated at room temperature for 1 h, followed by acidification (pH 2) and extraction in the same way as mentioned above. Eventually, the extracted compounds were reconstituted with 30 μL of methanol for MALDI-MS analyses. In the case of **1a**, the acidification step was omitted.

MALDI-MS Analyses

MALDI-TOF MS spectra were acquired using a Reflex-III mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany) equipped with a SCOUT 384™ probe ion source and a pulsed N_2 laser (337 nm, Model VSL-337ND, Laser Science, Boston, MA, USA) with energy of 400 μJ/pulse. The ions were accelerated under delayed extraction conditions in the positive ion mode. A leceroy 9384C 4 GHz digital storage oscilloscope was used for data acquisition. The analyses were carried out in the reflector mode in a mass range of m/z 100–700 with an accelerating voltage of 20 kV. The data was processed with the help of XMASS 5.1 program (Bruker Daltonics, Bremen, Germany) and 100 laser shots were summed for each spectrum. Sinapinic acid was used as matrix. A saturated solution of sinapinic acid in methanol and 0.1% aqueous TFA was mixed with the samples and subsequently spotted on the target for the analyses.

DU145 Whole Cell Assay

The assay was performed as described by Reichert *et al.*[16]

BPH Assay

The assay was performed in the same way as reported by Kattner *et al.*[5]

RESULTS AND DISCUSSION

The PAMPA %flux for **1** and **1a** is presented in Table I. The results showed that **1** with %flux of 6 shows poor membrane permeability (recovery almost 100%). On the contrary, **1a** with a relatively higher %flux of 55 showed enhanced permeability, however, its low recovery (11%) demonstrates a high affinity for the membrane. Consequently, more than 80% of the compound remained dissolved in the lipophilic membrane at the end of the experiment.

To understand these phenomena, the physicochemical parameters of **1** and **1a** were determined.

TABLE I Summary of permeation experiments for **1** and **1a** including PAMPA %flux, MALDI-MS analysis and biological activity in DU145 cells

			DU145 cells	
Compound	X[a]	PAMPA %flux (recovery in %)[b]	MALDI-MS analyses	IC_{50}[c-e] [μM]
1	COOH	6 (100)	Not detected	>10[f]
1a	$COOCH_3$	55 (11)	Detected with cleaved acid	4

[a] For the structure, see Figure 1. [b] The permeation of a compound through the membrane layer is described by the percentage permeation (%flux). The flux values were calculated considering the UV absorption of the acceptor compartment and that of a reference well with the same concentration containing no membrane barrier. [c] Mean value; tests were run in triplicate. The maximum standard deviation for IC_{50} is 20%. [d] Substrate: [^{3}H] androstenedione 5 nM. [e] Reference compound: Finasteride [0.039]. [f] Ref. 9.

The ester **1a** is more lipophilic (calculated logP: 5.62) than the acid **1** (logP: 5.31, more important logD at pH 7.4: 4.68). With a pKa value of 4.14 compound **1** is completely deprotonated at pH 7.4. The resulting ionic species show little affinity for the lipophilic membrane. Consequently, the permeation was low and the recovery was high indicating that no (or little) amount of the compound was present in the membrane.

Esterase Activity in DU 145 Cells

The putative esterase activity in DU145 cells was determined using 4-NPA as a substrate, which on cleavage, transforms into *p*-nitrophenol, a yellow product absorbing at 405 nm.

As shown in Figure 2, the DU145 cells are able to cleave the acetyl moiety from 4-NPA thereby releasing the corresponding product (*p*-nitrophenol). These results demonstrate the presence of esterase(s) in DU145 cells. The activity was found to be 6 μM release of *p*-nitrophenol per minute for a substrate concentration of 1 mM.

MALDI-MS Analyses

Limits of Detection (LOD) for 1 and 1a

The LODs for **1** and **1a** in the cell homogenates after LLE were 1.25 pmol/μL and 2.5 pmol/μL, respectively. At this level, the signal-to-noise ratio for the analytes (**1** and **1a**) was higher than 3.

Washing of Cells

After incubation of DU145 cells with **1** and **1a**, the cells were washed in order to minimize the compound adhering on the cell membrane. MALDI-analyses of the cell washings (1–4) showed a gradual decrease in the signal intensities of **1** and **1a** (data not shown). No compound was detected in the last washing above the detection limit. After every washing step the cells were checked for viability using a CASY cell counter (Scharfe System GmbH, Germany).

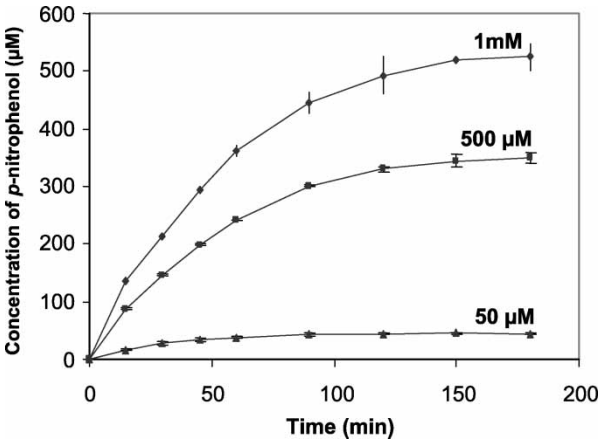

FIGURE 2 The plot of incubation time versus 4-nitrophenol concentration showing esterase activity in DU145 cells. The conversion of 4-NPA into 4-nitrophenol was read at 405 nm. Esterase activity: 6 μM/min for 1 mM substrate concentration.

Cell Permeation of 1 and 1a

The cell permeability was investigated using MALDI-MS. The analyses of the DU145 cells were performed with **1** after incubation (6 h). The cells were washed four times until no compound was detected in the last washing followed by homogenization and extraction (LLE). MALDI analysis of the homogenate after LLE showed absence of the acid above the detection limit (Table I) indicating a poor permeation. Further, **1** could be easily detected in the blank experiment carried out without cells.

The MALDI spectrum obtained after the incubation of DU145 cells with **1a** shows a peak at m/z 438 that could be assigned to **1a**, and suggested a good cell permeability (Figure 3). The esterase activity was further confirmed by the presence of the cleaved acid **1** at m/z 424 in the spectrum. The other peaks in the spectrum correspond to the signals produced by the matrix (sinapinic acid). The blank experiment performed without cells showed only ester **1a**.

MALDI results unambiguously showed that **1a** has a higher permeability (than **1**) and reveals that it cleaves to produce **1**. These results are in good accordance with PAMPA %flux values.

Biological Activity in DU145 Cells

Table I illustrates that **1** shows a moderate inhibitory activity ($IC_{50} > 10$ μM, Picard *et al.*[9]) in the cellular assay. Conversely, **1a** exhibits a significant increase in inhibition with an IC_{50} of 4 μM. Nevertheless, it does not reach the activity of the reference compound finasteride (IC_{50}: 0.039 μM). The higher activity of **1a** in the cellular system is obviously due to the cleavage of the ester by the esterase within the cells releasing the acid, **1**, which in turn inhibited the target (5αR). This observation accounts for the poor permeability of **1** thereby supporting the idea of a prodrug concept. Nevertheless, the possibility of the ester being the active species cannot be ruled out. Additionally, **1a** was found to be only a weak inhibitor of human type 2 isozyme in the BPH assay showing an inhibition of 20% at a concentration of 10 μM, whereas the corresponding acid **1** was found to be extremely potent (IC_{50}: 60 nM).

In conclusion, this work demonstrates that the carboxylic acid **1** has a poor membrane permeability when compared to its methyl ester **1a**, which cleaves to the corresponding acid **1** in a prostate cancer cell

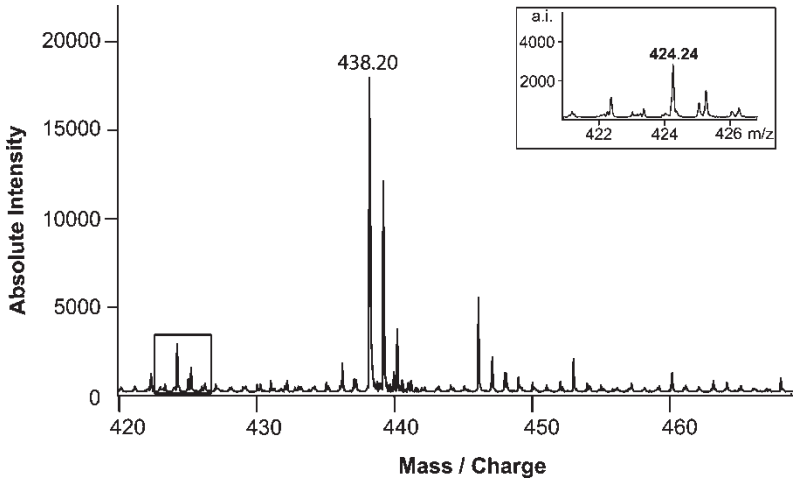

FIGURE 3 MALDI-MS spectrum after incubation of DU145 cells with **1a**. The spectrum shows the presence of **1a** (m/z 438) and **1** (m/z 424) in the homogenate after LLE. All the masses shown in the spectrum represent monoisotopic $[M + H]^+$-ions. Inset: A part of the spectrum zoomed to view the peak of **1** at m/z 424.

line (DU145). Therefore, the use of **1a** as a prodrug appears promising in enhancing the *in vivo* activity of **1** provided that ester cleavage does not occur on the periphery.

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