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Inhibition of the L-dopa transport system in human epidermal Langerhans cells by omeprazole and its analogues

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Summary. L-3,4-dihydroxyphenylalanine (L-dopa) transport into human Langerhans cells (LC) occurs by a saturable mediation. This plasma membrane agency is, due to its characteristics, distinguishable from systems transporting other neutral, cationic and anionic amino acids into other cells and serves to catalyze the flow of L-dopa, only, into LC. The uphill operation of this L-dopa transport system is believed to occur by down-gradient countermigration of H^+. Due to the uniqueness of the L-dopa transport system, the widely used analogue inhibition approach was not applicable. Instead we studied omeprazole and its analogues in our search for suitable inhibitory candidates. Omeprazole and most of its analogues were indeed inhibitory in the concentration range 1–100 μmol/L. Conspicuously, the compounds with strongest polarity were least inhibitory. The inhibitory pattern displayed by omeprazole and the other analogues on L-dopa uptake in LC corresponded to some extent to what has been observed previously for purified H^+,K^+-ATPase from tubulovesicles of the stomach. No effects of the inhibitors were registered on energy charge and lactate production of epidermal biopsies, nor were any gross alterations of ultrastructure of LC noticed.

Keywords: Epidermis – Omeprazole analogues – Langerhans cells – L-dopa transport – Lactate – Energy charge

Abbreviations: KRB, Krebs-Ringer-bicarbonate buffer; KRP, Krebs-Ringer-phosphate buffer; LC, Langerhans cells; L-dopa, L-dihydroxyphenylalanine; DTT, dithiotreitol; EC, energy charge

Introduction

Human Langerhans cells (LC) are dendritically-shaped bone marrow-derived monocytic cells, residing in stratified squamous epithelia like the epidermis. They compose only 1–3% of the total cell population of human epidermis but are -thanks to the fact that they are rich in dendrites- still able to supervise the microenvironment of epidermis. LC are characterized morphologically by their content of Birbeck's granules, and functionally by the fact that they constitutively or inductively express high levels of MCH class II molecules on their surface. LC are the critical cells for initiation of T-cell -dependent immune responses by epidermal cells and are therefore considered as antigen-presenting cells of the epidermis (Stingl et al., 1989). Based upon several phenotypic similarities between resident LC and monocytes/macrophages LC have usually been regarded as specialized macrophages. However, this belief may be modified, since a more dynamic view has been introduced showing that short-term cultured LC change their morphology, phenotype and functional capacities dramatically (Schuler and Steinman, 1985).

Recently we demonstrated that the plasma membrane of LC possesses a transport mechanism for L-3,4-dihydroxyphenylalanine (L-dopa) uptake, which is time- and concentration dependent. Further, the amino acid carrier is stereospecific and it appears to be very specialised, since L-dopa (which may be a derivative of the epidermal melanocytes) seems to be the only substrate amino acid that will be accepted by the carrier (Falck et al., 2003). The L-dopa transport into LC may be energized by a counterflow of protons across the plasma membrane which are produced by anaerobic glycolysis of LC in epidermis (Ronquist et al., 2003).

Enzyme-specific inhibitors are important biochemical tools. They can be used not only to identify and classify an enzyme or enzymatic system but also to give structural and functional information. In these respects, omeprazole and its derivatives have proved to be useful compounds in

the studies of the gastric H^+, K^+-ATPase responsible for proton production in tubulovesicles of the parietal cells of gastric mucosa (Fellenius et al., 1981; Olbe et al., 1982; Wallmark and Jarsten, 1983).

The carrier-mediated transport of L-dopa into LC, like the uptake of other amino acids into various cells, is characterized by the saturation phenomenon (Falck et al., 2003). Therefore, the shape of the uptake curve of L-dopa in LC implies for this mediated transport, as it does for enzymology, that the substrate is transiently bound by a mediating structure present in limiting supply. In other words, the kinetics of enzymatic catalysis is fundamentally the same whether it is a chemical change or a transport of the substrate that is catalyzed. Since omeprazole and its analogues are inhibitors of proton production (in another cell system) and protons are believed to be the motive force in concentrative L-dopa uptake in human LC (Falck et al., 2003), we found it justified to test whether omeprazole and its derivatives were influential on also L-dopa transport into LC.

The present study was accordingly designed to examine directly the possible inhibitory action of omeprazole and several of its analogues on L-dopa transport into LC. The results demonstrate that the amino acid carrier of LC is indeed sensitive to most of these compounds and provide direct evidence for inhibition of the L-dopa transport process into LC.

Materials and methods

Chemicals

Dithiotreitol (DTT), glutathione (reduced form) and porcine trypsin were obtained from Sigma Chemical Company (St. Louis, Mo., USA). All chemicals were of the purest grade commercially available. Omeprazole and those omeprazole-analogues with the pre-fix H were benevolently delivered from Astra Hässle, 431 83 Mölndal, Sweden. The omeprazole-analogues with the prefix B, were a generous gift from Byk Gulden, Lomberg Chemishe Fabrik GmbH, Byk-Gulden-Str. 2, 78467 Konstanz, Germany.

Biopsies

Thin split skin, consisting of epidermis and the upper dermis, was obtained without anaesthesia from volar forearm skin on healthy adults after informed consent had been obtained from each of these individuals. The study protocol was approved by the Ethics Committee of the University of Lund. The specimen sampling followed strictly established routines elaborated in our laboratory to avoid preanalytic sampling errors. The biopsies, except those processed for electron microscopy, were exposed to 0.05% trypsin in Krebs-Ringer-phosphate buffer (KRP) until separation could be performed (25–50 min). The epidermis was gently teased off the dermis with fine forceps and finally rinsed in KRP. Batches of 5 or 10 epidermal specimens from each person were frozen in liquid nitrogen, either directly after separation or after incubation in vitro, for determination of lactate or energy charge (EC).

Incubation procedures

1. L-dopa uptake studies. Generally, the uptake experiments involving epidermal biopsies with inhibitors were carried out in KRP or Krebs-Ringer-bicarbonate buffer (KRB) at pH 6.0 or at pH 8.1 (+37°C), and consisted of two parts.

   Part 1 comprised two subsequent preincubations with the inhibitor, each preincubation period usually lasting for 60 min at the most, due to lability of the inhibitor molecule in solution and a new preincubation with new inhibitor was started.

   Part 2 was the incubation with L-dopa, generally at 10 mmol/L and for 60 min, sometimes without and sometimes with the inhibitor, using the same concentration as in the preincubation. Also included in some of the experiments were one or two buffer washings of varying lengths between Part 1 and Part 2. On these occasions no inhibitor was present in Part 2.
2. Energy metabolic studies. At the end of incubation the biopsies intended for EC and lactate determinations were frozen in liquid nitrogen and kept at −70°C for up to 1 month until analysis. Incubation media for lactate analyses were also frozen and kept at −70°C until analysis.

Biochemical analyses

Biopsies and incubation media were analyzed separately for lactate. Each batch of biopsies was lyophilized, homogenized with a mortar and pestle and weighed.

Subsequently, the material was dissolved and extracted in 1 mol/L perchloric acid for 4 h at +23°C, followed by centrifugation for 20 min at +4°C and 1000 × g. The supernatant was neutralized according to two different procedures depending on the analysis to be carried out subsequently:

1. Adenylates and energy charge. The supernatant (perchloric acid extract) was treated with an equal volume of 1 mol/L N,N-dioctylmethylamine (DOMA) dissolved in chloroform and the neutral aqueous phase was recovered for analysis of adenylates.
2. Lactate. The supernatant (perchloric acid extract) was neutralized by the careful addition of solid $KHCO_3$ and potassium perchlorate was allowed to precipitate in the cold and was separated from the fluid.

Adenine-containing nucleotides were determined by HPLC-chromatography according to a previously published method (Hultman and Ronquist, 1984) and EC was calculated according to the equation of Atkinson (1968).

Lactate in biopsies and media was measured with a multistep reaction sequence, starting with conversion to pyruvate and hydrogen peroxide (H_2O_2) by lactate oxidase. In the presence of the H_2O_2 formed, peroxidase catalyzes the oxidative condensation of chromogen precursors (4-aminophenazone and 2,4,6 tribromo-3-hydroxybenzoic acid) giving rise to a quinone-imine dye, which was read at 540 nm in a spectrophotometer. There was a linear relationship between colour development and lactate concentration. This assay is substantially more sensitive than the conventional lactate dehydrogenase/NAD^+ method. 2,4,6 tribromo-3-hydroxybenzoic acid was synthesized according to Trinder and Webster (1984).

Fluorescence histochemistry

After the incubation procedure the biopsies were placed into 0.05% porcine trypsin in KRP at +4°C. The length of time for trypsin exposure, before separation of the epidermis was possible, was 25–50 min. The epidermis was gently teased off the dermis and processed according to the Falck/Hillarp formaldehyde method (Falck et al., 1982). Briefly, the epidermal sheets were placed on a slide, corneal surface downwards, dried overnight in a vacuum chamber, then exposed to formaldehyde gas for 1 h at +80°C and mounted in liquid paraffin. This method selectively visualizes a population of epidermal LC capable of taking up and accumulating L-dopa (Falck et al., 2003).

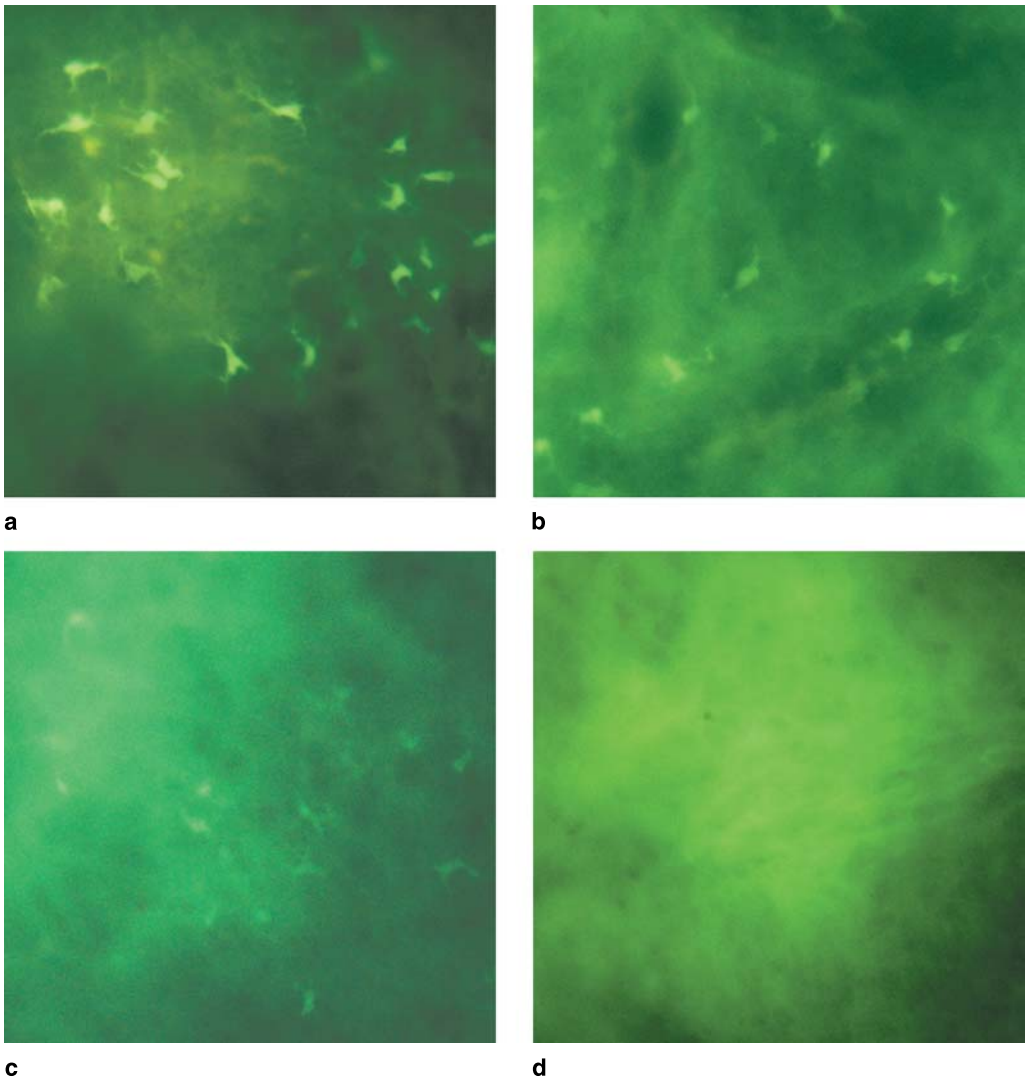

Fig. 1a–d. L-dopa uptake in Langerhans cells estimated by fluorescence intensity after incubation in absence and presence of different inhibitors, using an arbitrary scale containing 4 steps from no inhibition (0%) (**a**), weak inhibition (35%) (**b**), medium – strong inhibition (65%) (**c**), to maximal inhibition of L-dopa uptake, where no fluorescent cells are seen (100%) (**d**)

Biopsies were coded and viewed in the fluorescence microscope on at least two different occasions by two observers thus unaware of the type of experiment concerned. Intracellular L-dopa uptake was evaluated from the fluorescence intensity in accordance with a previous investigation (Falck et al., 2003).

Assay of inhibitory action on L-dopa uptake

The biopsy LC were examined regarding fluorescence intensity of L-dopa (Falck et al., 2003) after the incubation procedure and evaluated according to an arbitrary scale containing 4 steps from no inhibition of L-dopa uptake at all (inhibition = 0%), to maximal inhibition of L-dopa uptake (inhibition = 100%) (Fig. 1a–d).

Electron microscopy

The split skin biopsies were prefixed in 2.5% glutaraldehyde in 0.15 mol/L cacodylate buffer according to the isoosmolar fixation principle formulated by Falck et al. (1981) for optimal preservation of the volume of the various tissue components. For methodological details *cf.* (Falck et al., 1981).

Results

Inhibitory findings on L-dopa uptake

Following the incubation procedure given in Materials and methods, omeprazole, a well known inhibitor of the H^+-translocating ATPases (Egan and Murray, 2000), exerted an inhibitory action on L-dopa uptake as well (Table 1). It is also evident from the Table that the inhibitory effect of omeprazole was a function of time and concentration of inhibitor. Hence, the inhibition was distinct and already apparent at as low a concentration as

Table 1. Concentration and time-dependent inhibition of L-dopa uptake in LC by omeprazole at pH 6.0

Omeprazole, μmol/L	Incubation time, min	Inhibition, per cent	n
50	60	0	3
50	120	35	3
100	120	65	3
500	150	100	3

n, number of experiments performed on different occasions

50 μmol/L (Table 1). We also tested several omeprazole analogues provided by Astra Hässle, Mölndal, Sweden and Byk Gulden, Konstanz, Germany (Fig. 2). According to the suppliers, these analogues could be subdivided into two categories, *viz*. those which were irreversible and those which were reversible in their inhibition of the tubolovesicular H^+-translocation ATPase of parietal cells. Furthermore, it was evident that the substances under study were unstable in solution at neutral pH, so that a slightly acid pH was preferred. Among the 6 irreversible inhibitors available, we identified 5 with clear-cut inhibitory effects on L-dopa uptake in human LC (Table 2A). It could be noted in this context that the analogue without demonstrated inhibitory effect at pH 6.0 was the most polar one (Table 2A). The inhibitory, irreversible analogues were effective in the concentration range from

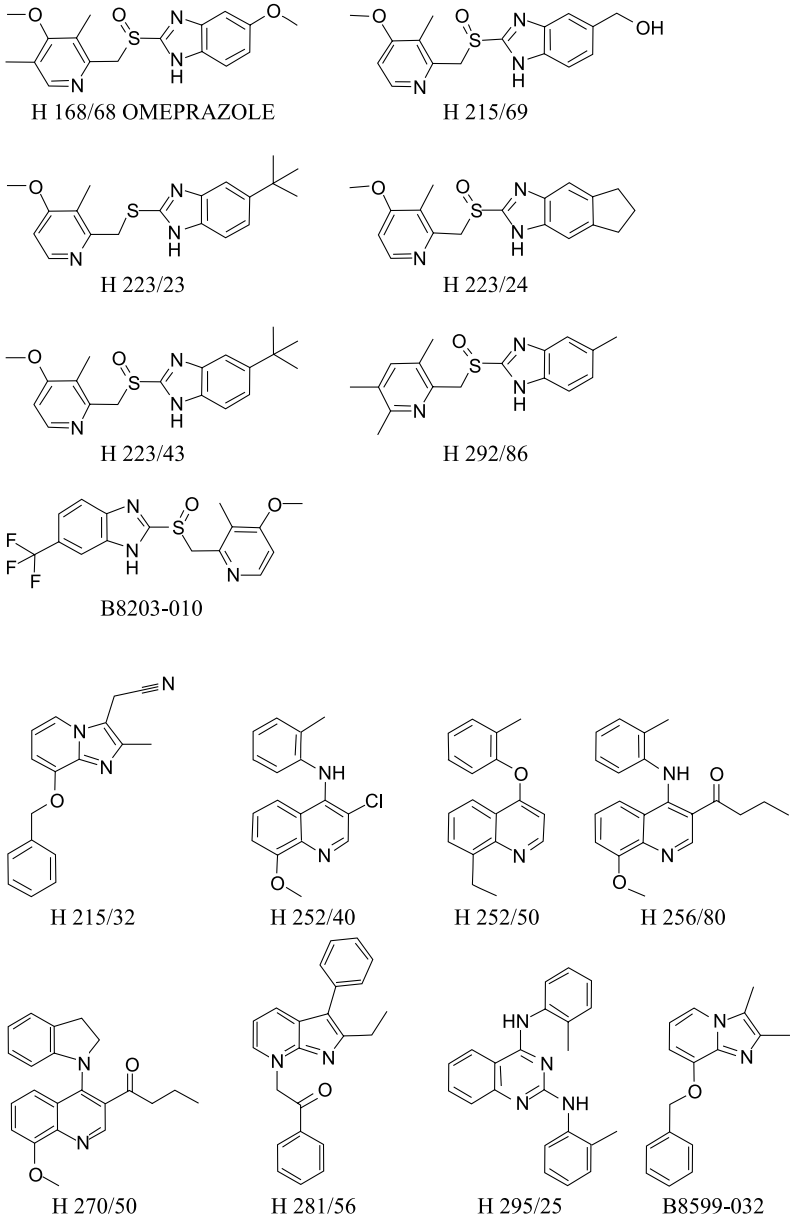

Fig. 2. Chemical formulas of omeprazole and its analogues

Table 2A. Inhibition of L-dopa uptake in LC by various (irreversible) omeprazole-analogues at different concentrations and at pH 6.0

Omeprazole-analogue	μmol/L	Inhibition, per cent	n
H 215/69	50	0	3
H 223/24	10	35–65	4
	50	65–100	11
H 223/23	10	100	3
	50	100	3
H 223/43	1	35–65	6
	5	65	3
	10	65–100	19
	50	100	5
B 8202-010	10	100	3
H 292/86	10	35–65	3
	50	35–65	3

n, number of experiments performed on different occasions

Table 2B. Inhibition of L-dopa uptake in LC by various (reversible) omeprazole-analogues at different concentrations and at pH 6.0

Omeprazole-analogue	μmol/L	Inhibition, per cent	n
H 215/32	50	0	3
B 8599-032	10	0	3
H 256/80	1	0–65	9
	10	35–100	7
	50	65–100	6
H 270/50	1	0	3
	10	100	5
	50	100	2
H 252/40	10	35	3
	50	35–65	3
H 252/50	10	35–65	4
	50	100	4
H 281/56	10	35	3
	50	35–65	3
H 295/25	1	35	3
	10	35–65	6
	50	100	3

n, number of experiments performed on different occasions

1–100 μmol/L. The same pattern was obtained with the reversible inhibitors. Hence, 6 out of 8 inhibitors were effective on L-dopa uptake in LC, similarly (Table 2B). Again, the two inefficient compounds at pH 6.0 were those with strongest polarity. Additionally, three of the irreversible and three of the reversible inhibitors were run at pH 8.1. All of them were efficient as inhibitors in this alkaline environment. It should be noted that the two compounds with high polarity and non-efficiency at a lower pH were indeed active as inhibitors at this higher pH (Tables 3A and 3B). The high-efficient, irreversible inhibitor H 223/43 was selected for dose/response

Table 3A. Inhibition of L-dopa uptake in LC by 3 (irreversible) omeprazole-analogues at pH 8.1

Omeprazole-analogue	μmol/L	Inhibition, per cent	n
H 215/69	100	0–35	3
H 223/24	100	100	3
H 223/43	100	65–100	3

n, number of experiments performed on different occasions

Table 3B. Inhibition of L-dopa uptake in LC by 3 (reversible) omeprazole-analogues at pH 8.1

Omeprazole-analogue	μmol/L	Inhibition, per cent	n
H 215/32	100	35	3
H 256/80	100	100	3
H 270/50	100	100	3

n, number of experiments performed on different occasions

Table 4. Concentration-dependent inhibition of L-dopa uptake in LC by H 223/43. Incubation time, 120 min

H223/43	Inhibition, per cent	n
1 μmol/L	0	6
10 μmol/L	65	6
50 μmol/L	100	3

n, number of experiments performed on different occasions

studies. Table 4 shows that a dose/response relationship existed. This molecule was also investigated with regard to its binding properties to LC by exposing it to one or two washings of different length. The inhibitory action was attenuated successively as a function of the duration of the washing time which varied from 30–300 min (Table 5). The results show that the inhibition was rather persistent and moderately influenced by the washing.

Table 5. Effect of duration of washing time on remaining inhibitory activity on L-dopa uptake in LC after preincubation (90 min) of LC with H 223/43 (50 μmol/L)

Duration of washing, min	Inhibition, per cent	n
30	100	3
60	65	3
180	65	6
240	35	6
300	0–35	6

n, number of experiments performed on different occasions

Table 6. EC values of incubated epidermal biopsies in absence (control) and presence of inhibitor, H 223/43

Type of experiment	Incubation time, min	EC, mean of 2 experiments*
control	60	0.78
H 223/43, 25 μmol/L	60	0.78
H 223/43, 10 μmol/L	240	0.89

* Each experiment comprised a batch of 10 epidermal biopsies

Table 7. Biopsy perchloric acid extract and medium lactate contents of incubated epidermal biopsies (240 min) in presence of the inhibitor, H 223/43 and in its absence (control)

Type of experiment	Biopsy acid extract, lactate, μmol/gdw		Incubation medium, lactate, μmol/L	
H 223/43, 100 μmol/L	40	mean	293	mean
H 223/43, 100 μmol/L	32	37	197	245
H 223/43, 100 μmol/L	38		245	
control	29	mean	218	mean
control	41	31	157	177
control	22		155	

Energy metabolic and ultrastructural findings on epidermal biopsies after incubation with and without inhibitors

There was no observable effect on EC by H 223/43 at 25 μmol/L. On the contrary, when incubation time was prolonged with a lower inhibitor concentration (10 μmol/L) the EC increased (Table 6). This increase as a function of time was consistent with a previous investigation with no inhibitor present (Ronquist et al., 2003). Also, four hours' incubation of biopsies in the presence of 100 μmol/L H 223/43 at pH 6.0 was not decisively influential on lactate production, neither in biopsy nor in incubation medium. Hence, H 223/43 did not seem to affect the glycolytic flow of epidermal cells (Table 7). We also checked the ultrastructure of LC exposed to 50 μmol/L of H 223/43 and made comparisons with those cells not exposed to the inhibitor but otherwise incubated similarly. There were no gross alterations found and no ultrastructural differences were observed between the two incubation conditions (Fig. 3a–b).

Discussion

The discovery of a trans-stimulation property associated with exodus of L-dopa from human LC has allowed the characterization of a bidirectional, carrier-mediated system for the transport of L-dopa across the human LC membrane (Falck et al., 2004). Demonstration of a trans-effect in a transport process provides strong evidence that the process is carrier-mediated, although not all carrier-mediated transport systems show trans-effects (Pisoni et al., 1985). Further evidence that this L-dopa transport system in human LC is carrier-mediated is indicated by its selectivity seen so far confining itself to one single amino acid, namely L-dopa and by its stereoselectivity for the L-isomer of this amino acid (Falck et al., 2003). The widely used

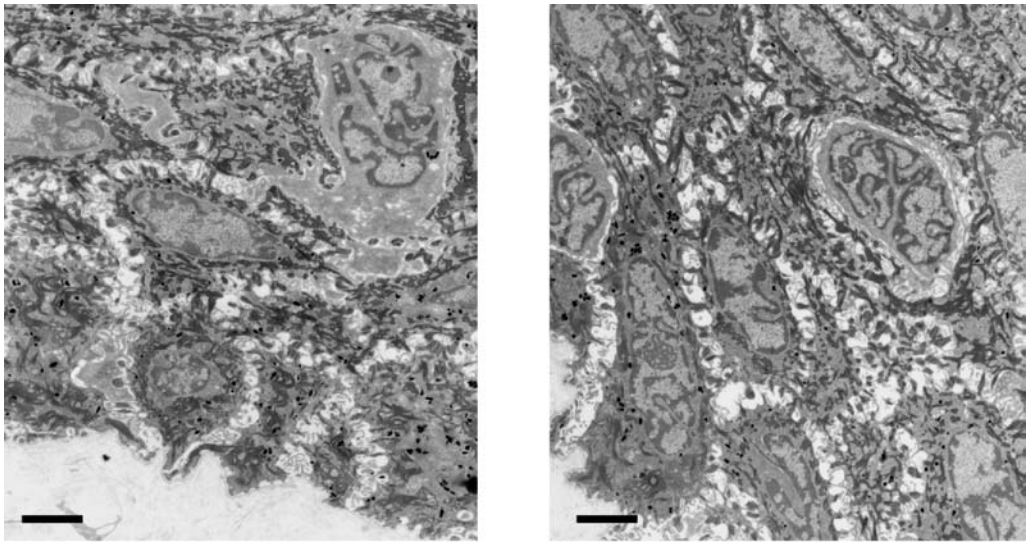

Fig. 3a–b. Electron micrograph showing the basal and spinous layers in a split biopsy incubated under control condition (**a**) and in presence of the omeprazole analogue H 223/43 (**b**). The gross morphology is similar in (**a**) and (**b**), and shows no changes in cell or nuclear morphology, desmosome apparatus or intercellular space. No signs of cell damage is present in either (**a**) or (**b**). (Higher magnification shows no organelle damage). Bar denotes 2 μm

analogue inhibition approach (Oxender and Christensen, 1963; Christensen, 1966) was not applicable in the present study, since the transport system of human LC seems to be unique for L-dopa since no influence of amino acids transported by other known systems was observed (Systems A, L, ASC, N, T, TAT 1) (Falck et al., 2003). Referring to the statements in the introduction regarding similarities between enzymatic catalysis and mediated transport of a substrate, we investigated the effects on L-dopa transport into human LC by H^+, K^+-ATPase inhibitors such as omeprazole and its analogues in an attempt to further characterize the L-dopa transport system in human LC. Omeprazole inhibits purified H^+, K^+-ATPase activity at an IC50 of 2–7.8 μmol/L at pH 6.1 (Wallmark and Jarsten, 1983; Keeling et al., 1985). A distinct inhibition of L-dopa transport into human LC was achieved at 50 μmol/L, *i.e.* at a concentration of almost the same order of magnitude as that used for enzymatic inhibition. The present study also demonstrates that most of its analogues were potent inhibitors of L-dopa transport in LC within the same concentration range. Therefore, it is plausible that the working principle is based on a common denominator, which is, however, not known. As regards omeprazole and its inhibitory action on the H^+, K^+-ATPase, it has to be converted to active inhibitor, the sulfenamide. This conversion is facilitated by a lowered pH (Lindberg et al., 1990) and the sulfenamide is bound covalently to the thiol group(s) of the H^+, K^+-ATPase molecule. Accordingly, the amount of active inhibitor formed for a given concentration of omeprazole is a function of incubation time and pH. However, we did not perform any systematic studies with successively lowered pHs.

As regards vacuolar H^+-ATPases there is a known sensitivity to sulfhydryl group reactive agents, such as N-ethylmaleimide (NEM), and this sensitivity is believed to be due to interaction with critical cysteine residues in the vicinity of the putative catalytic site in the cytoplasmatic domain of the enzyme (Zimniak et al., 1988; Moriyama and Nelson, 1987). Similarly, amino acid transport systems are inactivated by sulfhydryl-preferring reagents like NEM and p-chloromercuri-benzene sulfonate (PCMBS). Hence, NEM inactivated Na^+-dependent alanine transport in rat liver plasma membrane vesicles and it was concluded that one or perhaps several free thiol groups were essential for transport activity (Pola et al., 1990). Also, NEM caused a dose-dependent inhibition of taurine uptake by human brush border membrane vesicles (Dumaswala and Brown, 1996). However, such an inhibition was not obligatory and general but determined by type of cell and transport system. Hence, NEM clearly inhibited α-amino-isobutyric acid transport into glia (but not glioma) cells (Ronquist et al., 1976) and the low affinity lysine transporter in human erythrocytes was likewise inhibited opposite to the high affinity transporter that was not (Deves et al., 1993). We did not find any unambiguous inhibitory effect of NEM on L-dopa uptake in human LC (unpublished observation).

Based on the idea that the L-dopa transport system of LC could contain a thiol group, the intactness of which was essential for operative capability, we carried out experiments with two of the inhibitors in presence of dithiotreitol (DTT) (analogue H 223/24 and H 223/43) or glutathione (reduced form) (omeprazole and analogue H 223/43). However, in neither case were we able to see any abrogation of the inhibition (not shown in figures or tables). Therefore, we cannot claim that the observed inhibitory action of omeprazole and most of its analogues on L-dopa transport in LC was exerted via the inactivation of one or more essential thiol groups. Further, we have no firm evidence that LC contain an H^+-ATPase of the vacuolar H^+-ATPase type. Instead, we believe that the motive force for L-dopa uptake in LC is ongoing glycolysis in these cells generating protons that participate in an L-dopa/ H^+ antiport system across the membrane (Falck et al., 2003).

Because the active inhibitors were all less polar and therefore more hydrophobic it could be anticipated that they were able to penetrate the plasma membrane. Since the glycolytic enzyme 3-phosphoglyceraldehyde dehydrogenase is dependent on an intact thiol group for its activity, and our previous studies showed that iodoacetate could interfere with this enzyme (Ronquist et al., 2003), we were anxious to see whether omeprazole and its analogues could interfere with the glycolytic activity of LC via interaction with this essential thiol group. However, we did not observe any effects on EC values and lactate concentrations by the inhibitors and therefore we conclude that omeprazole and its analogues do not interfere with the glycolytic flow of LC.

Hence, the inhibition of tubulovesicular H^+, K^+-ATPase of the parietal cells by omeprazole and its analogues is preceded by two obligatory steps, the first being the pH-dependent transformation to highly active thiophilic sulfenamides inside the parietal cells, and the second being the covalent binding of the sulfenamide to the SH -group(s) of the H^+, K^+-ATPase and this binding is very rapid at pH 1 (Sachs et al., 1995). We have no grounds for the idea that the inhibition of L-dopa transport into LC would be preceded by the corresponding obligatory steps in the plasma membrane of (or inside) the LC. Thus, an acid dependency was not apparent, since a clear-cut inhibition of L-dopa transport was indeed registered even at pH 8.1 using six of the inhibitors. A corollary is

that a comparison between efficacious concentrations for inhibition of the H^+, K^+-ATPase and the L-dopa transport is not easily done. Generally, the concentration for a distinct inhibition of the H^+, K^+-ATPase was lower than for the corresponding effect on the L-dopa transport. This minor discrepancy could, however, well be explained by different mechanisms of action in the tubulovesicles of the parietal cells and in human LC. Since L-dopa transport is bi-directional in LC an observed inhibited uptake by omeprazole and its analogues could be due to either a reduced influx or an enhanced efflux. However, our inhibition assay comprised two parts, the first one involving two preincubations with the inhibitor and the second one involving incubation with L-dopa, sometimes without the inhibitor. This procedure strongly favours the view that omeprazole and its analogues were inhibitory to the uptake process and not stimulatory to the release process.

Accordingly, as long as the mechanism of action on inhibition of L-dopa transport into LC is not known, we have to interpret our data cautiously. This may be true especially for the highest concentration of omeprazole used ($500\,\mu$mol/L, Table 1), where toxic side effects may be contributory.

In conclusion, we have found, somewhat unexpectedly, a clear-cut inhibitory effect of omeprazole and most of its analogues on L-dopa uptake in LC. The mechanism of action of this inhibition is however not known.

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