Evidence that cycloleucine affects the high-affinity systems of amino acid uptake in cultured human fibroblasts

Madeleine FENEANT,* Nicole MOATTI,† Jean MACCARIO,† Marthe GAUTIER,*
Samia GUERROUI* and Alain LEMONNIER†

*Laboratoire Central de Biochimie et Unité de Recherches d'Hépatologie Infantile, INSERM U 56, Centre Hospitalier de Bicêtre, 94270 Le Kremlin-Bicêtre, France, and †Faculté des Sciences Pharmaceutiques et Biologiques de l'Université Paris-Sud, 92290 Chatenay-Malabry, France

(Received 7 June 1984/Accepted 9 August 1984)

The influence of cycloleucine on kinetic parameters of uptake of L-alanine, L-proline and L-leucine into cultured human fibroblasts was examined under initial-rate conditions with substrate concentrations of 0.05–10 mM and 5 mM-cycloleucine. Kinetic data obtained by computer analysis showed that, in the absence of cycloleucine, cell uptake was heterogeneous for each amino acid. L-Alanine and L-leucine entered by two transport systems with different affinities; L-proline was taken up by one saturable transport system plus a diffusion-like process. This heterogeneity disappeared in the presence of cycloleucine, since the high-affinity systems were no longer detectable. The remaining process had the same kinetic constants as the low-affinity system for alanine and leucine and a K_D similar to the diffusion constant for proline. The influence of cycloleucine on the amino acid uptake was not specific either to the amino acid concerned or to a particular transport system, since the three neutral amino acid-transport systems, A, ASC and L, were involved in these experiments. This influence was shown to be unaffected by the absence of Na^+ (for leucine uptake). ATP content of the cells was identical in the presence or in the absence of cycloleucine.

The use in humans of cycloleucine (1-amino-cyclopentanecarboxylic acid), a non-metabolizable amino acid, as a cytostatic agent, has resulted in a selective hyperaminoaciduria involving dibasic amino acids and cystine (Brown, 1967). A similar effect has been observed in rats (Goyer *et al.*, 1969; Lemonnier *et al.*, 1971). However, *in vitro*, this inhibition of tubular transport is not limited to dibasic amino acids and cystine, but is a more general phenomenon, as has been previously shown in rat kidney-cortex slices (Holtzapple *et al.*, 1970; Moatti *et al.*, 1976). In cultured human fibroblasts, cycloleucine is able to inhibit cellular accumulation of many naturally occurring amino acids, such as alanine, serine, proline, leucine and lysine (Moatti *et al.*, 1978). The characteristics of cycloleucine interaction seem to vary, not only between cell types, but also between luminal and

antiluminal membranes of epithelia (Craan & Bergeron, 1978).

In view of these somewhat contradictory results, we decided to re-examine the effects of cycloleucine, using cultured fibroblasts, a particularly well-adapted material for studies of transfer processes. In our preliminary report (Moatti *et al.*, 1978), the kinetic parameters of uptake were not defined. To gain new insight into the effects of cycloleucine, a study on the mechanisms of amino acid transport in this cell type was first carried out (Feneant *et al.*, 1981; Corriat *et al.*, 1983). In the last few years, considerable progress has been made in the characterization of the amino acid-transport systems in human fibroblasts. The three neutral amino acid carriers A, ASC and L are effective in this cell type. Gazzola *et al.* (1980) found L-proline, L- α -alanine and L-leucine to be

preferentially transported by systems A, ASC and L respectively. In this connection, these three amino acids have been chosen in order to establish their kinetic profiles of uptake in the presence and absence of cycloleucine.

Materials and methods

Cell cultures

Human fibroblasts were obtained from aponeurosis biopsies. Cells were grown at 37°C in Eagle's Minimum Essential Medium containing non-essential amino acids and supplemented with 10% (v/v) human serum and antibiotics (penicillin, 200 units/ml; streptomycin, 200 µg/ml) in a CO₂/air (1:19) atmosphere. All studies were performed on subcultures between passages 3 and 8.

Transport measurements

For the last passage of the experiment, cells were allowed to grow as monolayers on glass coverslips by the method of Foster & Pardee (1969). As we previously described (Feneant *et al.*, 1981), after 1 h of incubation in phosphate-buffered saline solution, pH 7.45, containing 0.1% glucose, each coverslip was transferred into an incubation vial containing 1.0 ml of buffer and the appropriate concentration of ¹⁴C-labelled amino acid (0.5 µCi/ml incubation mixture) in the absence or presence of 5 mM-cycloleucine. The concentrations of the amino acid tested were 2 mM for time-course determination, and 0.05 or 0.066 to 10 mM for kinetic studies. When Na⁺-free phosphate-buffered saline solution was required, an identical concentration of choline was used to replace the Na⁺. All incubations were carried out at 37°C in a metabolic shaker. Kinetic studies were performed after 1 or 2 min incubation (approximate initial-rate conditions). At the end of incubation, coverslips were quickly washed in three successive beakers each containing 20 ml of cold 0.9% NaCl, blotted on absorbent paper, and counted for radioactivity in a scintillation vial containing 15 ml of Bray (1960) solution and 0.5 ml of water. Protein content of the cell layer on each coverslip, measured by the method of Lowry *et al.* (1951), averaged 20 µg. We confirmed that, in this range of cell density, protein content was directly related to intracellular-fluid space. Therefore we expressed amino acid uptake in nmol/100 µg of protein.

A zero blank representing the extracellular label not removed by rinsing was obtained by dipping several coverslips for a few seconds in the incubation medium and then rinsing and counting them for radioactivity as described above.

ATP measurements

In each of 10 Petri dishes, 4 × 10⁵ cells were allowed to grow for 72 h. Then the growth medium was changed and experiments were performed on the day after. Petri dishes were rinsed three times with phosphate-buffered saline solution containing 0.1% glucose and incubated for 1 h in this buffer. Then the buffer was replaced by 10 ml of the same solution with or without cycloleucine. After incubation for 1 min, the cells were rinsed three times by cold 0.9% NaCl, and ATP was measured on each Petri dish. Then cells were harvested by trypsin treatment, as it has been shown to be a better method than scraping for measuring the ATP concentrations (Delhotal *et al.*, 1983). The cells from each Petri dish were exposed to 1 ml of trypsin solution (0.125%) until cell detachment. Then 10 ml of cold 0.9% NaCl was added, and cells were collected by centrifugation (150 g) for 10 min at 4°C. The supernatant was removed and ATP was measured on the cell pellets by bioluminescence by using a photometer (Pico ATP; Isa-Biologie, Cachan, France) and luciferin/luciferase. The whole manipulation was performed at 0°C. Each cell pellet was diluted (1:10, w/v) with 0.01 M-Mops (4-morpholinepropanesulphonic acid) buffer, pH 7.4. The cell suspension was vortex-mixed, then disrupted by dilution 1:10 in diethyl sulphoxide, and vortex-mixed again. After 30 s, successive dilutions were done in Mops. Luciferin/luciferase reagent (50 µl) was necessary for each photometric determination. Calibration was carried out with several concentrations of ATP (0.5–5 ng) in 250 µl of Mops buffer.

Mathematical procedures

Two mathematical procedures were used to determine the kinetic transport parameters; since the standard deviation of the velocity (*v*) was proportional to *v*, proper weighting was used in both methods and even for single-line plotting.

In the first mathematical procedure, previously described in detail (Feneant *et al.*, 1981), lines were plotted by the double-reciprocal method (Lineweaver & Burk, 1934), by weighted linear regression, with *v*² as the weighting factor of reciprocal velocity 1/*v*. When the presence of two systems with different affinities was shown by a break in the plot, the contribution of the high-affinity system to the uptake of high substrate concentrations was necessarily taken into account when calculating the kinetic constants of the low-affinity system. Therefore a straight line was adjusted along the points of the graph furthest to the right, in an area where the low-affinity system can be disregarded. The contribution of the high-affinity system was then subtracted from the

experimental velocities for the high substrate concentrations. This feathering technique is similar to that used in the treatment of exponential decays (Wagner, 1975).

The second procedure used to determine kinetic transport parameters was non-linear regression analysis (Metzler *et al.*, 1974). For the whole range of substrate concentrations tested, this computer program directly exploited the equations relating the experimental velocities to the substrate concentrations.

Different mathematical models were therefore tested, including:

$$v = V_{\max.} \frac{[S]}{K_m + [S]}, \quad v = V_{\max.1} \frac{[S]}{K_{m1} + [S]} + V_{\max.2} \frac{[S]}{K_{m2} + [S]}$$

$$v = V_{\max.} \frac{[S]}{K_m + [S]} + K_D [S], \quad v = V_{\max.1} \frac{[S]}{K_{m1} + [S]} + V_{\max.2} \frac{[S]}{K_{m2} + [S]} + K_D [S]$$

This non-linear analysis enabled us to take account of a diffusional process.

Materials

Eagle's Minimum Essential Medium (MEM 2111 plus 2mM-glutamine) was purchased from Eurobio (Paris, France) and phosphate-buffered saline solution (Dulbecco's) from Gibco (Paisley, Scotland, U.K.). L-[U-¹⁴C]Alanine, L-[U-¹⁴C]-leucine and L-[U-¹⁴C]proline were obtained from CEA (Gif sur Yvette, France). The unlabelled amino acids, cycloleucine and ATP were obtained from Sigma (St. Louis, MO, U.S.A.). Luciferin/luciferase was purchased from Dupont de Nemours (Paris, France).

Results

Effect of cycloleucine on the time course of uptake of L-α-alanine, L-proline and L-leucine (Fig. 1)

Cycloleucine (5mM) strongly inhibited the intracellular accumulation of 2mM-L-α-alanine, -L-proline and -L-leucine. This effect was already observed during the first few minutes of incubation. The percentage inhibitions were 61%, 68% and 59% at 5 min for L-α-alanine, L-proline and L-leucine respectively.

On the basis of the time course (Fig. 1), we assumed that 1 min could be taken as the approxi-

mate initial-rate condition for L-leucine transport, and 2 min for L-α-alanine and L-proline transport.

Effect of cycloleucine on the kinetic parameters of uptake of L-α-alanine, L-proline and L-leucine

With the same cell line for each amino acid, initial transport rates were measured over a wide concentration range (0.05–10mM) in the presence or absence of 5mM-cycloleucine.

L-α-Alanine uptake. (a) In the absence of cycloleucine. Data were plotted by the Lineweaver-Burk method (Fig. 2a). The break in the plot indicated that two saturable systems were involved in the L-α-alanine uptake. The kinetic parameters were calculated by computer, as described in the

Table 1. *Effect of cycloleucine on the kinetic parameters of uptake of L-α-alanine, L-proline and L-leucine*
Conditions of cell culture and preincubation were as described in the Materials and methods section. Coverslips were incubated in 1.0ml of phosphate-buffered saline solution with 0.1% glucose containing 0.05 or 0.066–10mM ¹⁴C-labelled L-amino acid. The initial rate of uptake was measured on each coverslip by taking the values of the uptake at 2 min (for L-α-alanine and L-proline: $V_{\max.}$, $\pm$ S.D., expressed as nmol/2 min per 100 μ g of protein; K_D expressed per 2 min) or at 1 min (for L-leucine; $V_{\max.}$, $\pm$ S.D., expressed as nmol/min per 100 μ g of protein). K_m values $\pm$ S.D. are expressed as mM. For each concentration 15 determinations were performed. See the text for the calculations of the kinetic constants of the systems.

	Cycloleucine absent	Cycloleucine (5mM) present
L-α-Alanine	$K_{m1} = 0.13 \pm 0.04$ $V_{\max.1} = 3.15 \pm 0.57$ $K_{m2} = 4.7 \pm 1.2$ $V_{\max.2} = 7.9 \pm 1.4$	Not detectable $K_m = 5.3 \pm 1.5$ $V_{\max.} = 9.4 \pm 2.3$
L-Proline	$K_m = 0.43 \pm 0.10$ $V_{\max.} = 0.31 \pm 0.18$ $K_D = 1.4$	Not detectable $K_D = 1.1$
L-Leucine	$K_{m1} = 0.011 \pm 0.013$ $V_{\max.1} = 1.18 \pm 0.19$ $K_{m2} = 4.0 \pm 1.1$ $V_{\max.2} = 6.8 \pm 1.4$	Not detectable $K_m = 3.7 \pm 0.6$ $V_{\max.} = 6.9 \pm 0.9$

Fig. 1. Effect of cycloleucine on the time course of uptake of L- α -alanine (a), L-proline (b) and L-leucine (c). Conditions of cell culture and incubation were as described in the Materials and methods section. Amino acid uptake was measured on each coverslip after incubation for the appropriate time in 1.0 ml of buffer containing 2 mM 14 C-labelled L-amino acid. The experimental points represent means ($\pm$ S.E.M.: bars) of about 20 determinations. (1) In the absence of cycloleucine; (2) in the presence of cycloleucine (5 mM).

Fig. 2. Double-reciprocal plots of the inhibition of L- α -alanine uptake by cycloleucine.

Conditions of cell culture and incubation were as described in the Materials and methods section. The initial rate of L- α -alanine uptake was measured for 2 min at substrate concentrations in 1.0 ml of buffer from 0.05 or 0.066 to 10 mM. The experimental points represent means of about 15 determinations. (a) In the absence of cycloleucine. Two straight lines define two apparent systems. See the text for the calculations of the kinetic parameters of the two transport systems. (b) In the presence of cycloleucine (5 mM). Note the different scale, owing to the strong inhibition of the uptake. All the points lie on one single straight line.

Materials and methods section. The K_m values were 0.13 ± 0.04 and 4.7 ± 1.2 mM for the high- and low-affinity systems respectively.

(b) In the presence of cycloleucine: Only one straight line could be drawn on the plot (Fig. 2b). The kinetic constants of the only system thus shown are similar to those of the low-affinity system defined in the preceding paragraph (Table 1).

L-Proline uptake. (a) In the absence of cycloleucine. The break in the plot (Fig. 3a) suggested the existence of two uptake processes. However, the mathematical treatment showed that only one saturable system and a diffusion-like process occurred. The best fit of the experimental data was obtained with the model:

$$v = V_{\max} \cdot \frac{[S]}{K_m + [S]} + K_D [S]$$

Fig. 3. Double-reciprocal plots of the inhibition of L-proline uptake by cycloleucine

Conditions of cell culture and incubation were as for Fig. 2. (a) In the absence of cycloleucine. Two straight lines define two apparent systems. See the text for the calculations defining one saturable system and a diffusion process. (b) In the presence of cycloleucine (5 mM). Note the different scale, owing to the strong inhibition of the uptake. A diffusional process occurs alone.

where $K_m = 0.43 \pm 0.10$ mM and $K_D = 1.4$ per 2 min.

Calculations of K_D were based on a cell volume of 0.47μ l/100 μ g of protein, measured as described by Foster & Pardee (1969).

(b) In the presence of cycloleucine. Proline uptake no longer seemed saturable in the range of concentrations tested (Fig. 3b). A diffusion-like process occurred. The slope of the line allowed us to calculate a K_D of 1.1 per 2 min.

L-Leucine uptake. (a) In the absence of cycloleucine. The break in the plot indicated (Fig. 4a) that two saturable systems were involved in L-leucine uptake: kinetic parameters were calculated by computer, as described above. One system

Fig. 4. Double-reciprocal plots of the inhibition of L-leucine uptake by cycloleucine

Conditions of cell culture and incubation were as described in the Materials and methods section. The initial rate of L-leucine uptake was measured for 1 min at substrate concentrations in 1.0 ml of buffer from 0.05 to 5 mM. The experimental points represent means of about 15 determinations. (a) In the absence of cycloleucine. Two straight lines define two apparent systems. See the text for the calculations of the kinetic parameters of the two transport systems. (b) In the presence of cycloleucine (5 mM). Note the different scale, owing to the strong inhibition of the uptake. All the points lie on one single straight line.

displayed very high affinity ($K_m = 0.011 \pm 0.013$ mM; the large s.d. of K_m may be explained by the very slight slope of the line). The low-affinity system had a K_m of 4.0 ± 1.1 mM.

(b) In the presence of cycloleucine. All the points were located on a single straight line (Fig. 4b). The kinetic parameters of this system closely resembled those of the low-affinity system (Table 1).

Na⁺ effect on the inhibition of L-leucine uptake by cycloleucine

Unlike the cellular transport of L- α -alanine and L-proline, that of L-leucine is known to be essentially Na⁺-independent, as confirmed by Gazzola *et al.* (1980). To ascertain the influence of

Table 2. Na^+ effect on the inhibition of L-leucine uptake by cycloleucine

Conditions of cell culture and preincubation were as described in the Materials and methods section. Each coverslip was incubated in 1.0 ml of appropriate buffer containing 0.05 mM-L-[^{14}C]leucine. Incubation time was 1 min. When necessary, the NaCl component of the incubation mixture was replaced by choline chloride. Each result is the mean $\pm$ S.E.M. for 20 determinations.

	Na^+ absent		Na^+ present	
	Uptake (nmol/min per 100 μg of protein)	Inhibition of uptake (%)	Uptake (nmol/min per 100 μg of protein)	Inhibition of uptake (%)
Control	1.25 ± 0.11		1.01 ± 0.07	
+ Cycloleucine (5 mM)	0.30 ± 0.03	76	0.27 ± 0.03	73

Na^+ on the action of cycloleucine, we measured the uptake of L-leucine and its inhibition by cycloleucine, with and without Na^+ in the incubation medium. To permit satisfactory testing of the high-affinity system, the leucine concentration chosen for this experiment was low (0.05 mM). Under these conditions, we confirmed that the absence of Na^+ does not alter the cellular uptake of L-leucine. We further noted that this absence did not affect the strong inhibition of L-leucine transport (80%) produced by cycloleucine (Table 2).

ATP measurements

The results are expressed as nmol of ATP/mg of protein (mean $\pm$ S.D.), and were 19.1 ± 2.1 and 21.5 ± 1.6 in the presence of 5 mM-cycloleucine and in its absence respectively. These results showed no significant alteration of the ATP content of the cells after 1 min incubation in the presence of cycloleucine.

Discussion

To gain new insight into the effects of cycloleucine on amino acid transport in human fibroblasts, we chose three substrates with different modes of uptake, shown by their kinetic parameters. These substrates also differ as regards the nature of their specific systems in this cell type (Gazzola *et al.*, 1980).

However, despite this heterogeneity, cycloleucine affects the cellular transport of all three amino acids in a similar way. Thus the uptake of L- α -alanine and L-leucine, which normally occurs by means of two systems, only involves one system in the presence of cycloleucine. Furthermore, the kinetic constants of this one system resemble those defined for the low-affinity transport system. As regards L-proline uptake, one saturable process is involved in the absence of the inhibitor. In the presence of cycloleucine, this system no longer seems effective, and only the diffusional process remains.

In other cell types than fibroblasts, cycloleucine is known to be transferred by the A and L systems (Robinson, 1976; Matthews & Zand, 1977). Le Cam & Freychet (1977) even assumed that the ASC system is also involved in the transport of cycloleucine in isolated rat hepatocytes. Then one can suggest that cycloleucine may be transferred by the same systems as for other amino acids in human fibroblasts, and consequently may be implicated in competition processes. However, the hypothesis of competition between cycloleucine and amino acids for the same carrier would not completely take into account the inhibition of amino acid transport observed here. Each of the three amino acids tested can be transported by any of the neutral amino acid carriers, A, ASC or L, but their respective affinities for each carrier are different. In the present case, only the high-affinity systems of the three amino acids, regardless of their specific characteristics (A, ASC, L), are selectively ineffective in the presence of cycloleucine. It remains conceivable that an amino acid analogue such as cycloleucine could competitively eliminate heterogeneity of transfer for the three amino acids and yet leave a different residual component of transport for each of the three, but it is worth noting that this component possessed the same kinetic constants as the respective low-affinity systems for each amino acid, measured in the absence of the inhibitor. The high-affinity system used by one amino acid may constitute a low-affinity system for another one. Consequently, it appears that the hypothesis of competition process does not explain by itself why only the high-affinity systems are affected.

Cycloleucine may also be involved in a different inhibition mechanism in relation to a type of transport, i.e. high-affinity processes. In this connection some sources of energy may be envisaged. However, the high-affinity transport system for L-leucine, which is Na^+ -independent, is affected by cycloleucine; at the same time we observe that the cycloleucine action is not Na^+ -dependent.

The ATP content of the cells is not modified after 1 min incubation with cycloleucine. Consequently, cycloleucine does not act through the Na⁺ pump and does not affect another important source of energy, i.e. ATP.

A hypothesis may be formulated on the basis of the known properties of cycloleucine. Caboche & Hatzfeld (1978) have clearly shown that cycloleucine, known to be *in vitro* an inhibitor of methionine adenosyltransferase (ATP:L-methionine S-adenosyltransferase, EC 2.5.1.6), had *in vivo* a toxicity correlated with a block of S-adenosyl-methionine biosynthesis and nucleic acid methylations. In this connection, we note that methionine adenosyltransferase may play a role in the uptake mechanisms, since Cox *et al.* (1974) observed a lower transfer of amino acids and sugars in methionine adenosyltransferase-deficient mutants of *Escherichia coli* than in normal organisms. This enzyme has been detected in cultured human fibroblasts (Bittles & Carson, 1981). Consequently, it may be suggested that cycloleucine acts on an essential mechanism of amino acid uptake by inhibition of a methylation process. This mechanism would be required for high-affinity transport systems to be effective. However, this hypothesis remains quite speculative, especially as the effects are observed within 1 min.

We gratefully acknowledge Mrs. M. Couturier and Mrs. G. Polini for technical assistance.

References

- Bittles, A. H. & Carson, N. A. J. (1981) *J. Inherited Metab. Dis.* **4**, 3–6
- Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–285
- Brown, R. R. (1967) *Science* **157**, 432–434
- Caboche, M. & Hatzfeld, J. (1978) *J. Cell. Physiol.* **97**, 361–370
- Corriat, A., Moatti, N. & Lemonnier, A. (1983) *In Vitro* **19**, 522–528
- Cox, G. S., Kaback, H. R. & Weissbach, H. (1974) *Arch. Biochem. Biophys.* **161**, 610–620
- Craan, A. G. & Bergeron, M. (1978) *Metab. Clin. Exp.* **27**, 1613–1625
- Delhotal, B., Lemonnier, F., Couturier, M. & Lemonnier, A. (1983) *Biochimie* **65**, 121–125
- Feneant, M., Moatti, N., Lemonnier, F. & Lemonnier, A. (1981) *J. Cell. Physiol.* **109**, 37–43
- Foster, D. O. & Pardee, A. B. (1969) *J. Biol. Chem.* **244**, 2675–2681
- Gazzola, G. C., Dall'Asta, V. & Guidotti, G. G. (1980) *J. Biol. Chem.* **255**, 929–936
- Goyer, R. A., Reynolds, J. O. & Elston, R. C. (1969) *Proc. Soc. Exp. Biol. Med.* **130**, 860–863
- Holtzapple, P., Rea, C., Genel, M. & Segal, S. (1970) *J. Lab. Clin. Med.* **75**, 818–825
- Le Cam, A. & Freychet, P. (1977) *J. Biol. Chem.* **252**, 148–156
- Lemonnier, A., Moatti, N., Charpentier, C. & Levilain, P. (1971) *Clin. Chim. Acta* **33**, 351–357
- Lineweaver, H. & Burk, D. (1934) *J. Am. Chem. Soc.* **56**, 658–666
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Matthews, R. H. & Zand, R. (1977) *Biochemistry* **16**, 3820–3824
- Metzler, C. M., Elfving, G. L. & McEwen, A. J. (1974) *Biometrics* **30**, 562–563
- Moatti, N., Lemonnier, A. & Barthou, F. (1976) *Biomedicine* **25**, 18–22
- Moatti, N., Feneant, M., Lemonnier, F., Gautier, M. & Lemonnier, A. (1978) *Clin. Chim. Acta* **86**, 245–249
- Robinson, J. H. (1976) *J. Cell. Physiol.* **89**, 101–110
- Wagner, J. G. (1975) *Fundamentals of Clinical Pharmacokinetics*, pp. 59–62, Drug Intelligence Publications, Hamilton, IL