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INHIBITION OF L-FUCOKINASE FROM RAT LIVER BY L-FUCOSE ANALOGUES *IN VITRO*

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By investigating the effects of more than 15 different L-fucose analogues on the activity of L-fucokinase (EC 2.7.1.52) from rat liver *in vitro*, certain structural requirements for potent inhibition of this enzyme were established. Of the novel compounds, 4,6-dideoxy-L-xylo-hexopyranose (**4**) and methyl 4,6-dideoxy-4-iodo-L-glucopyranose (**9**) were found to be competitive inhibitors with K_i-values of 0.5 mM and 5.0 mM respectively. Thus 4,6-dideoxy-L-xylo-hexopyranose is a better inhibitor of L-fucokinase than methyl-α-L-fucoside (**1**). Uptake of L-fucose into rat hepatoma cells is reduced by 52% in the presence of the deoxy derivative (**4**), leading to a decrease of 45% in the incorporation of L-fucose into total cellular glycoproteins.

Keywords: Rat liver; L-fucokinase; L-fucose analogues; inhibition; glycosylation.

INTRODUCTION

L-Fucose is an important sugar constituent of glycoconjugates and is frequently bound at the terminal site of the oligosaccharide portion. When located at this position, it may participate in biological processes such as blood group determination, clearance of serum glycoproteins, cell-cell-recognition and neuronal differentation.[1–3] Recent investigations have shown a significant influence of fucosylated glycoconjugates on surface antigen variation of different malignant cells.[4–7] Feizi for example has identified L-fucose as a constituent of oncogenic antigens.[8] Fucosylation of glycoconjugates can be impaired either by inhibiting fucosidases or by inhibiting the biosynthesis of GDP-L-fucose.[9] The initial step for the biosynthesis of GDP-L-fucose, the activated nucleotide sugar for

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Abbreviations: Fuc-1-P, β-fucosyl-1-phosphate; GDP-L-Fuc, guanosinediphospho-L-fucose.

fucosyltransferases, is the phosphorylation of L-fucose by L-fucokinase.[10,11] For the specific inibition of the fucosylation of glycoconjugates, we investigated the effects of novel synthetic fucose analogues on L-fucokinase from rat liver *in vitro* and on the fucosylation of glycoproteins in Morris hepatoma 7777 cells. Among the fucose analogues, those with lipophilic side chains (**3** and **11**) were synthesized as candidates for cell membrane penetration, while the azido derivative (**7**) was designed as a photoreactive substrate. Others such as L-galactose and D-arabinose were tested to check the steric tolerance of L-fucokinase.

MATERIALS AND METHODS

Chemicals

Chemicals of analytical grade were from Merck (Darmstadt, Germany). Nucleotides, phosphoenolpyruvate and pyruvate kinase were from Boehringer Mannheim (Mannheim, Germany). L-Fucose was from Pfannstiel Laboratories Inc. (Waukegan, Illinois). L-[1-^{14}C]-Fucose (48.6 mCi/mmol) was purchased from NEN (Bad Homburg, Germany) Liquid scintillation counting was performed in a Packard (Zürich, Switzerland) liquid scintillation counter with QS 501 from Zinsser Analytik (Frankfurt/Main, Germany) as scintillation liquid.

Dulbecco's modification of Eagles's medium (DMEM) was from Biochrom KG (Berlin, Germany). Insulin (4 I.E./l) and dexamethasone (0.4 mg/l) were from Merck (Darmstadt, Germany). Streptomycin (100 mg/l) and Penicillin (1×10^5 I.U.) from Boehringer Mannheim (Mannheim, Germany) and 10% heat-inactivated horse serum were added to the medium.

Fucose analogues were synthesized in Thiem's group according to procedures in the literature or recently published procedures.[12]

In Vitro Investigation of L-Fucokinase

L-Fucokinase activity was measured in a crude homogenate of rat liver. For preparation of the homogenate, livers from Wistar rats were removed under light ether anesthesia, minced and transfered to 2.5 volumes of chilled homogenizing medium containing 0.2 mol/l Tris-HCl, 2 mmol/l $MgCl_2$, pH 7.4. The tissue was homogenized in a Dounce homogenizer with 25 strokes of a loose fitting pestle. The incubation mixture (350 μl) contained 8.6 mmol/l ATP, 4.3 mmol/l GTP, 8.3 mmol/l PEP and 11 U/ml pyruvate kinase, 200 μl homogenate corresponding to 20 mg protein as determined by the method of Lowry *et al.*[13], and varying amounts of fucose analogues for inhibition studies. The reaction was started by adding 50 nCi

of ^{14}C-L-fucose and the mixture was incubated at 37°C for 30 min. The reaction was stopped by addition of 500 μl ethanol (96%) followed by heating at 65°C for 5 min to precipitate the proteins. Portions (100 μl) of the clear supernatant were subjected to descending paper chromatography (Schleicher and Schüll 2043b paper) using the solvent system ethanol; 1 mol/l ammonium acetate (pH 4.5) (5:2). Ten microliters UMP (2%) were applied to the chromatogram as an internal standard, because this nucleotide can be visualized under UV light. After running time of between 16 h and 20 h, the chromatogram was dried and the amount of radioactivity was determined by cutting the chromatogram into 0.5–2.0 cm wide strips and counting in 10 ml of QS 501 scintillation mixture.

For the determination of the K_i-values the substrate concentration was varied by addition of unlabeled L-fucose. Knowing the total amount of radioactivity added as well as the concentration of the unlabelled sugar, it was possible to calculate the amount of metabolite formed during the incubation period. K_i-values were derived from a Dixon plot and the type of inhibition was derived from a Lineweaver-Burk plot.

Cell-labeling Experiments

Morris hepatoma 7777 cells were cultured in DMEM containing 10% heat-inactivated horse serum. After preincubation with 10 mmol/l 4,6-dideoxy-L-xylo-hexopyranose (**4**) and subsequent labeling with 3 μCi ^{14}C-L-fucose in small tissue culture plates (5.5 cm in diameter) the medium was removed. The cells were washed two times with ice cold PBS (phosphate-buffered saline) and suspended in a final volume of 1 ml of PBS.

Protein was determined according to Lowry *et al.*[13] in cell suspensions. Protein-bound radioactivity was determined according to Mans and Novell[14] in the medium and in the cell suspension. For uptake studies, radioactivity was measured in cell suspensions.

RESULTS

Inhibition of GDP-L-Fucose Biosynthesis in a Rat Liver Homogenate

^{14}C-L-Fucose, when incubated in a rat liver homogenate, is metabolized to β-L-fucosyl-1-phosphate and GDP-L-fucose which can easily be separated by paper chromatography. The reaction is linear over a 30 min period and follows Michaelis-Menten kinetics under these conditions. After 30 min at 37°C, 73.2% L-fucose, 24.0% L-fucose-1-phosphate and 2.8% GDP-L-fucose are found (see Figure 1a).

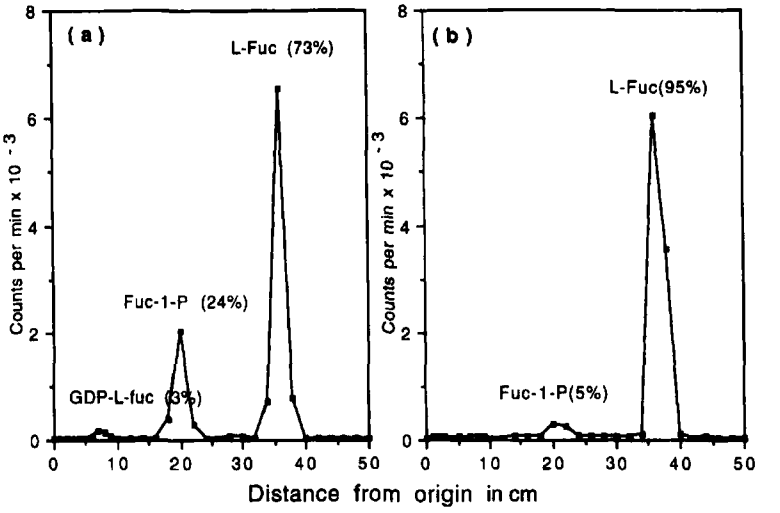

FIGURE 1 L-Fucose and its metabolites in a rat liver homogenate. The homogenate was prepared as described under *Methods*. (a) Control (b) Addition of 4,6-dideoxy-4-iodo-L-glucopyranose (10 mM). Incubation time was 30 min. Separation was performed in a solvent system consisting of ethanol; 1 M ammonium acetate pH 4.5 (7:3). For further details see *Methods*.

Addition of 10 mM 4,6-dideoxy-4-iodo-L-glucopyranose (**5**) reduces the formation of L-fucose metabolites from 26.8% to 5.5% (see Figure 1b) which corresponds to 80% inhibition of fucokinase activity in this system. Table I summarizes the inhibition data of fucokinase *in vitro* by various fucose analogues (see Figure 2).

Characterization of the Inhibition of Fucokinase by 4,6-Dideoxy-L-xylo-hexopyranose (4) and 4,6-Dideoxy-4-iodo-L-glucopyranose (5)

Figure 3a shows the Dixon plot of the fucokinase inhibition by 4,6-dideoxy-L-xylo-hexopyranose (**4**) which gives a K_i-value of 0.5 mmol/l. The Lineweaver-Burk-plot (Figure 3b) reveals that the inhibition is competitive. Inhibition of fucokinase by 4,6-dideoxy-4-iodo-L-glucopyranose (**5**) was also shown to be competitive with a K_i-value of 5.0 mmol/l.

Inhibition of Fucosylation in Hepatoma 7777 Cells by 4,6-Dideoxy-L-xylo-hexopyranose (4)

To obtain more information on the potential biological role of 4,6-dideoxy-L-xylo-hexopyranose (**4**) we examined the influence of 4,6-dideoxy-L-xylo-hexopyranose

TABLE I Influence of fucose analogues* on enzyme activity of L-fucokinase from rat liver

	compound	*inhibitions***
1	Methyl 6-deoxy-α-L-galactopyranoside (Methyl d-L-fucoside)	89% K_i = 1.1 mmol/l
2	1,1,1-Trichlorethyl 6-deoxy-α-L-galacto-pyranoside*	87% K_i = 5.5 mmol/l
3	Octyl 6 deoxy-α-L-galactopyranoside	40% 19% (1 mmol/l)
variation at C-4		
4	4,6-Dideoxy-L-xylo-hexopyranose (4-Deoxy-L-fucose)	74% K_i = 0.5 mmol/l
5	4,6-Dideoxy-4-iodo-L-glucopyranose	75% K_i = 5.0 mmol/l
6	4,6-Dideoxy-4-fluoro-L-gluco-pyranose	30%
7	4,6-Dideoxy-4-azido-α-L-gluco-pyranose	9%
8	Methyl 4,6-dideoxy-α-L-xylo-hexopyranoside	9%
9	Methyl 4,6-dideoxy-4-iodo-α-L-glucopyranoside	54%
variation at C-3		
10	Methyl 3,6-dideoxy-α-L-xylo-hexopyranoside (Methyl 3-deoxy-fucoside)	17%
11	Methyl 6-deoxy-2-O-dodecanoyl-α-L-galacto-pyranoside	37%
steric and confor-mational variations		
12	Methyl 6-deoxy-α-L-mannopyranoside	n.i.
13	D-Arabinose	84%
14	4-Deoxy-D-threo-pentapyranose	4%
15	L-Galactose	n.i.
16	L-Arabinose	n.i.
17	D-Fucose	n.i.
18	D-Galactose	n.i.

*Synthesized by S.C.T. Svensson during a postdoctoral fellowship in Thiem's group in 1988. **The inhibition is calculated as percentage of reduction of the precursor formed in relationship to an assay system without any supplements. The concentration of inhibitor in the assay is usually 10 mmol/l unless otherwise indicated separately. n.i. = no inhibition.

on hepatoma 7777 cells in cell culture experiments. Experiments with 10 mmol/l (**4**) showed no change of viability after 14 h, 24 h and 48 h as detected by the trypan blue method.

Incorporation of ^{14}C-L-fucose into total cellular glycoproteins was reduced by 45% after 6 h preincubation with 10 mM (**4**). At the same time, uptake of ^{14}C-L-fucose into cells is reduced by 51% (see Table II). Incorporation into secreted fucoproteins is reduced by 22% under the conditions described.

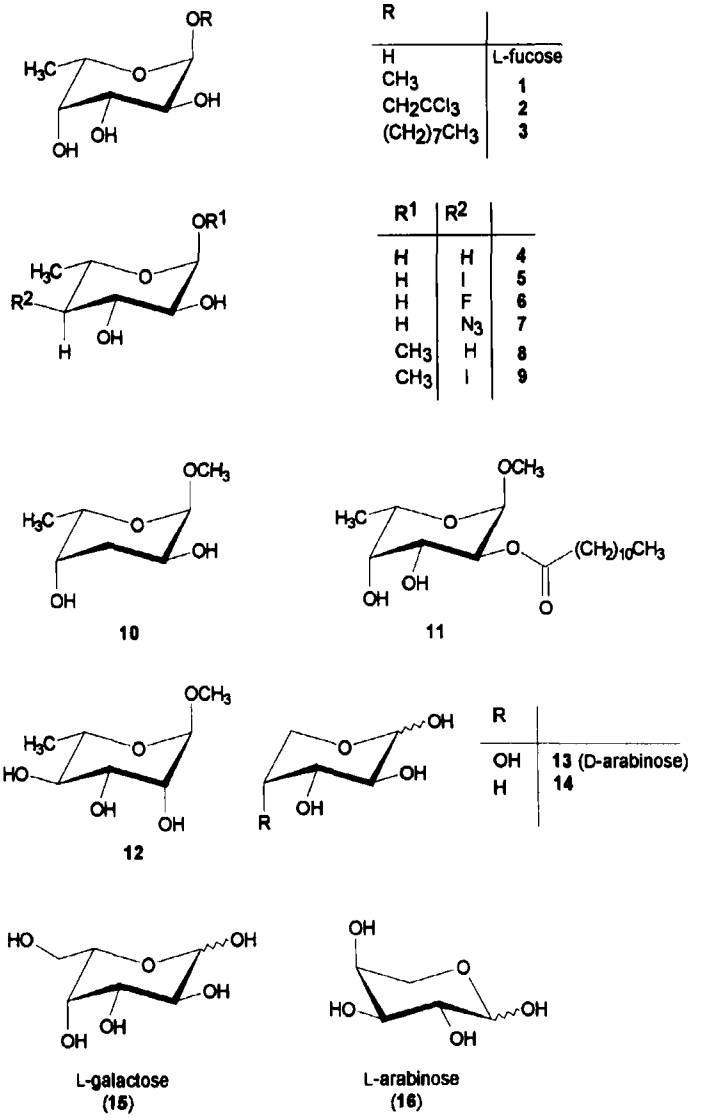

FIGURE 2 Structures of fucose analogues tested.

TABLE II Influence of 4,6-dideoxy-L-xylo-hexopyranose (14) on uptake and incorporation of L-fucose in Morris hepatoma 7777 cells. Cells were treated with 10 mmol/l of inhibitor with different preincubation times as indicated and then labeled with ^{14}C-L-fucose. For further details see Experimental Procedures

Measured parameter	*Preincubation time*				
	control	*1 h*	*2 h*	*4 h*	*6 h*
uptake (cpm/mg cellular protein $\times 10^{-4}$)	10.7	7.9	6.5	6.4	5.2
incorporation (cpm/mg cellular protein $\times 10^{-4}$)	4.0	3.4	2.3	2.7	2.2

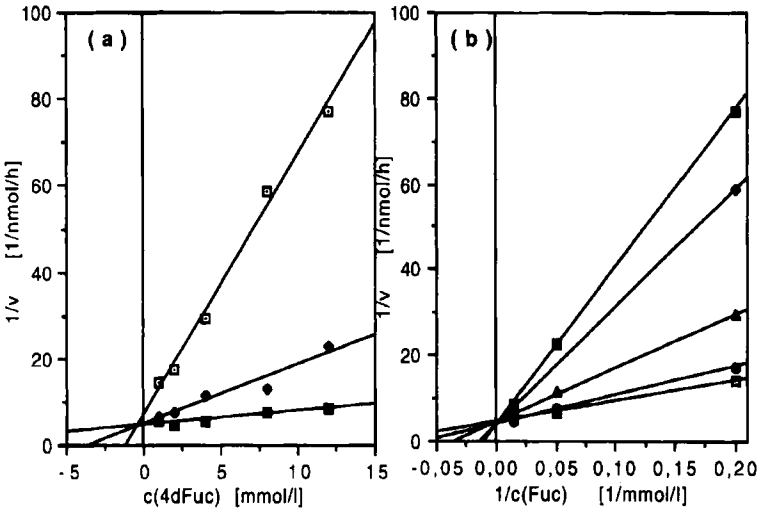

FIGURE 3 Inhibition of L-fucokinase from rat liver *in vitro* by 4,6-dideoxy-L-xylo-hexopyranose (4,4 dFuc). (a) Dixon-plot: the concentrations of the substrate were 5 μmol/l (□), 20 μmol/l (♦) and 65 μmol/l (■). (b) Lineweaver-Burk plot: the concentrations of the inhibitor were 1 mmol/l (□), 2 mmol/l (●), 4 mmol/l (▲), 8 mmol/l (♦) and 12 mmol/l (■).

DISCUSSION

The cell free system used in this study allows the specific measurement of L-fucokinase activity in a liver homogenate using ^{14}C-L-fucose as precursor. L-fucokinase from rat liver is inhibited *in vitro* by various L-fucose analogues, resulting in decreased formation of GDP-L-fucose. The most effective inhibitor is 4,6-dideoxy-L-xylo-hexopyranose (**4**) with a K_i-value of 0.5 mmol/l. Thus (**4**) is a better inhibitor of L-fucokinase than the hitherto used methyl α-L-fucoside (**1**) which has a K_i-value of 1 mmol/l.[15]

4,6-Dideoxy-L-xylo-hexopyranose (**4**) showed no influence on the viability of rat hepatoma cells in our experiments. Other authors, however, report a reduction of cell growth by different fucose analogues, e.g. reduction in the growth of mouse leukemia cells by 2-deoxy-L-fucose. They also found impairment of incorporation of L-fucose into cellular glycoproteins. On the other hand they failed to monitor the inhibition of L-fucose uptake.[16] Inhibition of the fucosylation of glycoproteins by 10 mM (**4**) in hepatoma 7777 cells is more probably due to a decreased uptake of L-fucose into the cells than inhibition of fucoprotein biosynthesis. We suggest that (**4**) competes with L-fucose during the transport of L-fucose through the membrane.

Certain structural requirements for effective inhibitors of L-fucokinase can be derived from an analysis of the data for the *in vitro* inhibition of tho enzyme:

- Inhibitor molecules can be α-glycosylated at C-1 (compounds **1**, **2**, **3**, **8**, **9**, **10**, **11** and **12**). Bulky substituents lead to diminished inhibitor potency (as in **3**).
- Some variations at C-4 of L-fucose as in compounds (**4**), (**5**), (**6**) and (**7**) also lead to good inhibitors. Thus, the 4-hydroxyl group seems not to be involved in substrate binding. In all these analogues the substituent in the 4-position is equatorially bound resulting in an L-gluco configuration. We suggest that more potent inhibition is possible with axial 4-substitution representing L-fucose analogues with the original L-galacto configuration.
- Inhibitory effects resulting from substitution at C-1 and C-4 (**8**, **9**) do not seem to add to already improved inhibition potency. It should be noted that the methyl 4-deoxy-α-L-fucoside (**8**) is not inhibitory, whereas methyl α-L-fucoside (**1**) and the 4-deoxy derivative (**4**) do inhibit L-fucokinase activity.
- L-Fucokinase is not inhibited by D-fucose, D-galactose and L-arabinose, indicating that the enzyme is fairly well able to discriminate between the different enantiomers.
- Derivatization at C-6 is ambivalent. L-Galactose does not inhibit L-fucokinase. D-Arabinose (which may be viewed as a homo-L-fucose in which the terminal methyl group is substituted by a hydrogen atom and bears $^1C_4$-configuration as in L-fucose) does not F(14) inhibit. However, 4-deoxy-D-arabinose is slightly inhibitory. These results therefore reveal certain equivocal aspects of L-fucokinase inhibition, since the structure-activity relationships described are far from clear. This is the subject of further investigation.

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