The structural basis of the inhibition of human α -mannosidases by azafuranose analogues of mannose

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Eight pyrrolidine, five pyrrolizidine and one indolizidine analogue(s) of the known α -mannosidase inhibitor, the azafuranose, 1,4-dideoxy-1,4-imino-D-mannitol (DIM), have been tested for inhibition of the multiple forms of α -mannosidase in human liver *in vitro*. Substitution of the ring nitrogen markedly decreased or abolished inhibition, but loss of the C-6 hydroxy group, as in 6-deoxy-DIM and 6-deoxy-6-fluoro-DIM, enhanced inhibition, particularly of the lysosomal α -mannosidase. Addition of the anomeric substituent $-\text{CH}_2\text{OH}$ decreased inhibition. To be a potent inhibitor of the lysosomal, Golgi II and neutral α -mannosidases, a polyhydroxylated pyrrolidine must have the same substituents and chirality as mannofuranose at C-2, C-3, C-4 and C-5. These four chiral centres can also be part of a

polyhydroxylated indolizidine, e.g. swainsonine, but not of a pyrrolizidine, e.g. cyclized DIM, ring-contracted swainsonine or 1,7-diepi-australine. DIM did not inhibit lysosomal α -mannosidase intracellularly, but both 6-deoxy-DIM and 6-deoxy-6-fluoro-DIM caused accumulation of partially catabolized glycans in normal human fibroblasts. Analysis of these induced storage products by h.p.l.c. showed that both compounds also inhibited Golgi α -mannosidase II and that 6-deoxy-6-fluoro-DIM was also a good inhibitor of the endoplasmic reticulum α -mannosidase and specific lysosomal α (1-6)-mannosidase. None of the mannofuranose analogues appeared to inhibit Golgi α -mannosidase I.

INTRODUCTION

Amino-sugar glycosidase inhibitors are polyhydroxylated piperidines, pyrrolidines, indolizidines and pyrrolizidines, which can be regarded as analogues of monosaccharides in which the ring oxygen has been replaced by nitrogen (Fellows and Nash, 1990; Winchester and Fleet, 1992). They inhibit glycosidases by mimicking the natural substrates and are useful for studying the mechanism of action of these enzymes. The modulation of glycosidase activity by amino-sugars *in vivo* to alter the digestion of sugars, the glycosylation of glycoproteins or the catabolism of glycoconjugates has been shown to have therapeutic potential. About 25 naturally occurring amino-sugars have been isolated (Fellows et al., 1992), and several hundred have been synthesized to achieve greater potency or specificity of inhibition. Most mammalian glycosidases occur as isoenzymes, many of which show marked selectivity of inhibition by amino-sugars. The structurally, functionally and genetically distinct multiple forms of mammalian α -D-mannosidase (EC 3.2.1.24) have characteristic patterns of inhibition by different amino-sugar glycosidase inhibitors (Winchester, 1984; Bischoff and Kornfeld, 1984; Cenci de Bello et al., 1989; Fairbanks et al., 1991). Deoxymannojirimycin (1,5-dideoxy-1,5-imino-D-mannitol), an azapyranose analogue of mannose, selectively inhibits Golgi α -mannosidase I at micromolar concentrations (Bischoff and Kornfeld, 1984), and can be used to block the processing of glycoproteins at the high-mannose stage in cells in culture (Fuhrmann et al., 1984). In contrast, DIM (1,4-dideoxy-1,4-imino-D-mannitol), an azafuranose analogue of mannose (Fleet et al., 1984), is a good inhibitor of lysosomal and Golgi II α -

mannosidases (Palamarczyk et al., 1985), but does not inhibit Golgi α -mannosidase I. The polyhydroxylated indolizidine, swainsonine, in which the configuration of the secondary hydroxy groups is identical to that in DIM and mannofuranose, is an even more potent inhibitor of the same α -mannosidases as DIM (Tulsiani et al., 1982; Elbein et al., 1982; Cenci di Bello et al., 1989). To understand the structural basis of this specificity of inhibition, several derivatives and analogues of DIM have been synthesized and their inhibition of the multiple forms of human α -mannosidase investigated *in vitro* and in cells in culture.

MATERIALS AND METHODS

Source of enzymes

Post-mortem human liver, which had been stored at -20°C , was homogenized in a Potter–Elvehjem homogenizer in distilled water (50%, w/v). The homogenate was centrifuged at 37000 g for 30 min in an MSE 18 centrifuge. The resultant supernatant was used as the source of glycosidases. Lysosomal glycosidases were separated from the non-lysosomal activities by affinity chromatography on concanavalin A–Sepharose-4B (Phillips et al., 1976). An extract of fibroblasts from a patient with genetic α -mannosidosis was used as a source of Golgi α -mannosidase II (Cenci di Bello et al., 1989).

Enzyme assays

Glycosidases were assayed at their optimum pH values using the appropriate fluorogenic 4-methylumbelliferyl glycoside substrate (Melford Laboratories, Ipswich, Suffolk, U.K.) at a final con-

Abbreviation used: DIM, 1,4-dideoxy-1,4-imino-D-mannitol.

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Figure 1 Structures of DIM and australine analogues

centration of 0.5 mM in the presence or the absence of 1 mM inhibitor (Cenci di Bello et al., 1989).

Inhibitors

The structures and trivial names of the polyhydroxylated pyrrolidines and pyrrolizidines tested are shown in Figure 1. DIM (1), swainsonine [(1*S*,2*R*,8*R*,8*aR*)-1,2,8-trihydroxyoctahydroindolizine] (2) and ring-contracted swainsonine [(1*S*,2*R*,7*R*,7*aR*)-1,2,7-trihoxypyrrolizidine] (10) were synthesized as described previously (Carpenter et al., 1989). *N*-Methylation, -butylation, -hexanoylation and -benzylation of DIM to give compounds (3), (4), (5) and (6) was carried out essentially as described previously for the analogue 1,4-dideoxy-1,4-imino-*L*-allitol (Al Daher et al., 1989). 6-Deoxy-DIM (7) and 6-deoxy-6-fluoro-DIM (8) were prepared from *D*-mannose (N. M. Carpenter, G. W. J. Fleet, I. Cenci di Bello and B. Winchester, unpublished work). Cyclized DIM [(1*S*,2*R*,6*R*,7*S*)-1,2,6,7-tetrahydroxypyrrolizidine] (9) (Fairbanks et al., 1991) α -homo-DIM (11) (Myerscough et al., 1992) and 1,7-diepi-

australine (12) (Choi et al., 1991) were prepared from *D*-glycero-*D*-talo-heptonolactone. 7-Deoxy-1-epi-australine (13) was prepared from diacetone glucose (S. Choi, G. W. J. Fleet and B. Winchester, unpublished work). 1-Epi-australine (14), which had been isolated from *Castanospermum australe* (Nash et al., 1990), was kindly provided by Dr. R. Nash, Royal Botanical Gardens, Kew, Surrey, U.K. Details of unpublished methods are available on application to G. W. J. F.

Cell culture

Normal human fibroblasts were grown as monolayers in 25 cm² flasks in Ham's F-10 nutrient mixture, supplemented with 10% (v/v) fetal calf serum, glutamine (0.15 μ g/ml), penicillin (100 i.u./ml) and streptomycin (100 μ g/ml) (Imperial Laboratories, Andover, Hants., U.K.), in an incubator at 37 °C in an atmosphere of 95% oxygen and 5% CO₂. The effects of the inhibitors on normal human fibroblasts were determined by incubating cell monolayers in growth medium containing the inhibitor at a final concentration of 1 mM. After 7 days the

medium was removed, and the cells were washed three times with cold PBS to remove any remaining inhibitor or medium and harvested by trypsinization with 0.25% trypsin in PBS. The cells were then recovered by centrifugation in an MSE bench-top centrifuge at 13000 *g* for 3 min, washed twice with cold PBS to remove any trypsin and re-centrifuged.

Isolation of neutral oligosaccharides from human normal fibroblasts

Cell pellets were resuspended in 0.5 ml of distilled, deionized water. The neutral oligosaccharides were released by sonicating the cells in an MSE Ultrasonic disintegrator for 2×15 s pulses at 20 kHz. The cell debris was then removed by centrifugation in an Eppendorf bench-top centrifuge 5414 at 13000 *g* for 10 min. The cell lysates containing the induced oligosaccharides were passed through a Duolite MB 6113 mixed-bed resin (BDH) to remove the charged material. The low-molecular-mass neutral oligosaccharides were separated from glycoproteins by centrifugation of the cell extract in a Centrifree micropartition system (Amicon UK, Ltd.) at 2000 *g* for 15 min. The resulting filtrate, containing protein-free neutral oligosaccharides, was analysed.

Labelling of neutral oligosaccharides by reduction with tritiated sodium borohydride

Radiolabelling of neutral oligosaccharides was carried out by a modification of the methods described initially by Mellis and Baenziger (1981) and Hall and Patrick (1989). Briefly, cell extracts containing neutral oligosaccharides were dried down under nitrogen at 40 °C and then resuspended in 200 mM sodium borate buffer, pH 10.5. Tritiated sodium borohydride was dissolved in 15 mM NaOH and added to the oligosaccharide solution to a final concentration of 20 mM. Samples were then incubated for 3 h at 30 °C. Reduction was stopped by the addition of 50 μ l of 5% (v/v) acetic acid, and the reaction mixtures were then dried down under nitrogen. This was followed by two further washes with methanol (2×250 μ l).

Analysis of labelled neutral oligosaccharides

The radiolabelled neutral oligosaccharides were analysed by h.p.t.l.c. and by h.p.l.c. as described previously (Al Daher et al., 1991, 1992).

Digestion of oligosaccharide substrate

Man α (1-6)[Man α (1-3)]Man α (1-6)[Man α (1-3)]Man β (1-4)-GlcNAc was kindly made available by Dr. C. Warren (Lovett Laboratories, Massachusetts General Hospital, Boston, MA, U.S.A.) and used in assays as described previously (Al Daher et al., 1991).

Metabolic labelling of storage products in cells in culture

Fibroblasts were grown in the presence of D-[2-³H]mannose (13.6 Ci/mmol) (Amersham International, Amersham, Bucks., U.K.) at 20 μ Ci/ml of growth medium. Oligosaccharides were extracted and reduced with sodium borohydride for analysis by h.p.l.c. and h.p.t.l.c.

RESULTS AND DISCUSSION

Inhibition of human liver α -mannosidases *in vitro*

The ability of the derivatives of DIM (1) to inhibit glycosidases was tested initially by assaying the soluble glycosidases in an extract of human liver with a panel of eleven 4-methylumbelliferyl glycosides in the presence and absence of the compound (1 mM). Several derivatives were found to be specific inhibitors of α -mannosidase. They were subsequently tested to see which α -mannosidase isoenzymes were inhibited (Table 1). Some compounds were found to be inhibitors of other glycosidases. The structural basis for their specificity of inhibition is discussed for each compound.

DIM (1)

DIM was found to be a specific and potent inhibitor of all three forms of human α -mannosidase *in vitro*. It has been shown previously that DIM inhibits jack-bean α -mannosidase competitively *in vitro* (Fleet et al., 1984) as well as the processing α -mannosidase II and lysosomal α -mannosidase from rat liver (Palamarczyk et al., 1985). It also prevents the formation of complex asparagine-linked glycans in MDCK cells (Palamarczyk et al., 1985) and macrophages (Daniel et al., 1989) by inhibition of the processing α -mannosidase II.

DIM is the azafuranose analogue of 1-deoxymannose, having the same chirality at carbons 2, 3, 4 and 5 as mannose. Its inhibition of α -mannosidase is presumably due to recognition of these chiral centres by the enzyme. Further evidence that the azafuranose structure is a potent inhibitor of α -mannosidase is provided by swainsonine (2), which is the most potent known inhibitor of α -mannosidase (Dorling et al., 1980; Cenci di Bello et al., 1989). The chirality at C-1, C-2, C-8 and C-8a of the

Table 1 Effects of compounds 1–14 on human α -mannosidases

The lysosomal and neutral enzymes are the α -mannosidase activities in a water-soluble extract that bind and do not bind respectively to concanavalin A–Sepharose. The Golgi α -mannosidase II is the residual α -mannosidase activity assayed with a synthetic substrate at pH 5.75 in fibroblasts from a patient with genetic α -mannosidosis (Cenci di Bello et al., 1989). Abbreviations: ND, not detected; I_{50} , concn. causing 50% inhibition.

Compound		Inhibition at concentration of 1 mM (%)		
No.	Name	Lysosomal	Neutral	Golgi II
(1)	DIM (pK_a 7.5)	89 (K_i 13 μ M)	84 (I_{50} 200 μ M)	96 (I_{50} 100 μ M)
(2)	Swainsonine (pK_a 7.4)	100 (K_i 70 nM)	89 (K_i 4 μ M)	98 (I_{50} 40 nM)
(3)	<i>N</i> -Methyl-DIM	14	45	42
(4)	<i>N</i> -Butyl-DIM	5	14	61
(5)	<i>N</i> -Hexanoyl-DIM	0	0	9
(6)	<i>N</i> -Benzyl-DIM	34	44	72
(7)	6-Deoxy-DIM	100 (K_i 1.3 μ M)	85 (I_{50} 300 μ M)	91 (I_{50} 60 μ M)
(8)	6-Deoxy-6-fluoro-DIM	98 (K_i 1.5 μ M)	91 (I_{50} 25 μ M)	93 (I_{50} 30 μ M)
(9)	Cyclized DIM	20	5	32
(10)	Ring-contracted swainsonine	50	35	44
(11)	α -Homo-DIM	71	71	92 (I_{50} 35 μ M)
(12)	1,7-Diepi-australine	7	0	19
(13)	7-Deoxy-1-epi- australine	0	3	0
(14)	1-Epi-australine	13	ND	ND

indolizine is the same as at C-2, C-3, C-4 and C-5 of the pyrrolidine. A correspondence in specificity of inhibition of glycosidases between other azafuranoses and their corresponding indolizidines has been observed previously (Cenci di Bello et al., 1989).

6-Deoxy-DIM (7) and 6-deoxy-6-fluoro-DIM (8)

The lack of a hydroxy group at C-7 in swainsonine corresponding to the C-6 hydroxy group in mannose and DIM suggests that such a group is not necessary for recognition by α -mannosidases. This was confirmed by the very powerful inhibition of all three α -mannosidases by 6-deoxy-DIM (7) and 6-deoxy-6-fluoro-DIM (8). In fact, the deoxy derivatives are more potent competitive inhibitors of the lysosomal α -mannosidase than the parent compound. 6-Deoxy-DIM has been shown previously to be a good inhibitor of jack-bean α -mannosidase (Eis et al., 1985). Both 6-deoxy-DIM (7) and 6-deoxy-6-fluoro-DIM (8) are moderately good inhibitors of β -galactosidase (82 and 78% inhibition respectively) and α -L-arabinosidase (72 and 65% inhibition respectively), suggesting that they inhibit the broad-specificity soluble β -galactosidase (Chester et al., 1976). Alternatively, removal of the C-6 hydroxy group may permit the compounds to bind to the active sites of the specific β -galactosidase and α -arabinosidase enzymes.

N-Substituted derivatives

Substitution of the ring nitrogen of DIM with a methyl (3), butyl (4), hexanoyl (5) or benzyl (6) group markedly decreases or abolishes inhibition of the lysosomal and neutral α -mannosidases. In contrast, inhibition of the Golgi α -mannosidase is less affected, except by *N*-hexanoyl-DIM (5). *N*-Benzyl-DIM (6) inhibits β -D-glucosidase by 70%, whereas DIM is inactive towards this enzyme. Benzylation of the ring nitrogen of a pyrrolidine amino-sugar glycosidase inhibitor has been shown previously to alter the specificity of inhibition (Al Daher et al., 1989).

Cyclized DIM (9) and ring-contracted swainsonine (10)

The pyrrolizidine compound (9) can be considered as a cyclized form of DIM (1), or more accurately of *N*-methyl-DIM (3). It is a weak inhibitor of the Golgi α -mannosidase and does not inhibit the other two forms of α -mannosidase. The combination of substitution of the ring nitrogen and a change in spatial arrangement of the C-6 and C-7 hydroxy groups due to the formation of the second pyrrolidine ring clearly lowers the affinity of the active sites for the compound. Similarly, compound (10) can be regarded as the pyrrolizidine analogue of swainsonine (2), i.e. a ring-contracted swainsonine in which the six-membered piperidine ring has been contracted to a five-membered ring. Its inhibition of all three α -mannosidases is markedly decreased compared with swainsonine. The transition from an indolizidine (2) to a pyrrolizidine (10) must affect the stereochemistry of the hydroxy groups in a crucial manner. Ring-contracted swainsonine (10), like its open-chain analogue 6-deoxy-DIM (7), is a moderately good inhibitor of β -galactosidase (69%) and α -L-arabinosidase (46%).

α -Homo-DIM (11)

The addition of a hydroxymethyl group to C-1 of DIM (1) to form α -homo-DIM (11) lowers the inhibition of the lysosomal

and neutral α -mannosidases considerably, but has very little effect on the inhibition of the Golgi α -mannosidase. α -Homomannojirimycin has a very similar potency of inhibition towards the three forms of human α -mannosidase as deoxymannojirimycin (Bruce et al., 1989). These observations suggest that recognition of an α -anomeric substituent is not important for binding of inhibitors and substrates to the active sites of human α -mannosidases.

Australine analogues (12), (13) and (14)

The substitution of a hydroxymethyl group on position C-3 of ring-contracted swainsonine, equivalent to an α -anomeric substitution of the C-1 of the furanose analogue, generates an epimer, 1,7-diepi-australine (12), of the naturally occurring pyrrolizidine australine (15). This molecule (12) retains the same stereochemistry at C-1, C-2, C-7a and C-7 as DIM and all the monocyclic derivatives, but is non-inhibitory towards the α -mannosidases. However, it was a good inhibitor of β -galactosidase (91%), β -glucosidase (61%) and β -D-xylosidase (46%) and a weak inhibitor of α -L-fucosidase (41%). This is the same specificity of inhibition as shown by 6-deoxy-DIM (7) and ring-contracted swainsonine (10), confirming that the stereochemistry of the trihydroxylated pyrrolizidine probably complements that of the active sites of these enzymes. Removal or inversion of the configuration of the C-7 hydroxy group of (12) produced 7-deoxy-1-epi-australine (13) and 1-epi-australine (14) respectively. Neither of these compounds inhibited the α -mannosidases, but 1-epi-australine does inhibit β -hexosaminidase (66%), β -xylosidase (64%), α -galactosidase (53%) and β -glucosidase (48%). The parent compound australine (15) is a good inhibitor of α -glucosidases (Tropea et al., 1989).

It is possible to draw some conclusions about the structural features of these compounds that contribute to the inhibition *in vitro* of the different forms of human α -mannosidase. The azafuranose analogue of mannose, DIM (1), is a powerful inhibitory molecule, more potent than the corresponding azapyranose, deoxymannojirimycin for all three α -mannosidases *in vitro*. The values of K_i for inhibition of lysosomal α -mannosidase by DIM and deoxymannojirimycin are 13 μ M and 750 μ M respectively (Fairbanks et al., 1992). Potent inhibition requires the same chirality as mannofuranose at C-2, C-3, C-4 and C-5. The C-6 hydroxy group is not necessary for inhibition, as shown by the more potent inhibition by 6-deoxy-DIM (7) and 6-deoxy-6-fluoro-DIM (8). These four chiral centres can be part of an indolizidine structure, e.g. swainsonine (2), but not of a pyrrolizidine, e.g. cyclized DIM (9) and ring-contracted swainsonine (10). Presumably the orientation of the C-5 hydroxy group (C-8 hydroxy in swainsonine) induced by the bicyclic compounds more closely approaches the active-site configuration in the indolizidine than in the pyrrolizidine, although such changes may also affect the pK_a of the amine. Swainsonine is the most potent inhibitor of α -mannosidases known, with K_i values for the acidic and neutral enzymes of 70 nM and 4 μ M respectively. *N*-Alkylation of DIM generally abolishes inhibition, but the Golgi activity is less affected than the other two activities. Notwithstanding swainsonine, the decrease in inhibition on *N*-substitution may be due to steric hindrance or alteration of the pK_a of the ring nitrogen. Whichever is the correct effect, it probably also contributes to the weaker inhibition of the bicyclic compounds. An α -anomeric substituent, as in α -homo-DIM (11) decreases the inhibition slightly, suggesting that interaction with an anomeric group is not important in the binding of the inhibitors or substrate to the α -mannosidases. A comparable β -anomeric substituent is probably necessary for the inhibition of

β -mannosidase, because none of the 1-deoxy-azafuranose analogues, including swainsonine, inhibits β -mannosidase.

Inhibition of hydrolysis of oligomannosyl substrate by DIM derivatives

Human lysosomal α -mannosidase hydrolyses natural substrate of composition $\text{Man}_2\text{GlcNAc}$ in an ordered manner *in vitro* (Al Daher et al., 1991). Those derivatives of DIM (1) which were shown to be potent inhibitors of the hydrolysis of the synthetic substrate 4-methylumbelliferyl α -D-mannopyranoside by human lysosomal α -mannosidase, also inhibited the breakdown of the natural substrate $\text{Man}\alpha(1-6)[\text{Man}\alpha(1-3)]\text{Man}\alpha(1-6)[\text{Man}\alpha(1-3)]\text{Man}\beta(1-4)\text{GlcNAc}$ (Figure 2). None of the specific breakdown product, $\text{Man}\alpha(1-6)[\text{Man}\alpha(1-3)]\text{Man}\alpha(1-6)\text{Man}\beta(1-4)\text{GlcNAc}$, was detected after 4 h in digestions which contained DIM (1) (lane 3), 6-deoxy-DIM (7) (lane 4) or 6-deoxy-6-fluoro-DIM (8) (lane 5). In contrast, breakdown was observed in digestions containing deoxymannojirimycin (lane 2) and *N*-methyl-DIM (3) (lane 6), which are weak inhibitors of lysosomal α -mannosidase towards the synthetic substrate. Thus there is a good correlation between the inhibition by amino-sugars of the hydrolysis of natural and synthetic substrates.

Inhibition of α -mannosidases in cells in culture

Normal human fibroblasts were maintained in the presence of several DIM derivatives (1 mM) for 2 h to see if the compounds

Figure 2 H.p.t.l.c. of digestion of the natural substrate $\text{Man}_2\text{GlcNAc}$ in the presence of inhibitors of α -mannosidase

Radiolabelled $\text{Man}\alpha(1-6)[\text{Man}\alpha(1-3)]\text{Man}\alpha(1-6)[\text{Man}\alpha(1-3)]\text{Man}\beta(1-4)\text{GlcNAc}$ was digested with lysosomal α -mannosidase for 4 h in the absence (lane 1) and in the presence of (1 mM) deoxymannojirimycin (lane 2), DIM (1) (lane 3), 6-deoxy-DIM (7) (lane 4), 6-deoxy-6-fluoro DIM (8) (lane 5) or *N*-methyl-DIM (3) (lane 6).

Table 2 Intracellular inhibition of human fibroblast α -mannosidases by the inhibitors DIM (1), swainsonine (2), methyl-DIM (3), 6-deoxy-DIM (7) and 6-deoxy-6-fluoro-DIM (8)

Human normal fibroblasts were incubated for 2 h in growth medium not containing any inhibitor (control) or containing a final concentration of 0.1 mM swainsonine (2) or 1 mM of the other inhibitors.

Inhibitor	Protein (mg/ml)	Activity (munits/ml)	Specific activity (munits/mg)	Inhibition (%)
None (control)	0.190	0.80	4.2	0
(1)	0.190	0.71	3.7	12
(2)	0.192	0.16	0.83	80
(3)	0.185	0.79	4.3	2
(7)	0.180	0.38	2.1	50
(8)	0.192	0.42	2.2	48

inhibited lysosomal α -mannosidase intracellularly (Table 2). The degree of inhibition of the intracellular lysosomal α -mannosidase appeared to reflect the potency of inhibition *in vitro*. This suggests that the structural differences among the inhibitors do not profoundly affect their uptake over this period of time. Subsequently, the cells were grown for 7 days in contact with the inhibitors to see if the uptake of the inhibitors caused the accumulation of oligosaccharide storage products. Soluble neutral oligosaccharides were isolated from the cells and analysed by h.p.t.l.c. and h.p.l.c. after reduction. Oligosaccharides were also extracted from cells grown in the absence of any inhibitor and from cells grown in the presence of 100 μM swainsonine, which has been shown previously to induce the accumulation of mannose-rich oligosaccharides (Cenci di Bello et al., 1983). A culture of cells from a patient with genetic α -mannosidosis was also analysed for comparison.

It can be seen (Figure 3) that both 6-deoxy-DIM (7) and 6-deoxy-6-fluoro-DIM (8) cause extensive accumulation of oligosaccharides, but DIM is ineffective at this concentration, as has been reported previously (Cenci di Bello et al., 1989; Daniel et al., 1989). The main component induced by (7) and (8) has the same mobility as the $\text{Man}_5\text{GlcNAc}$ standard. The excessive accumulation of this compound suggests that the processing Golgi α -mannosidase II has been inhibited, causing the formation of hybrid glycans at the expense of complex glycans. Such accumulation is consistent with inhibition of the degradation of this compound *in vitro* by 6-deoxy-DIM (7) and 6-deoxy-6-fluoro-DIM (8), and shows that the lysosomal breakdown of hybrid asparagine-linked glycans is inhibited. The presence of components of composition $\text{Man}_5\text{GlcNAc}$ and $\text{Man}_2\text{GlcNAc}$ indicates that the lysosomal catabolism of glycans derived from high-mannose and complex asparagine-linked glycans respectively has also been disrupted. Thus the lysosomal α -mannosidase has been effectively inhibited intracellularly. Although the identity of some of the induced storage products can be deduced from h.p.t.l.c., more detailed information on the structures of the oligosaccharides was obtained from h.p.l.c.

Comparison of the retention times for authentic samples of oligosaccharides with the times for the induced components

Figure 3 H.p.t.l.c. of neutral oligosaccharides in normal human fibroblasts grown in the presence of α -mannosidase inhibitors

Cells were grown for 7 days in the presence of inhibitors. Oligosaccharides were extracted, partially purified, reduced and then analysed by h.p.t.l.c. Lane 1, normal cells; lane 2, cell from a subject with genetic α -mannosidosis; lane 3, swainsonine; lane 4, DIM (1); lane 5, 6-deoxy-6-fluoro-DIM (8); lane 6, 6-deoxy-DIM (7); lane 7, mixture of authentic standards of composition $\text{Man}_5\text{GlcNAc}$, $\text{Man}_4\text{GlcNAc}$ and $\text{Man}_3\text{GlcNAc}$; lane 8, $\text{Man}_5\text{GlcNAc}$ standard; lane 9, $\text{Man}\alpha(1-3)[\text{Man}\alpha(1-6)]\text{Man}\beta(1-4)\text{GlcNAc}$; lane 10, $\text{Man}\beta(1-4)\text{GlcNAc}$. The radioactive band denoted with an asterisk in lanes 1–6 was seen in normal fibroblasts reduced with tritiated sodium borohydride. This band was not seen when fibroblasts were metabolically labelled with [^3H]mannose and reduced with unlabelled sodium borohydride.

Figure 4 H.p.l.c. of neutral oligosaccharides in fibroblasts grown in the presence of α -mannosidase inhibitors

(a) Normal cells grown in the absence of any inhibitor, (b) fibroblasts from a patient with genetic α -mannosidosis, (c) normal fibroblasts with swainsonine, (d) normal fibroblasts with DIM (1), (e) normal fibroblasts with 6-deoxy-DIM (6) and (f) normal fibroblasts with 6-deoxy-6-fluoro-DIM (7). The oligosaccharides in (b) and (c) were metabolically labelled. In (a) the peak X is an artifact of the reduction process and was present in all mixtures of oligosaccharides that were labelled by reduction with NaBH_4 . This peak was not present when normal cells were labelled metabolically in the absence of inhibitors. It is therefore not present in (b) and (c). The structures of certain oligosaccharides were established by comparison with retention times of authentic standards and are represented by the following scheme: $\bullet$ — $\bullet$, $\text{Man}\alpha(1-2)\text{Man}$; $\bullet$ — $\bullet$, $\text{Man}\alpha(1-3)\text{Man}$; $\bullet$ — $\bullet$, $\text{Man}\alpha(1-6)\text{Man}$.

permitted identification of all the major components (Figure 4). The peak marked 'X' is an artifact of the reduction procedure and is not present when the induced storage products are labelled

metabolically with [^3H]mannose. The structure of the most abundant storage product induced by (7) and (8) was $\text{Man}\alpha(1-6)[\text{Man}\alpha(1-3)]\text{Man}\alpha(1-6)[\text{Man}\alpha(1-3)]\text{Man}\beta(1-4)\text{GlcNAc}$, confirming inhibition of processing Golgi α -mannosidase II. This is in agreement with the effects of (7) and (8) on Golgi α -mannosidase II *in vitro* (Table 1). Deoxy-DIM (7) and 6-deoxy-6-fluoro-DIM (8) appear to differ slightly in their intracellular inhibition of other α -mannosidases at this concentration. There is relatively more material of composition $\text{Man}_6\text{GlcNAc}$ in cells grown in the presence of 6-deoxy-6-fluoro-DIM (8), suggesting that this compound is a better intracellular inhibitor of the endoplasmic reticulum α -mannosidase than is 6-deoxy DIM (7). *In vitro*, both compounds inhibit the neutral α -mannosidase, which is believed to be generated from the endoplasmic reticulum enzyme by proteolysis (Bischoff et al., 1986). The presence of peaks in the h.p.l.c. profile corresponding to oligosaccharides of composition $\text{Man}_6\text{GlcNAc}$, together with the abundance of $\text{Man}_5\text{GlcNAc}$, indicates that (7) and (8) have not inhibited Golgi α -mannosidase I appreciably. The smallest storage products, of composition $\text{Man}_2\text{GlcNAc}$ and $\text{Man}_3\text{GlcNAc}$, must arise from the breakdown of complex asparagine-linked glycans formed before the inhibition of Golgi α -mannosidase II. The $\text{Man}_3\text{GlcNAc}$ induced by both compounds had the structure $\text{Man}\alpha(1-3)[\text{Man}\alpha(1-6)]\text{Man}\beta(1-4)\text{GlcNAc}$. Although the two possible $\text{Man}_3\text{GlcNAc}$ isomers are not resolved by h.p.l.c. under these conditions, h.p.t.l.c. showed that the $\text{Man}_3\text{GlcNAc}$ induced by 6-fluoro-6-deoxy-DIM (8) was almost exclusively $\text{Man}\alpha(1-6)\text{Man}\beta(1-4)\text{GlcNAc}$, whereas a mixture of $\text{Man}\alpha(1-6)\text{Man}\beta(1-4)\text{GlcNAc}$ and $\text{Man}\alpha(1-3)\text{Man}\beta(1-4)\text{GlcNAc}$ was induced by 6-deoxy-DIM (7). Human lysosomal α -mannosidase converts $\text{Man}\alpha(1-3)[\text{Man}\alpha(1-6)]\text{Man}\beta(1-4)\text{GlcNAc}$ predominantly to $\text{Man}\alpha(1-6)\text{Man}\beta(1-4)\text{GlcNAc}$ (Cenci di Bello et al., 1983; Al Daher et al., 1991; Daniel et al., 1992). In genetic α -mannosidosis, which is due to a deficiency of this enzyme, $\text{Man}\alpha(1-3)[\text{Man}\alpha(1-6)]\text{Man}\beta(1-4)\text{GlcNAc}$ is converted to $\text{Man}\alpha(1-3)\text{Man}\beta(1-4)\text{GlcNAc}$ by an alternative pathway catalysed by a specific $\alpha(1-6)$ -mannosidase. Thus the absence of $\text{Man}\alpha(1-3)\text{Man}\beta(1-4)\text{GlcNAc}$ in the presence of (8) indicates that this compound inhibits the lysosomal $\alpha(1-6)$ -mannosidase as well as the major lysosomal α -mannosidase. In contrast, the appearance of $\text{Man}\alpha(1-3)\text{Man}\beta(1-4)\text{GlcNAc}$ in the presence of (7) suggests that the specific $\alpha(1-6)$ -mannosidase is not strongly inhibited by this azafuranose.

Thus 6-deoxy-6-fluoro-DIM (8) is generally a better intracellular inhibitor than 6-deoxy-DIM (7) of those α -mannosidases that are susceptible to inhibition by azafuranose analogues of mannose, namely the major lysosomal enzyme and the specific lysosomal $\alpha(1-6)$ -mannosidase, and the endoplasmic reticulum and Golgi II α -mannosidases. This must be due to the fluorine atom enhancing the uptake or retention of 6-deoxy-6-fluoro-DIM in cells or its efficacy in a cellular environment, because it has a very similar potency to 6-deoxy-DIM *in vitro*. The specificity of inhibition of human α -mannosidases by 6-deoxy-6-fluoro-DIM (8) is similar to that of swainsonine. The specificity of inhibition of human α -mannosidases by these amino-sugars supports our previous suggestions concerning the structural basis of the selectivity of inhibition of α -mannosidases (Cenci di Bello et al., 1989; Fairbanks et al., 1992). These compounds enlarge the range of amino-sugar inhibitors of glycosidases that can be used to selectively modify glycosylation of glycoproteins for therapeutic or biotechnological applications (Winchester and Fleet, 1992).

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