Structure–activity relationship of swainsonine

Inhibition of human α -mannosidases by swainsonine analogues

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The inhibitory properties of a series of synthetic epimers and analogues of swainsonine towards the multiple forms of human α -mannosidases were studied *in vitro* and in cells in culture. Of the five epimers tested, only the 8a-epimer and 8,8a-diepimer of swainsonine were specific and competitive inhibitors (K_i values of 7.5×10^{-5} and 2×10^{-6} M respectively) of lysosomal α -mannosidases *in vitro* and induced storage of mannose-rich oligosaccharides in human fibroblasts in culture. The structures of these storage products indicated that processing α -mannosidases had also been inhibited. This was consistent with the observed inhibition *in vitro* of these enzymes by these compounds. In contrast, the 8-epimer, 1,8-diepimer and 2,8a-diepimer of swainsonine had no appreciable effect on any α -mannosidases. The corresponding open-chain analogues of swainsonine, namely 1,4-dideoxy-1,4-imino-D-mannitol, of the 8a-epimer, namely 1,4-dideoxy-1,4-imino-D-talitol, and of the 8,8a-diepimer, namely 1,4-dideoxy-1,4-imino-L-allitol, were weaker competitive inhibitors of lysosomal α -mannosidase, with K_i values of 1.3×10^{-5} , 1.2×10^{-4} and 1.2×10^{-4} M respectively. These analogues also proved less effective at inducing oligosaccharide accumulation and in disturbing glycoprotein processing. These compounds offer the opportunity to determine which alterations in the chirality of the swainsonine molecule affect its inhibitory specificity. A comparison of their biological activities has identified reagents that will be useful for studying steps in the biosynthesis and catabolism of glycoproteins and that may be of potential value in chemotherapy.

INTRODUCTION

Many naturally occurring or synthetic polyhydroxylated indolizidine, piperidine and pyrrolidine alkaloids are specific and potent inhibitors of mammalian glycosidases (Fellows, 1986; Elbein, 1987). They are used extensively to inhibit processing glycosidases to assess the effect of changes in glycosylation on the properties and functions of glycoproteins (Schwarz & Datema, 1984; Fuhrmann *et al.*, 1985). Changes in glycosylation are associated with many disease processes, and the therapeutic value of these compounds as anti-metastatic (Humphries *et al.*, 1986), anti-tumour-proliferation (Dennis, 1986) and anti-viral (Tyms *et al.*, 1987; Gruters *et al.*, 1987) agents is currently being tested. Many of these compounds are lysosomotropic on account of their weakly basic nature, and have been used to induce reversible deficiencies of lysosomal glycosidases *in vivo* (Huxtable & Dorling, 1982; Saul *et al.*, 1985) and in cells in culture (Cenci di Bello *et al.*, 1983). This can produce a phenocopy of the lysosomal storage disease that results from a genetic deficiency of the same enzyme. Such model systems can be used to study the dynamics of storage-product accumulation and their dispersal on restoration of enzymic activity on withdrawal of the inhibitor (Cenci di Bello *et al.*, 1983), and to identify reversible and

irreversible steps in pathogenetic mechanisms (Huxtable & Dorling, 1982).

Mammalian α -mannosidase exists in genetically and structurally distinct multiple forms, which differ in their pH optima and subcellular locations (Winchester, 1984). Swainsonine (1*S*,2*R*,8*R*,8a*R*)-1,2,8-trihydroxyoctahydroindolizine is a known potent and specific inhibitor of lysosomal and some of the processing forms of α -mannosidase (Dorling *et al.*, 1980; Tulsiani *et al.*, 1982; Cenci di Bello *et al.*, 1983). To gain some understanding of the specificity of this inhibition and to identify novel reagents for studying the metabolism of glycoproteins, the inhibitory effects of a series of isomers and analogues of swainsonine towards the different forms of human α -mannosidase have been investigated.

MATERIALS AND METHODS

Tissue

Post-mortem human liver, which had been stored at -20°C until required, was homogenized (50% w/v) in 10 mM-sodium phosphate buffer, pH 6.0, in a Potter–Elvehjem homogenizer and then centrifuged at 37000 g for 30 min in an MSE 18 centrifuge. The resultant supernatant was used as the source of enzymic activities.

Abbreviations used: DIM, 1,4-dideoxy-1,4-imino-D-mannitol; DIT, 1,4-dideoxy-1,4-imino-D-talitol; DIA, 1,4-dideoxy-1,4-imino-L-allitol.

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Fig. 1. Structures of swainsonine and analogues

Cell culture

Normal human fibroblasts and fibroblasts from a patient with mannosidosis were grown and extracts were prepared as described previously (Burditt *et al.*, 1978). The uptake of swainsonine and analogues into fibroblasts in culture and the measurement of internalized inhibitor was carried out as before (Chotai *et al.*, 1983).

Swainsonine analogues

Swainsonine was kindly provided by Professor P. Dorling, Murdoch University, Perth, Western Australia, Australia. The 8-epimer (Iimura *et al.*, 1986), 8a-epimer (Tadano *et al.*, 1987), 1,8-diepimer, 2,8a-diepimer and 8,8a-diepimer of swainsonine (Tadano *et al.*, 1988), 1,4-dideoxy-1,4-imino-D-mannitol (DIM) (Bashyal *et al.*, 1987) and 1,4-dideoxy-1,4-imino-D-talitol (DIT) (Fleet *et al.*, 1988) were all synthesized and characterized in our laboratories as described previously. 1,4-Dideoxy-1,4-imino-L-allitol (DIA) was prepared from L-gulonolactone by the same method as that used to synthesize 1,4-dideoxy-1,4-imino-D-allitol from D-gulonolactone (Fleet & Son, 1988). Their structures are shown in Fig. 1.

Enzyme assays

Glycosidase activities in extracts of normal human liver and fibroblasts were assayed by using the appropriate fluorogenic 4-methylumbelliferyl glycoside substrate (Koch-Light, Haverhill, Suffolk, U.K.) at a concentration of 0.5 mM at the pH optimum of each enzyme (Burditt *et al.*, 1980). Human liver acidic (lysosomal) and neutral (cytosolic) α -mannosidases were similarly assayed at their pH optima of 4.0 and 6.5, after separation by affinity chromatography on concanavalin

A-Sepharose (Pharmacia, Milton Keynes, Bucks., U.K.) (Phillips *et al.*, 1976). The residual intermediate (Golgi) α -mannosidase II activity in extracts of mannosidosis fibroblasts was assayed at pH 5.75 with the synthetic substrate. Preliminary screening for inhibitory properties was achieved by assaying the multiple forms of α -D-mannosidase and ten other glycosidases in the presence and in the absence of a 1 mM solution of each compound. The nature of the inhibition of the α -D-mannosidase and the value of the K_i were determined by using the Dixon graphical procedure.

Isolation and analysis of neutral oligosaccharides from fibroblasts

Neutral oligosaccharides were isolated from normal human fibroblasts grown in the presence of inhibitors and from urine and fibroblasts from a patient with genetic mannosidosis and analysed by t.l.c. as previously described (Cenci di Bello *et al.*, 1983). In some experiments the relative proportions of the induced storage products were estimated by densitometry with the use of a Joyce-Loebl Chromoscan 3 scanning densitometer.

RESULTS AND DISCUSSION

Inhibition of human liver and fibroblast acidic α -D-mannosidase

Swainsonine completely inhibited the acidic (lysosomal) α -mannosidase in a competitive manner with a K_i of 70 nM at pH 4.0. Although other swainsonine analogues were also specific competitive inhibitors of this form of α -mannosidase, changes in the configuration of

Table 1. Inhibition of human α -D-mannosidases *in vitro*

Values given are representative of experiments carried out two to six times. Abbreviation: N.D., not determined.

Compound	Inhibition by 1 mM compound (%)		
	Lysosomal enzyme (pH 4)	Golgi enzyme (pH 5.75)	Cytosolic enzyme (pH 6.5)
Swainsonine	100	98	89
8a-Epimer of swainsonine	93	96	14
8,8a-Diepimer of swainsonine	100	97	46
8-Epimer of swainsonine	15	N.D.	N.D.
1,8-Diepimer of swainsonine	20	37	21
2,8-Diepimer of swainsonine	15	2	3
1,4-Dideoxy-1,4-imino-D-mannitol (DIM)	59	96	28*
1,4-Dideoxy-1,4-imino-D-talitol (DIT)	80	64	58
1,4-Dideoxy-1,4-imino-L-allitol (DIA)	84	89	53

* 100 μ M inhibitor.

the hydroxy groups and the bridge hydrogen atom (8a) of swainsonine had a marked effect on the efficacy of inhibition (Table 1 and Fig. 1). The 8-epimer was a very poor inhibitor of α -mannosidase. In contrast, alteration of the configuration at the bridge 8a position did not destroy the ability to inhibit and even appeared to contribute to an inhibitory structure in the 8,8a-diepimer. However, the 1,8- and 2,8a-diepipimers of swainsonine were inactive towards α -mannosidase. The 2-epimer and the 2,8-diepimer have previously been shown not to inhibit α -mannosidase (Elbein *et al.*, 1987). From these observations, it is concluded that, for the effective inhibition of acidic α -mannosidase, the configuration of the 1- and 2-hydroxy groups in swainsonine epimers must be 1*S* and 2*R*. However, this is not the total requirement, because the 8-epimer and 8-deoxy-swainsonine (Colegate *et al.*, 1985), which also possess this configuration, do not inhibit α -mannosidase.

DIM, the azafuranose analogue of mannose and an open-chain analogue of swainsonine, is a powerful inhibitor of acidic α -mannosidase (Fleet *et al.*, 1984). Therefore the corresponding azafuranose analogues of the two active swainsonine epimers, the 8a-epimer ('D-talo') and the 8,8a-diepimer ('L-allo'), were similarly tested. Both DIT and DIA were specific competitive inhibitors of acidic α -mannosidase. This confirmed that it is the configuration of the substituents on atoms 1, 2, 8a and 8, corresponding to atoms 2, 3, 4 and 5 in mannose, that determines the inhibitory properties of swainsonine.

Inhibition of other acidic glycosidases

Swainsonine is a specific inhibitor of α -D-mannosidase

at nanomolar concentrations, but at the higher concentration of 1 mM α -glucosidase, β -glucuronidase, β -galactosidase, β -xylosidase and α -arabinosidase were inhibited by over 50 % at their optimal pH values. The effect on the last three enzymes suggests that the broad-specificity cytosolic β -galactosidase is inhibited. With the exception of the 8a-epimer and the 2,8a-diepimer of swainsonine, which inhibited β -galactosidase by 40 % and 30 % respectively, and DIA, which inhibited the activities of β -glucosidase, α -fucosidase and β -hexosaminidase by about 50 %, the other compounds, at 1 mM concentration, did not inhibit other glycosidases by more than 10–20 %. In most cases the glycosidase corresponding to the configuration has not been described in human tissues or the substrate is not available, for example the 1,8-diepimer or 'L-galacto'-swainsonine. Therefore it is not possible to assess whether specificity of inhibition of glycosidases can be predicted from the configuration of isomers. Elbein *et al.* (1987) demonstrated that the 2-epimer, or 'D-gluco'-swainsonine, was a good inhibitor of fungal α -glucosidase amyloglucosidase (K_i 5×10^{-5} M), but interestingly, inhibited Golgi α -mannosidase II *in vivo*. This group also showed that the 2,8-diepimer ('L-ido'-swainsonine) was a moderate inhibitor of β -glucosidase (K_i 5×10^{-4} M). None of the compounds inhibited β -mannosidase by more than 5 %, except for the 8,8a-diepimer of swainsonine and its open-chain analogue, DIA, which causes approx. 25 % inhibition. This demonstrates very clearly that the enzymic mechanisms for the hydrolysis of the α - and β -mannosidic linkages are quite distinct.

Effect of pH on the inhibition of acidic and neutral α -mannosidases

The inhibition of glycosidases by indolizidine alkaloids (Saul *et al.*, 1984) and pyrrolidine alkaloids (Palamarczyk *et al.*, 1985) is pH-dependent. This could be due to the effect of pH on the state of ionization of the inhibitor or of the enzyme. In the case of crude preparations of human α -mannosidases, there could also be a differential effect on the multiple forms of α -mannosidase, which have distinct pH optima, subcellular locations and functions (Winchester, 1984). To clarify this effect, the lysosomal (acidic) and cytosolic (neutral) α -mannosidases that are present in aqueous extract of human liver were separated by affinity chromatography on concanavalin A-Sepharose (Phillips *et al.*, 1976). The inhibition of lysosomal α -mannosidase by swainsonine and the 8a-epimer and 8,8a-diepimer of swainsonine and their open-chain analogues, DIM, DIT and DIA, increased with pH, reaching a limiting value at pH 6.0 (Fig. 2a). This observation suggests that the inhibition depends on the ionization of a group with a pK between 4.1 and 4.6. The values of pK for the ring nitrogen atom in swainsonine and that in DIM are 7.4 (Dorling *et al.*, 1980) and 7.5 respectively. The values of pK for the nitrogen atom in the other swainsonine and azafuranose isomers that have also been shown to be inhibitory are likely to be similar. Thus the inhibitors are positively charged throughout the pH range over which the inhibition varies. Therefore it is concluded that the pH-dependence of inhibition is determined by the state of ionization of a group(s) on the enzyme rather than on the inhibitors. A carboxy group has been implicated in the catalytic mechanism of α -mannosidase (De Prijcker

Fig. 2. Effect of pH on inhibition of lysosomal and neutral α -D-mannosidases

Human liver lysosomal (a) and neutral (b) α -D-mannosidases, which had been separated by affinity chromatography on concanavalin A-Sepharose, were treated with the following: ●, swainsonine, 1 μ M in (a) and 10 μ M in (b); ○, DIM, 0.2 mM in (a) and (b); ■, 8a-epimer of swainsonine, 1 mM in (a) and (b); □, DIT, 0.25 mM in (a) and 1 mM in (b); ▲, 8,8a-diepimer of swainsonine, 1 mM in (a) and (b); △, DIA, 1 mM in (a) and (b).

et al., 1974; Legler & Julich, 1984). Formation of an ion-pair between an active-site carboxylate anion and the charged inhibitor could explain the pH-dependence of inhibition. This explanation would predict that inhibition would decrease at pH values above 6.5, where the inhibitor would begin to ionize to the neutral form. It is difficult to test this hypothesis because acidic α -mannosidase has very low activity at pH 6.5 or above. However, a consistent decrease in the percentage inhibition of acidic α -mannosidase was observed above pH 6.0 (Fig. 2a).

Only swainsonine and to a much lesser extent the 8,8a-diepimer and the azafuranoses inhibited neutral α -mannosidase (Fig. 2b). Inhibition increased with pH up to pH 6.5 and then remained constant or decreased slightly, suggesting that both the ionized and un-ionized forms of the inhibitors can inhibit this enzyme. This indicates that lysosomal and cytosolic α -mannosidases have different mechanisms of action, as would have been expected from our previous kinetic studies (Hirani & Winchester, 1979).

Inhibition of Golgi α -mannosidase II *in vitro*

Fibroblasts from patients with the lysosomal storage disease mannosidosis are deficient in acidic α -mannosidase. The residual activity in these cells towards the synthetic substrate has an optimal pH of 5.75 and is due to Golgi α -mannosidase II. Table 1 summarizes the effect *in vitro* of the various inhibitors on this Golgi α -mannosidase activity as well as on the lysosomal and cytosolic forms of the enzyme. The Golgi α -mannosidase II is particularly sensitive to inhibition *in vitro*. The formation of an ion-pair between the protonated inhibitor and a carboxylate group on the enzyme may also explain the susceptibility of this enzyme to inhibition.

Inhibition of α -mannosidases *in vivo*

Swainsonine is taken up into fibroblasts in culture in a concentration-, time- and temperature-dependent manner (Chotai *et al.*, 1983). The very high intracellular concentration of swainsonine that results has been attributed to its weakly basic nature, leading to its accumulation within lysosomes and other subcellular compartments with a low pH. The swainsonine-induced accumulation of mannose-rich oligosaccharides in lysosomes of such cells and their dispersal on removal of the inhibitor provide experimental evidence for the lysosomotropic nature of swainsonine (Cenci di Bello *et al.*, 1983). The structures of the induced storage products show that swainsonine has also altered the processing of the glycoproteins by inhibiting the Golgi α -mannosidase II and the endoplasmic-reticulum (cytosolic) α -mannosidase form or the Golgi α -mannosidase I (Cenci di Bello *et al.*, 1983; Winchester, 1984).

These observations are consistent with the inhibition of α -mannosidase *in vitro*, by swainsonine, reported in the present paper. Thus, if a swainsonine analogue inhibits lysosomal α -mannosidase *in vitro* and is lysosomotropic, it should lead to accumulation of oligomannosides. By comparing the pattern of storage products induced in fibroblasts by swainsonine isomers and analogues with the known patterns produced in swainsonine and genetically induced mannosidosis, it is possible to deduce the specificity of inhibition of these compounds *in vivo*. Preliminary experiments showed that the 8a-epimer and 8,8a-diepimer of swainsonine and DIM, DIT and DIA were taken up into normal fibroblasts from the culture medium in a concentration- and time-dependent manner similar to that observed for swainsonine. Therefore cells were grown in the presence of these inhibitors for 1 week and analysed for the induction of storage products (Fig. 3). Clearly the 8,8a-diepimer and 8a-epimer of swainsonine, which inhibited the lysosomal α -mannosidase *in vitro*, caused accumulation of oligosaccharides, indicating that this enzyme was also affected *in vivo*. In contrast, the 1,8-diepimer of swainsonine,

Fig. 3. T.L.C. of storage products induced in human fibroblasts by swainsonine and analogues

Neutral oligosaccharides were isolated from fibroblasts and analysed by t.l.c. as described in the Materials and methods section. Normal cells were grown in the presence of the swainsonine epimers (1 mM) for 7 days. The standards M_2G ($Man_2GlcNAc$) and M_3G ($Man_3GlcNAc$) are authentic samples of oligosaccharides isolated from the urine of a patient with mannosidosis.

which was non-inhibitory *in vitro*, did not induce storage. The pattern of storage material induced by the 8,8a-diepimer of swainsonine was very similar to that produced by swainsonine. The presence of $Man_5GlcNAc$ and higher homologues suggests that Golgi α -mannosidase II and one of the early processing α -(1 $\rightarrow$ 2)-mannosidases have been inhibited. This is consistent with the specificity of inhibition of this compound observed *in vitro*. The major storage products induced by the 8a-epimer of swainsonine were $Man_2GlcNAc$, $Man_3GlcNAc$ and a component with a mobility corresponding to an oligosaccharide with a composition of $Man_4GlcNAc$. This suggests that the 8a-epimer of swainsonine does not inhibit the processing α -mannosidase *in vivo* despite its effect on Golgi α -mannosidase II *in vitro*. An explanation of this observation could be that Golgi α -mannosidase II is blocked but that processing is diverted to a minor uninhibited pathway that can catalyse the hydrolysis of $Man_5GlcNAc$. Such an activity has been described in BHK (baby-hamster kidney) cells (Monis *et al.*, 1987). By implication, the 8a-epimer of swainsonine has no effect on this activity whereas swainsonine and its 8,8a-diepimer are inhibitory towards this enzyme. Complete structural analysis of the $Man_4GlcNAc$ oligosaccharide could provide information about the specificity of this postulated novel pathway. We obtained evidence for a novel lysosomal α -(1 $\rightarrow$ 6)-mannosidase by similar experiments with swainsonine (Cenci di Bello *et al.*, 1983).

Analysis of the storage products induced by the

azafuranose analogues also revealed interesting differences in the specificities of the α -mannosidase inhibitors *in vivo* (Fig. 4). DIM, the analogue of swainsonine, was not a very effective inducer of storage products, and a higher concentration (5 mM) and a longer contact time had to be used. The main oligosaccharides produced were $Man_2GlcNAc$ and $Man_3GlcNAc$, but smaller amounts of $Man_5GlcNAc$, $Man_6GlcNAc$ and particularly $Man_7GlcNAc$ were detected, suggesting that Golgi α -mannosidase I or the endoplasmic-reticulum α -mannosidase had been inhibited. A similar conclusion about the effect of this compound on the processing of glycoproteins has been reached by other groups (Palamarczyk *et al.*, 1985; Daniel *et al.*, 1987). $Man_2GlcNAc$ and $Man_3GlcNAc$ were also the main storage products detected in cells grown in the presence of DIT, with only barely detectable amounts of higher homologues. This suggests that the processing α -mannosidases were not markedly affected by this inhibitor, which is in agreement with the studies performed *in vitro*. The predominant storage product induced by DIA was $Man_2GlcNAc$, but significant amounts of two smaller oligosaccharides of greater mobility were also present. The latter may arise by the inhibition of lysosomal β -mannosidase, which DIA inhibited *in vitro*. Only small amounts of larger oligosaccharides analogous to those present in genetic α -mannosidosis were detected. The preponderance of $Man_2GlcNAc$ over $Man_3GlcNAc$ in cells treated with the azafuranoses is analogous to the situation in genetic mannosidosis. This suggests that

Fig. 4. Densitometric analysis of t.l.c. of storage products induced by azafuranose analogues

Experimental details were as indicated in Fig. 3 legend. (a) DIM-treated cells; (b) DIT-treated cells; (c) DIA-treated cells; (d) mannosidosis cells; (e) mannosidosis urine. $M_{2-8}G$ represent $Man_{2-8}GlcNAc$.

these compounds, unlike swainsonine and its 8,8a-diepimer, do not inhibit the minor lysosomal α -mannosidase, which is unaffected in genetic mannosidosis.

CONCLUSIONS

Several interesting and novel inhibitors of lysosomal and processing α -mannosidases have been described in

this paper. They will be useful in elucidating more steps in the post-translational modification of glycoproteins and their catabolism. In general, it is possible to predict their specificity of inhibition *in vivo* from their action *in vitro*. However, the kinetic experiments do not give any clues as to the lysosomotropic nature of the compound or its ability to penetrate the plasma membrane or other intracellular membranes. Although the pK values of the ring nitrogen atoms of the indolizidine and pyrrolidine alkaloids are similar, the swainsonine epimers were more effective in inducing accumulation of partially catabolized glycans. The structural basis of this observation needs to be investigated in view of the potential chemotherapeutic value of these compounds.

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