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Kifunensine, a Potent Inhibitor of the Glycoprotein Processing Mannosidase I*

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Kifunensine, produced by the actinomycete *Kitasatosporia kifunense* 9482, is an alkaloid that corresponds to a cyclic oxamide derivative of 1-amino mannojirimycin. This compound was reported to be a weak inhibitor of jack bean α-mannosidase (IC_{50} of 1.2×10^{-4} M) (Kayakiri, H., Takese, S., Shibata, T., Okamoto, M., Terano, H., Hashimoto, M., Tada, T., and Koda, S. (1989) *J. Org. Chem.* 54, 4015–4016). We also found that kifunensine was a poor inhibitor of jack bean and mung bean aryl-α-mannosidases, but it was a very potent inhibitor of the plant glycoprotein processing enzyme, mannosidase I (IC_{50} of $2–5 \times 10^{-8}$ M), when [^{3}H]mannose-labeled $Man_9GlcNAc$ was used as substrate. However, kifunensine was inactive toward the plant mannosidase II.

Studies with rat liver microsomes also indicated that kifunensine inhibited the Golgi mannosidase I, but probably does not inhibit the endoplasmic reticulum mannosidase. Kifunensine was tested in cell culture by examining its ability to inhibit processing of the influenza viral glycoproteins in Madin-Darby canine kidney cells. Thus, when kifunensine was placed in the incubation medium at concentrations of 1 μg/ml or higher, it caused a complete shift in the structure of the *N*-linked oligosaccharides from complex chains to $Man_9(GlcNAc)_2$ structures, in keeping with its inhibition of mannosidase I. On the other hand, even at 50 μg/ml, deoxymannojirimycin did not prevent the formation of all complex chains. Thus kifunensine appears to be one of the most effective glycoprotein processing inhibitors observed thus far, and knowledge of its structure may lead to the development of potent inhibitors for other processing enzymes.

There are currently a number of inhibitors known that act on each of the glycosidases in the processing pathway of *N*-linked oligosaccharides (1, 2). However, most of these compounds suffer from one or two major flaws, *i.e.* either they do not have the high degree of specificity or they lack the potency that one would desire in an ideal inhibitor. Thus, castanospermine (3, 4) and deoxynojirimycin (5) inhibit glucosidase I, but they are also effective against glucosidase II (6) as well as lysosomal α-glucosidase (7), intestinal sucrase (8), and so on. Swainsonine, a potent inhibitor of the processing mannosidase II (9, 10) also inhibits lysosomal and other aryl-mannosidases (11, 12). These other activities make some of the *in vivo* studies on the effects of altered oligosaccharide processing on glycoprotein function open to question. Although the mannosidase I inhibitor, deoxymannojirimycin, appears to be quite specific for the Golgi mannosidase I, fairly high concentrations of this compound are required for inhibition ($IC_{50} = 1 \times 10^{-5}$ M) (13). In addition, deoxymannojirimycin is a fairly poor inhibitor of the plant mannosidase I (14), and thus may not act on all enzymes of this class.

In order to find better inhibitors of these enzymes, we have continued testing various compounds that might have the desired characteristics. In this report, we describe the activity of kifunensine (Fig. 1), a compound isolated from an actinomycete and shown to be a weak inhibitor of aryl-mannosidase (15). We show here that kifunensine is a potent inhibitor of the plant mannosidase I *in vitro* and also inhibits the processing of the influenza viral glycoproteins in MDCK[1] cells. Against the purified mannosidase I, kifunensine showed an IC_{50} of about $2–5 \times 10^{-8}$ M. The activity of kifunensine should make it a valuable addition to the growing arsenal of glycosidase inhibitors.

EXPERIMENTAL PROCEDURES

Materials

[2-^{3}H]Mannose (27 Ci/mmol) was purchased from Du Pont-New England Nuclear. Endo-β-*N*-acetylglucosaminidase H (Endo H from *Streptomyces griseus*) and [4,5-^{3}H]leucine were from ICN Biochemicals. Pronase (from *S. griseus*) was obtained from Calbiochem and 1-deoxymannojirimycin was from Genzyme. Concanavalin A-Sepharose, amyloglucosidase (from *Aspergillus niger*), β-glucosidase (from almonds), α-galactosidase (from *A. niger*), β-galactosidase (from jack beans), α-mannosidase (from jack beans), β-*N*-acetylhexosaminidase (from jack beans), and all of the *p*-nitrophenylglycoside substrates were purchased from Sigma. β-Mannosidase was purified from *A. niger* as described previously (16). Mannosidase I (14), mannosidase II (17), and aryl-α-mannosidases were purified from mung bean seedlings. [^{3}H]Mannose-labeled $Man_9GlcNAc$, for use as a substrate for mannosidase I, was prepared in influenza virus-infected MDCK cells labeled with [2-^{3}H]mannose in the presence of the mannosidase I inhibitor deoxymannojirimycin (18). [^{3}H]Mannose-labeled $GlcNAcMan_5GlcNAc$, for use as a substrate for mannosidase II, was produced from the mannose-labeled $Man_9GlcNAc$ by treatment with purified mannosidase I followed by incubation with a partially purified GlcNAc transferase I and UDP-GlcNAc (19). Tissue culture materials were obtained from either Flow Laboratories, Corning Glass Works, or HyClone. Bio-Gel P-4, Bio-Gel P-2, and Dowex 50 H^+ (100–200 mesh) were from Bio-Rad. Kifunensine was a kindly provided by Drs. M. Yamashita and M. Iwami, Fujisawa Pharmaceutical Co., Ibaraki, Japan. Swainsonine was isolated from *Astragalus lentiginosus* as described previously (20). All other chemicals were from reliable sources and were of the best grade available.

Rat liver (Sprague-Dawley male) microsomes and cytosol fractions were prepared according to the method described by Ragab *et al.* (21). Light membranes, rich in mannosidase I activity, were isolated by Percoll gradient fractionation (22) of the 410,000 × *g* pellet (21).

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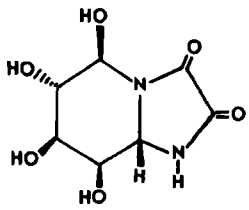
[1] The abbreviations used are: MDCK, Madin-Darby canine kidney; Endo H, endoglucosaminidase H; MES, 4-morpholine-ethanesulfonic acid; ER, endoplasmic reticulum.



KIFUNENSINE

FIG. 1. **Structure of kifunensine.**

Enzyme Assays

Aryl-α-mannosidases were assayed using the colorimetric substrate, *p*-nitrophenyl-α-D-mannopyranoside, as substrate in the following reaction mixture: 100 mM sodium acetate buffer, pH 5.0, 1 mM *p*-nitrophenyl-α-D-mannoside, and various amounts of enzyme in a final volume of 0.5 ml. When kifunensine or other inhibitors were tested, various amounts of each compound were preincubated with the enzyme in an ice bath for several minutes, and then the reaction was initiated by the addition of *p*-nitrophenyl-α-D-mannoside and warming the tubes to 37 °C. After various times of incubation, the reaction was stopped by the addition of 2.5 ml of 0.4 M glycine buffer, pH 10.4, and the amount of *p*-nitrophenol liberated was measured at 410 nm.

Mannosidase I and mannosidase II were assayed by measuring the amount of [^{3}H]mannose released from [^{3}H]mannose-labeled $Man_9GlcNAc$ and $GlcNAc\text{-}Man_5GlcNAc$ using a concanavalin A-Sepharose binding assay or a gel filtration assay, as described previously (18). Mannosidase I (14) and mannosidase II (17) were purified from mung bean seedlings as described. Incubations for the assay of mannosidase I contained the following components, all in a final volume of 0.5 ml: 100 mM MES buffer, pH 6.0, 0.1% Triton X-100, 5 mM $CaCl_2$, 25,000 cpm of $Man_9GlcNAc$, various amounts of kifunensine (or other inhibitors), and enzyme. Incubations were usually for 1 h and were stopped and deproteinized by the addition of a mixture of perchloric acid and trichloroacetic acid. The amount of radioactivity in the liberated mannose was determined by passing the assay mixtures through columns of concanavalin A-Sepharose and measuring the radioactivity in the washes of these columns. The mannosidase II assay mixtures were similar to those of mannosidase I, except that the substrate used in this case was [^{3}H]mannose-labeled $GlcNAc\text{-}Man_5GlcNAc$, and the $CaCl_2$ was omitted from the incubation mixtures. After deproteinization, the liberated mannose was determined either by the concanavalin A binding assay or by gel filtration on columns of Bio-Gel P-4.

Effect of Kifunensine on the Processing of Influenza Viral Glycoproteins

Madin-Darby canine kidney (MDCK) cells were grown to confluence in modified Eagle's medium containing 10% fetal calf serum and the confluent cells were infected with influenza virus. Various amounts of kifunensine were then added to the infected cell cultures to determine the effect of this inhibitor on the processing of the influenza viral glycoproteins. Thus, after a preincubation of 1 h with the inhibitor, [2-^{3}H]mannose (20 μCi/ml) was added to label the viral glycoproteins. Infected cells were allowed to incubate with inhibitor and radioisotope for about 40 h in order to produce mature virus. The turbid suspension, containing lysed cells and virus, was removed and any cells still adhering to the plastic were removed by scraping with a plastic paddle in the presence of phosphate-buffered saline. The combined turbid suspension and scraped cells were pooled and centrifuged at 10,000 × *g* for 30 min to remove cell debris and whole cells. The supernatant liquid from this centrifugation was then centrifuged overnight at 100,000 × *g* to pellet the virus.

The cell pellets and the viral pellets were suspended in 20 mM Tris buffer, pH 7.5, containing 1 mM $CaCl_2$, and digested exhaustively with pronase to produce glycopeptides (3). The glycopeptides were isolated by chromatography on columns of Bio-Gel P-4, and were then treated with endoglucosaminidase H to determine the percentage of susceptible glycopeptides produced at various levels of kifunensine. After digestion with Endo H, susceptible oligosaccharides (*i.e.* hybrid and high mannose structures were separated from resistant (*i.e.* complex chains) glycopeptides by chromatography on columns of Bio-Gel P-4.

Characterization of Oligosaccharides Formed in the Presence of Inhibitor

The structure of the oligosaccharide(s) produced in the presence of kifunensine was determined by a combination of chromatographic and enzymatic methods.

Chromatographic Methods—The radiolabeled oligosaccharides released by Endo H were chromatographed on a long, calibrated column of Bio-Gel P-4 (1.5 × 150 cm, 200–400 mesh), equilibrated in 0.1% acetic acid. A variety of standard oligosaccharides were run on this column for calibration. The various labeled oligosaccharides and glycopeptides were also chromatographed on small columns of concanavalin A-Sepharose (1.5 ml of gel in a Pasteur pipette) to compare the elution profiles to those of control cell oligosaccharides. Samples of known radioactivity were applied to the columns and the columns were washed well with equilibration buffer (20 mM Tris buffer, pH 7.2, containing 200 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$) to remove any unbound material. Fractions of 0.5 ml were collected and examined for radioactivity. Material bound to the column was then eluted, first by washing the column with 10 mM α-methylglucoside in the equilibration buffer to remove biantennary and hybrid structures, and then with 200 mM α-methylmannoside in the equilibration buffer to elute the high mannose structures. Fractions of 0.5 ml were collected and examined for their radioactive content.

Enzymatic Methods—Kifunensine-induced oligosaccharides were digested with jack bean α-mannosidase to determine whether they were high mannose structures or whether they contained other sugars, such as terminal glucose or GlcNAc residues. After digestion, the products were examined by gel filtration on the calibrated Bio-Gel P-4 column.

Effect of Kifunensine on Protein Synthesis and the Formation of Lipid-linked Saccharides

The effect of this inhibitor on protein synthesis and lipid-linked saccharide formation was examined in uninfected MDCK cells. Confluent monolayers were preincubated for 1 h in the presence of various amounts of kifunensine (0.1–100 μg/ml) in modified Eagle's medium containing 2% fetal calf serum. The cells were then incubated with [4,5-^{3}H]leucine (20 μCi/ml) to label the cell proteins, or with [2-^{3}H]mannose (10 μCi/ml) to label the lipid-linked saccharides. After incubation of cells with inhibitor and isotope for the appropriate time (usually 1 h), the monolayers were washed well with phosphate-buffered saline, and the cells were scraped from the plates in saline and placed in tubes for further analysis.

For the analysis of lipid-linked saccharides, the cell suspensions were sequentially extracted with $CHCl_3$:CH_3OH:H_2O (1:1:1) and then with $CHCl_3$:CH_3OH:H_2O (10:10:3) to isolate the lipid-linked monosaccharides and lipid-linked oligosaccharides (23). For analysis of protein synthesis, cell suspensions were sonicated and then extracted with 20% trichloroacetic acid. Bovine serum albumin (5 mg) was added to the extracts to help precipitate the proteins. After standing overnight at 0 °C, the precipitated protein was isolated by centrifugation, washed twice with 5% trichloroacetic acid, once with absolute methanol, twice with 50% methanol, and once again with absolute methanol. The samples were then digested with Pronase to determine their radioactive content.

RESULTS

Activity of Kifunensine against Aryl-α-mannosidases—Kifunensine was first tested against jack bean α-mannosidase as well as the aryl-mannosidases isolated and purified from mung bean seedlings to see whether it would inhibit these enzymes. Fig. 2 shows an inhibition curve on jack bean α-mannosidase comparing kifunensine to swainsonine and to mannostatin A. It can be seen that swainsonine and mannostatin A were powerful inhibitors of this enzyme, showing IC_{50} values of about $1\text{–}2 \times 10^{-8}$ M. On the other hand, kifunensine showed almost no activity on this enzyme at the lower concentrations shown in the figure (*i.e.* up to about 1 μg/ml). Thus, like deoxymannojirimycin, kifunensine only showed reasonable inhibition in the millimolar range (data not shown). Kifunensine also did not inhibit the mung bean aryl-α-mannosidases, except again at reasonably high concentrations (data not shown). Kifunensine was also tested as a

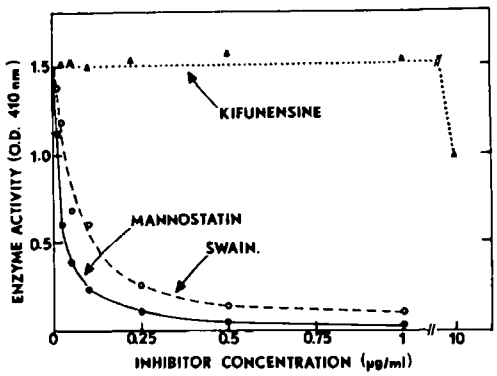


FIG. 2. **Comparison of inhibition of jack bean aryl-α-mannosidase by kifunensine, swainsonine (*SWAIN.*) and mannostatin A.** Various amounts of the inhibitors shown were preincubated with jack bean α-mannosidase for several minutes and then the reaction was initiated by addition of the substrate, *p*-nitrophenyl-α-mannoside. After an incubation of 20 min, the reaction was stopped by the addition of 2.5 ml of glycine buffer, pH 10.4, and the formation of *p*-nitrophenol was measured at 410 nm.

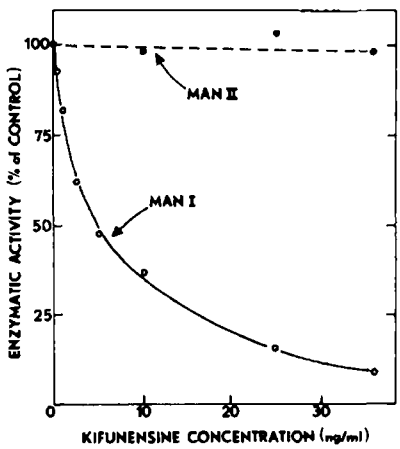


FIG. 3. **Effect of kifunensine concentration on the activities of mung bean purified mannosidase I (*MANI*) and mannosidase II (*MAN II*).** Mannosidase I was assayed as described by monitoring the release of labeled mannose from the [^{3}H]mannose-labeled $Man_9GlcNAc$, and mannosidase II by measuring mannose release from [^{3}H]mannose-labeled GlcNAc-Man_5-GlcNAc. The incubation mixtures were as described in the text and contained the indicated amounts of kifunensine. Incubations were for 1 h.

possible inhibitor of other aryl-glycosidases, including β-mannosidase, amyloglucosidase (an α-glucosidase), and α- and β-galactosidase. This compound was essentially inactive against all of these enzymes, even at concentrations as high as 50 μg/ml. However, it did show some activity toward the β-glucosidase, where 50% inhibition was seen at about 10 μg/ml.

Effect of Kifunensine on the Glycoprotein Processing Enzymes, Mannosidase I, and Mannosidase II—Kifunensine was tested as an inhibitor of the purified plant glycoprotein procesing enzymes, mannosidase I and mannosidase II, as shown in Fig. 3. For these studies, we used the radiolabeled oligosaccharides $Man_9GlcNAc$ and GlcNAc-Man_5GlcNAc as substrates. Fig. 3 shows the effect of increasing concentrations of this compound on the activities of these two enzymes, and clearly demonstrates that kifunensine is a very potent inhibitor of the plant mannosidase I, but has very little effect on mannosidase II. Thus, 50% inhibition of mannosidase I occurred at a concentration of about 4 ng/ml kifunensine, which gives an IC_{50} of about 2×10^{-8} M. This makes kifunensine one of the most potent processing inhibitors, and indicates that this compound is much more effective than deoxymannojirimycin, the other mannosidase I inhibitor.

Effect of Kifunensine on Rat Liver Processing Enzymes—In order to be certain that kifunensine also affected the animal processing mannosidases, we examined the effects of increasing amounts of this compound on mannosidase processing in rat liver microsomes and in the light membrane fraction. We also examined processing in the soluble enzyme fraction of rat liver. In these experiments, the radiolabeled $Man_9GlcNAc$ was used as the substrate, and the experiments were done in the presence of 2 μg/ml swainsonine in order to inhibit any lysosomal α-mannosidase that might be present in the preparations. The results of this experiment are shown in Fig. 4. It can be seen that the release of radioactive mannose was strongly inhibited by increasing amounts of kifunensine in both the light membranes and in the microsomes. In the light membranous fraction which includes rough endoplasmic reticulum, Golgi bodies, and plasma membranes, the IC_{50} for kifunensine was about 1×10^{-7} M. However, even at high concentrations of inhibitor, it was not possible to inhibit more than 80% indicating that these extracts had kifunensine and swainsonine resistant α-mannosidase activity. Interestingly enough, no inhibition of mannose release was observed with the soluble enzyme fraction even at fairly high concentrations of inhibitor. Since the soluble α-mannosidase activity has been reported to be similar to the ER processing α-mannosidase (24), these data suggest that kifunensine is a potent inhibitor of Golgi mannosidase I, but does not affect the ER mannosidase.

We also did similar studies with a microsomal enzyme preparation from Madin-Darby canine kidney cells to determine what effect kifunensine would have on the processing of $Man_9GlcNAc$ in that system. Again the experiments were done in the presence of swainsonine in order to block out the action of lysosomal α-mannosidase. In this case also, increasing amounts of kifunensine caused an increased inhibition of mannose release from the high mannose substrate, and the IC_{50} was estimated to be about 2×10^{-8} M. However, in contrast to the rat liver extracts, the release of mannose from $Man_9GlcNAc$ could be almost completely inhibited (*i.e.* > 97%) at a kifunensine concentration of 2×10^{-7} M (data not shown). These results suggest that the ER α-mannosidase is not present in MDCK cells. *In vivo* studies on glycoprotein

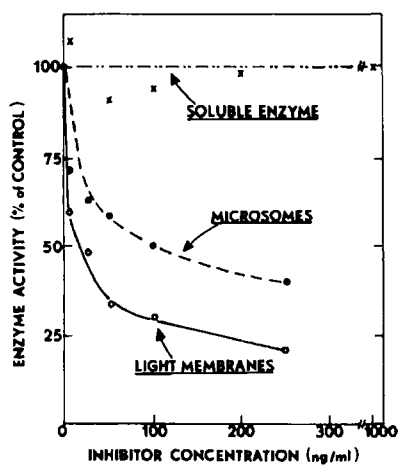


FIG. 4. **Effect of kifunensine concentration on the release of labeled mannose from $Man_9GlcNAc$ by rat liver enzyme fractions.** Rat liver microsomes, rat liver light membranes and rat liver "soluble" enzyme were prepared as described in the text. Each of these fractions was incubated in the presence of various amounts of kifunensine and the release of labeled mannose from [^{3}H]mannose-labeled $Man_9GlcNAc$ was measured.

processing, described below, also indicate that this enzyme is absent from these cells.

Effect of Kifunensine on Glycoprotein Processing in Cell Culture—Since kifunensine was a very strong inhibitor of both the purified mannosidase I from plants, and the membrane-bound mannosidase I of animal cells, it was of interest to determine how it would affect glycoprotein processing in cell culture. For these studies, we infected MDCK cells with influenza virus and then examined the effect of increasing concentrations of inhibitor on the processing of the viral glycoproteins (3). For comparison, we also did these studies in the presence of deoxymannojirimycin, since this compound is also an inhibitor of mannosidase I (25). Thus, after infection, various amounts of kifunensine (or deoxymannojirimycin) were added to the infected cell monolayers, and then [2-^{3}H]mannose was added to label the viral glycoproteins. The viral glycoproteins were digested with pronase and the resulting glycopeptides were separated on columns of Bio-Gel P-4. Fig. 5 shows an example of these profiles and compares the glycopeptides of control virus (upper profile) to those of virus raised in the presence of 100 ng/ml (5×10^{-7} M) kifunensine (*middle profile*), or 1 μg/ml kifunensine (5×10^{-6} M; *lower profile*).

It can be seen from the control incubations (Fig. 5, *upper profile, solid lines*) that the complex types of glycopeptides did not separate well from the high mannose glycopeptides on this short Bio-Gel P-4 column, and they emerged close together in a rather broad peak. Thus, the entire glycopeptide peak was pooled, treated with Endo H to cleave high-mannose (and hybrid) glycopeptides, and the entire digest was reapplied to the same column. As shown by the *dotted line* (Fig. 5, *upper profile*), after treatment of the control glycopeptides with Endo H, the high mannose oligosaccharides migrated more slowly on the column since they were smaller in size, and they could now be resolved from the complex types of glycopeptides. It can be seen from the lower profiles that the viral glycopeptides from cells incubated in kifunensine were very different from those of control cells. Thus, at 100 ng/ml of inhibitor, there was a great decrease in the amount of complex glycopeptides produced (Fig. 5, *middle profile, first peak*), and a great increase in the amount of high mannose oligosaccharides. This was even more evident at higher concentrations of kifunensine (*lower profile*) where there was almost a complete absence of complex oligosaccharides, and essentially all of the radioactivity was in a sharp peak that was characterized as high-mannose oligosaccharides.

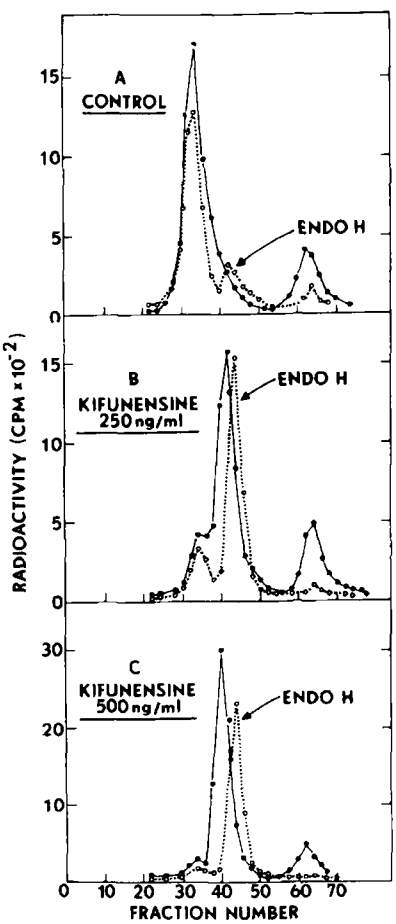


FIG. 5. **Effect of kifunensine on the oligosaccharide composition of the influenza viral hemagglutinin.** Infected MDCK cells were placed in 100 ng/ml or 1 μg/ml kifunensine, and after 1 h [2-^{3}H]mannose was added to label the viral glycoproteins. Control flasks (*upper profile*) were done in the absence of inhibitor. Glycopeptides were prepared as described in the text and isolated on the Bio-Gel P-4 column as shown by the *solid line* in these profiles. The glycopeptide peak was pooled, digested with endoglucosaminidase H, and rechromatographed on the column. Aliquots of each fraction were removed for the determination of radioactivity.

Table I summarizes the data from a series of experiments in which virus was raised in various concentrations of kifunensine, deoxymannojirimycin, or both, and the complex and high mannose structures were separated by gel filtration. It can be seen from the table that increasing concentrations of kifunensine caused a decrease in the amount of radioactivity in peak 1 with a great increase in the amount of radioactivity in peak 2. Thus at 1 μg/ml of this inhibitor, more than 95% of the total radioactivity incorporated into viral glycoproteins was in the high mannose oligosaccharides (*i.e.* peak 2). On the other hand, when deoxymannojirimycin was used as the inhibitor, there was still a significant amount of radioactivity found in the complex glycopeptides, even at 50 μg/ml of this compound. However, when small amounts of kifunensine were added to the incubations along with 25 μg/ml deoxymannojirimycin, the formation of complex chains was almost completely inhibited. It should be noted that in the presence of higher concentrations of kifunensine, there was a considerable increase in the amount of radioactive mannose found in the

TABLE I

Effect of kifunensine and deoxymannojirimycin on the formation of endoglucosaminidase H-sensitive oligosaccharides

Various amounts of the inhibitors shown were incubated with influenza virus-infected MDCK cells as described in the text. The resulting [^{3}H]mannose-labeled glycopeptides were isolated on columns of Bio-Gel P-4, and their susceptibility to digestion by endoglucosaminidase H was examined.

Concentration of inhibitor	Radioactivity (cpm × 10^{-1})	
	Peak 1 (Endo H-resistant)	Peak 2 (Endo H-sensitive)
Kifunensine		
0 (control)	1509	945
10 ng/ml	1506	1007
100 ng/ml	800	2043
1 μg/ml	121	2478
10 μg/ml	21	3421
Deoxymannojirimycin		
1 μg/ml	1229	1198
10 μg/ml	961	1742
50 μg/ml	361	2015
Deoxymannojirimycin (25 μg/ml) Plus Kifunensine		
100 ng/ml	187	2699
5 μg/ml	58	3738
25 μg/ml	46	3269

glycopeptides. This is undoubtedly due to the fact that the oligosaccharides produced in the presence of kifunensine are not processed and therefore have 9, instead of 3, mannose residues.

The oligosaccharide produced in the presence of 1 μg/ml kifunensine and treated with Endo H was sized on a long, calibrated column of Bio-Gel P-4 as shown in Fig. 6. The elution position of this oligosaccharide was identical to that of the $Man_9GlcNAc$ standard (*upper profile*). The oligosaccharide was treated with jack bean α-mannosidase to determine its susceptibility to this enzyme. As shown by the lower profile, the oligosaccharide was completely susceptible to this enzyme and gave rise mostly to free mannose. However, several other faster migrating peaks were also seen. The peak emerging at fraction 160 or so is probably Man-β-GlcNAc that results from complete digestion of $Man_9GlcNAc$ with α-mannosidase. The other two peaks probably result from incomplete digestion with α-mannosidase, or they could come from small amounts of high-mannose oligosaccharides that still have a blocking glucose on the 3-branch. Similar results were obtained with the major oligosaccharide produced in the presence of 50 μg/ml deoxymannojirimycin. These results indicate that most, if not all, of the oligosaccharides produced in the presence of kifunensine are high-mannose structures of the $Man_9(GlcNAc)_2$ structure.

Identification of Oligosaccharide Structures by Chromatography on Columns of Concanavalin A-Sepharose—We compared the binding to columns of concanavalin A-Sepharose of control cell glycopeptides and oligosaccharides (isolated as in Fig. 5) and those produced in the presence of kifunensine, as additional proof that this inhibitor gives rise only to high-mannose structures. Fig. 7 shows the elution profiles of these oligosaccharides from the concanavalin A-Sepharose columns. The uppermost profile is that of the Endo H-resistant peak from control virus (*i.e.* peak 1 from Bio-Gel P-4 columns). It can be seen that this peak does not bind to concanavalin A-Sepharose and all of the radioactivity emerges in the wash. This is the expected behavior for triantennary and tetraantennary complex chains. The middle profile is that given by peak 2 of the control virus, after treatment with Endo H. In this case, some of the radioactivity did not bind to the column and was found in the wash, but there was also radioactivity that bound to the column and was eluted with α-methylglucoside, as well as a large peak of radioactivity that required α-methylmannoside for elution. The peak of radioactivity emerging in the wash is probably contamination from peak 1 since there was not sufficient separation of peak 1 and 2 on the Bio-Gel column, and since peak 1 contained much larger amounts of radioactivity. The rather broad peak of radioactivity emerging in the α-methylglucoside elution is probably due to hybrid types of *N*-linked structures and perhaps some biantennary complex chains, while the large peak that elutes with α-methylmannoside is probably mostly high-mannose chains. The lowest profile shows the results

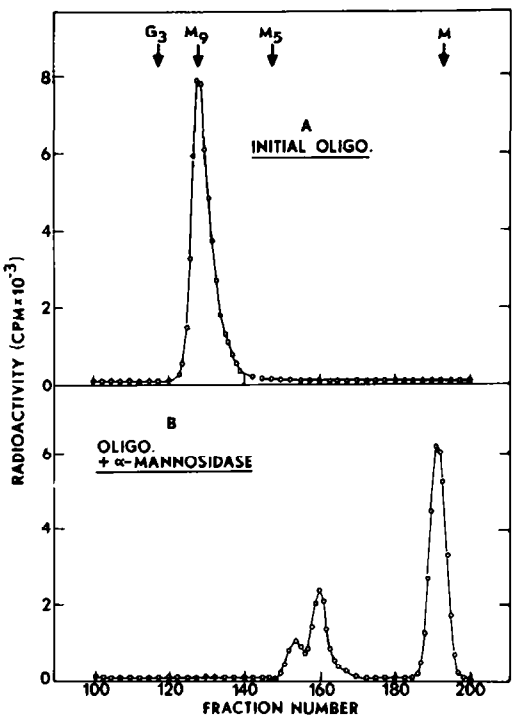


FIG. 6. **Identification of oligosaccharide produced in the presence of kifunensine.** The oligosaccharide (*OLIGO.*) produced at 10 ug/ml kifunensine was isolated from the Bio-Gel P-4 column and chromatographed on a long (1.5 × 150 cm), calibrated column of Bio-Gel P-4 (200–400 mesh). The *upper profile* shows the migration of the intact oligosaccharide. This oligosaccharide was treated with jack bean α-mannosidase, and the digestion was reapplied to the same Bio-Gel P-4 column. Aliquots of each fraction were removed for the determination of radioactivity. Standard oligosaccharides shown by the *arrows* are as follows: G_3, $Glc_3Man_9GlcNAc$; M_9, $Man_9GlcNAc$; M_5, $Man_5GlcNAc$; *M*, mannose.

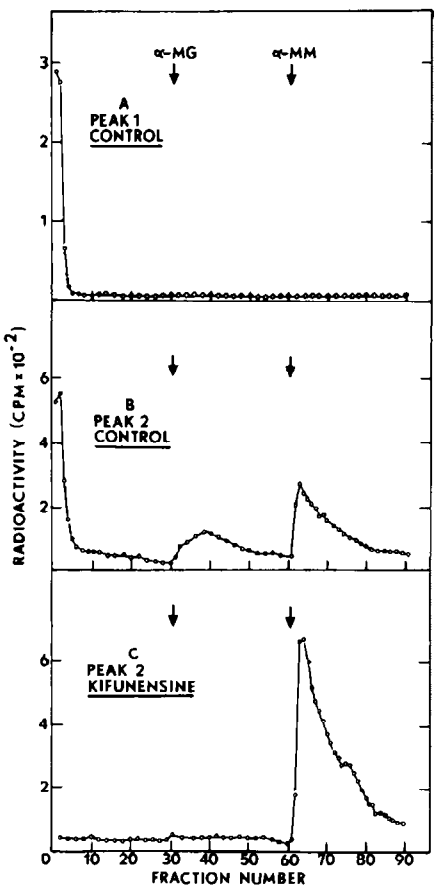


FIG. 7. **Identification of glycopeptides and oligosaccharides by chromatography on columns of concanavalin A-Sepharose.** Peak 1 (Endo H-resistant) and peak 2 (Endo H-sensitive) from control cells (see Fig. 5, Bio-Gel P-4 columns) were applied to 1.5-cm columns of concanavalin A-Sepharose and the columns were washed with equilibration buffer. Columns were then eluted, first with 10 mM α-methylglucoside (*α-MG*) and then with 200 mM α-methylmannoside (*α-MM*). Fractions were collected from each treatment, and their radioactive content was determined. The lowest curve shows the profile obtained from the kifunensine-induced oligosaccharide on the concanavalin A column. In this case, peak 1 was completely devoid of activity (see Table I), and all the radioactivity was in peak 2.

obtained with the oligosaccharide peak produced in the presence of 10 μg/ml kifunensine. It can be seen that in this case, all of the radioactivity bound very tightly to the column, and all of it was eluted at 200 mM α-methylmannoside. This is strong evidence that kifunensine prevents the formation of any hybrid or complex types of oligosaccharides, and gives rise exclusively to high mannose structures.

Effect of Kifunensine on the Formation of Lipid-linked Saccharides and Protein Synthesis—Since kifunensine was a potent inhibitor of glycoprotein processing, it was important to determine whether it had any effect on the synthesis of proteins and whether it inhibited the formation of lipid-linked saccharides. For these studies, uninfected MDCK cells were incubated with various amounts of kifunensine and then labeled, either with [2-^{3}H]mannose (for lipid-linked saccharides), or with [4,5-^{3}H]leucine (for protein). The amount of radioactivity incorporated into protein was determined after trichloroacetic acid precipitation of the protein and pronase digestion, while incorporation of radioactivity into lipid-linked saccharides was determined after extraction with $CHCl_3$:CH_3OH as described under "Experimental Procedures."

Kifunensine had no effect on the incorporation of leucine into protein at concentrations ranging from 1 to 100 μg/ml during a 2-h incubation (data not shown). Similar results were obtained with regard to the formation of lipid-linked saccharides (data not shown). Thus, this inhibitor appears not to affect glycosylation or synthesis of the protein itself, at least at concentrations that completely inhibit glycoprotein processing reactions.

DISCUSSION

The results described in this paper show that kifunensine is a potent and quite specific inhibitor of mannosidase I, but has essentially no effect on mannosidase II or other aryl-α-mannosidases. Although these *in vitro* studies were done with purified plant processing mannosidases, it was also shown that kifunensine had a strong inhibitory effect on the release of mannose from [^{3}H]mannose-labeled $Man_9GlcNAc$ by rat liver microsomes and by a light membrane fraction isolated from rat liver. This latter enzyme fraction contains a mixture of endoplasmic reticulum, Golgi, and plasma membranes. All of the experiments with these membranes were done in the presence of sufficient amounts of swainsonine so that any contaminating lysosomal (or other) aryl-α-mannosidase would be inhibited. Interestingly enough, while there was a very marked inhibition of the membrane processing mannosidase activity at low concentrations of kifunensine (IC_{50} of about 1×10^{-7} M), this inhibition leveled off at about 20% of control values (*i.e.* about 80% inhibition), even at high concentrations of this inhibitor (10 μg/ml). We assume that this residual 20% activity is due to the presence of the ER α-mannosidase, which has been shown to be resistant to inhibition by deoxymannojirimycin (26). From this data, it would appear that the ER α-mannosidase is also resistant to kifunensine. This would fit with our observation that the soluble rat liver mannosidase activity, which has been shown to be similar immunologically to the ER mannosidase (24), is also not inhibited by kifunensine. Thus, kifunensine appears to also be a potent inhibitor of the animal Golgi mannosidase I, but not the ER processing mannosidase.

Kifunensine also proved to be an excellent inhibitor of glycoprotein processing in cell culture studies. In these studies, we examined the processing of the influenza viral glycoproteins in MDCK cells. We have used this system for other processing inhibitors (3, 27), since the viral hemagglutinin is an *N*-linked glycoprotein that has seven or eight oligosaccharide chains, of which about five are of the complex type (28). In the presence of 1 μg/ml, or higher amounts, of kifunensine, more than 95% of the oligosaccharide chains were of the high-mannose type, as determined by their tight binding to columns of concanavalin A, and also by their migration behavior on columns of Bio-Gel P-4. When the kifunensine-induced oligosaccharide was chromatographed on a long calibrated column of Bio-Gel P-4, it emerged in the same position as a $Man_9GlcNAc$ standard. In addition, this oligosaccharide was almost completely susceptible to digestion by jack bean α-mannosidase, indicating it was essentially a high-mannose, unsubstituted oligosaccharide. These data are also consistent with kifunensine being a potent and quite specific inhibitor of the Golgi processing mannosidase I.

Kifunensine represents a new and interesting structure for a glycoprotein processing inhibitor (see Fig. 8 for the structures of the various glycoprotein processing inhibitors discussed here) since it can be considered to be a cyclic oxamide derivative of 1-aminodeoxymannojirimycin (15). However, kifunensine is 50–100 times more effective than deoxymannojirimycin (see Fig. 8) towards mannosidase I. Although kifunensine bears some resemblance to the indolizidine ring structures of swainsonine (29) and castanospermine (30), it is a more complicated and more highly substituted structure in that it has a bridgehead nitrogen as well as another nitrogen in the 5-membered ring, four asymmetric hydroxyl groups and two carbonyl groups. Some of these other groups may help in enhancing the interaction of the inhibitor with the enzyme. In addition, the unusual 5-membered ring may cause the compound to adopt a particularly favorable conformation with respect to the active site of the enzyme. An important aspect to the isolation and characterization of new glycosidase inhibitors is to have a repertoire of inhibitors from which one

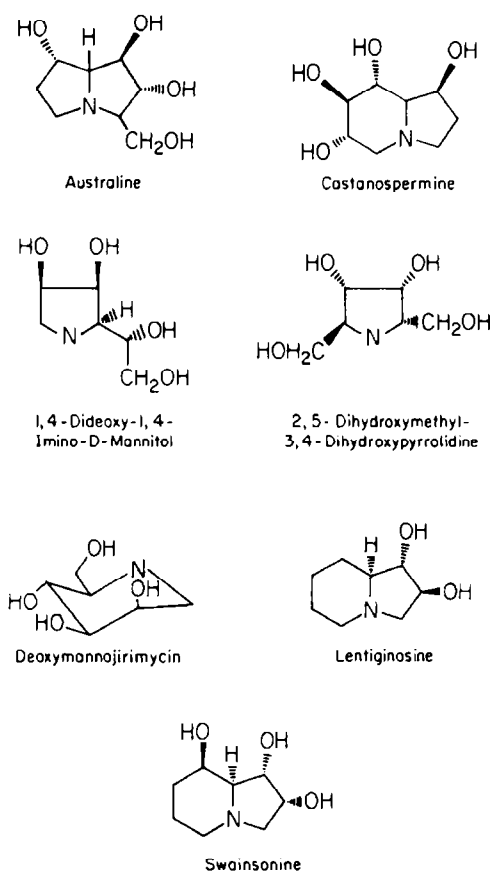


FIG. 8. **Structures of the various processing inhibitors.**

can derive information on the necessary and optimum structural requirements for a compound to be a potent and specific glycosidase inhibitor. It would appear from the structure and activity of kifunensine that the more structural information that a molecule contains, the more likely it is to be a better inhibitor, as long, of course, as it contains the basic essentials for inhibition.

What are the basic essentials that all of the glycosidase inhibitors do contain? All of these inhibitors do have a nitrogen atom in their structure, and with the exception of mannostatin (31), the nitrogen is in the ring frequently as a bridgehead in the fused ring systems. Mannostatin A is the only glycoprotein processing inhibitor known thus far that does not contain the endocyclic amine but has an exocyclic amino group rather than a ring nitrogen. This compound, however, has been found to be a very potent inhibitor of mannosidase II, showing similar activity to swainsonine.[2] Thus, under some circumstances, an amino group protruding from the ring is sufficient for activity. In addition to the nitrogen, all of the inhibitors are ring structures but some such as castanospermine (30) and swainsonine (29) contain a fused 6-membered and 5-membered ring system, whereas others, such as australine (33) are pyrrolizidine alkaloids with two 5-membered ring systems. There are also several naturally occurring and synthetic compounds that have a single 6-membered or 5-membered ring system, and these may also be good inhibitors. Deoxymannojirimycin, already mentioned above (25), resembles mannose with a nitrogen in the ring in place of oxygen, and it is a good inhibitor of mannosidase I. 2,5-Dihydroxymethyl-3,4-dihydroxypyrrolidine is a pyrrolidine alkaloid (a 5-membered ring system) that is a reasonable inhibitor of α-glucosidase, and also inhibits glycoprotein processing, presumably at the glucosidase I stage (34). 1,4-Dideoxy-1,4-imino-D-mannitol is a synthetic compound that also has a 5-membered ring structure with a nitrogen in the ring, and it inhibits α-mannosidase and the glycoprotein processing mannosidase I (35). Finally, the third requirement for activity appears to be at least two and probably three hydroxyl groups that have the same configuration as the sugar that is a substrate for the enzyme in question. All of the above inhibitors have at least three asymmetric centers, but one inhibitor called lentiginosine was recently isolated and characterized (32), and it has an indolizidine ring system but only two hydroxyl groups. However, this compound was much less effective as an inhibitor of amyloglucosidase than were either castanospermine or australine.

Clearly, two things will be necessary before we have enough information to be able to predict structure-function relations or before we can hope to design more specific inhibitors. One is to have a more varied repertoire of inhibitors representing structures of different complexities. The second is to be able to look at the three-dimensional structure of these compounds so that they can be compared to each other in space. Both of these goals can be attained by identifying new inhibitors of glycosidases and by computer modeling of these structures to determine their relation to each other and to the sugars that they mimic.

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