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THE EFFECT OF 1-DEOXYMANNOJIRIMYCIN ON RAT LIVER α-MANNOSIDASES

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SUMMARY: The mannose analogue, 1-deoxymannojirimycin, has been tested for its effect on five α-mannosidase activities present in rat liver and shown to be a specific inhibitor of Golgi α-mannosidase I at low μmolar concentrations. Golgi α-mannosidases I and II were assayed in a highly purified Golgi membrane preparation. Endoplasmic reticulum α-mannosidase activity was measured in a rough endoplasmic reticulum detergent extract. A purified soluble α-mannosidase activity which we believe is derived from the endoplasmic reticulum during tissue homogenization was also tested. And finally, the lysosomal or acidic α-mannosidase was measured in a postnuclear supernatant fraction obtained from rat liver. The results presented here show that 1-deoxymannojirimycin inhibits only Golgi α-mannosidase I, which is consistent with its effect on oligosaccharide processing *in vivo* (Fuhrmann et al. Nature 1984 307:755-758).

Many proteins synthesized on membrane bound polysomes and translocated into the lumen of the rough endoplasmic reticulum acquire N-linked oligosaccharide structures by the *en bloc* transfer of $Glc_3Man_9GlcNAc_2$ from a lipid-linked precursor to Asn-X-Thr(Ser) sites on the nascent protein (for review, see ref. 1). Several enzymes capable of removing sugars from this high mannose-type oligosaccharide have been identified and partially purified from rat liver. The glucose residues are removed in the ER[1] by two enzymes, glucosidase I and II (2,3). An α-mannosidase activity has been found in the ER that can remove up to four α-1,2-linked mannoses from a $Man_9GlcNAc$ (4). Golgi α-mannosidase I is also capable of removing four α-1,2-linked mannoses from $Man_9GlcNAc$ (5,6). Golgi α-mannosidase II hydrolyzes α-1,3- and α-1,6-linked mannoses from the $GlcNAcMan_5GlcNAc_2$ intermediate and thereby initiates the

[1] Abbreviations: ER, endoplasmic reticulum; pnp-Man, p-nitrophenyl-α-D-mannopyranoside; dMM, 1-deoxymannojirimycin.

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formation of complex-type oligosaccharides (7,8). Some N-linked oligosaccharides remain high mannose while others are converted to complex-type oligosaccharides during their transport through the Golgi apparatus by the addition of N-acetylglucosamine, galactose, sialic acid, and fucose to the oligosaccharide (1).

A number of inhibitors of the processing glycosidases have been discovered which prevent the conversion from high mannose-type to complex-type oligosaccharides. 1-Deoxynorjirimycin (9) and castanospermine (10) inhibit the removal of glucose from $Glc_3Man_9GlcNAc$, and bromoconduritol (11) inhibits the removal of glucose from $Glc_1Man_9GlcNAc_2$ units. Swainsonine inhibits Golgi α-mannosidase II (12) and causes the accumulation of hybrid oligosaccharides *in vivo* (13). A relatively new drug, 1-deoxymannojirimycin (dMM) (14), has been shown to prevent the *in vivo* conversion of high mannose-type to complex-type oligosaccharides on IgM in hybridoma cells. The authors propose that dMM inhibits Golgi α-mannosidase I activity. In this paper, we have tested directly the effect of dMM on all of the known rat liver α-mannosidases and have shown that the inhibitor is relatively specific for Golgi α-mannosidase I. This information will be very useful in designing experiments in which dMM is used to block the normal oligosaccharide processing in cells or for studying the various α-mannosidase activities *in vitro*.

EXPERIMENTAL PROCEDURES:

Materials - Materials were obtained from the following sources: Sprague Dawley male rats (100-150 grams body weight), Chappel Breeders; enzyme grade sucrose, Schwarz/Mann Inc; yeast hexokinase, Boehringer-Mannheim; AG1-X8 formate (200-400 mesh), Bio-Rad Laboratories; Scinti Verse I scintillation mixture, Fisher; pnp-Man and ATP, Sigma. dMM was kindly provided by Dr. Gunter Legler of the University of Cologne. Swainsonine was kindly provided by Dr. Philip Stahl of Washington University and Dr. Oscar Touster of Vanderbilt University. [2-^{3}H]Mannose-labeled high mannose oligosaccharides were isolated as previously described (15) and separated into $Glc_1Man_9GlcNAc$, $Man_9GlcNAc$, $Man_8GlcNAc$, $Man_7GlcNAc$, $Man_6GlcNAc$, and $Man_5GlcNAc$ by high pressure liquid chromatography (16). [2-^{3}H]Mannose-labeled $GlcNAcMan_5GlcNAc_2$-peptide was prepared as described (7).

Subcellular Fractionation - Rat liver rough ER was isolated from fed male rats by a modification (4) of the procedure of Kreibach et al. (17). ER α-mannosidase activity was solubilized from the rough membranes with sodium deoxycholate as described (4). Rat liver Golgi membranes were prepared by the method of Leelevathi et al. (18) with the modifications of Tabas and Kornfeld (5).

Golgi α-mannosidase I activity was solubilized from Golgi membranes as described (5).

Other Enzyme Sources - The soluble α-mannosidase was purified 700 fold from a high speed supernatant of a rat liver homogenate following the procedure of Shoup and Touster (19) with several modifications. Details of the purification will be presented elsewhere. The lysosomal enzyme activity was measured in the postnuclear supernatant fraction obtained in the Golgi membrane subcellular fractionation.

Enzyme Assays - Golgi α-mannosidase I activity was measured using [2-^{3}H]mannose-labeled $Man_9GlcNAc$ or $Man_8GlcNAc$ as a substrate and Golgi α-mannosidase II activity was measured using [2-^{3}H]mannose-labeled $GlcNAcMan_5GlcNAc_2$-peptide as previously described (5) except that the latter assay was carried out in 40mM citrate-phosphate buffer pH 6.0, 0.1% Triton X-100. Golgi α-mannosidase II activity towards 4mM pnp-Man was measured in the same buffer. Released p-nitrophenol was measured by its absorbance at 410 nm after adding 0.2M Na_2CO_3. ER α-mannosidase activity towards 2mM pnp-Man or [2-^{3}H]mannose-labeled $Man_9GlcNAc$ was assayed as described (4). Soluble α-mannosidase was measured under the same conditions as the ER α-mannosidase. Lysosomal α-mannosidase activity was measured using 4mM pnp-Man or [2-^{3}H]mannose-labeled $Man_9GlcNAc$ in 50mM sodium acetate pH 4.5, 0.1% Triton X-100, 4mg/ml BSA. Whenever dMM or swainsonine was included in any of the above assays, the enzyme sample was preincubated with the inhibitor for 2 min. before adding the substrate. In assays using pnp-Man as substrate, a unit is defined as 1 nmole of p-nitrophenol formed per minute. Variation from the average in duplicate assays was approximately 6%. In assays using labeled oligosaccharide or glycopeptide as substrate, a unit is defined as 1% of the input [2-^{3}H]mannose radioactivity liberated per minute. Variation from the average in duplicate assays was 6-10%.

Protein determination - Protein concentrations were determined by the method of Lowry et al. (20) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION:

The inhibitor, dMM, was first tested for its effect on Golgi α-mannosidase I. Golgi membranes were prepared from rat liver by sucrose density gradient sedimentation and washed in a 0.4M NaCl buffer to remove adsorbed proteins. The Golgi α-mannosidase I activity was solubilized from an aliquot of the washed membranes with Triton X-100. Both the membranes and the detergent extract were assayed for Golgi α-mannosidase I activity in the presence of increasing concentrations of dMM. As seen in Figure 1, the Golgi α-mannosidase I activity is very sensitive to dMM with 50% inhibition occuring at 1-2μM. In contrast, Golgi α-mannosidase II activity toward its physiologic substrate, $GlcNAcMan_5GlcNAc_2$-peptide,[2] or the artificial substrate pnp-Man, is relatively

[2] $GlcNAcMan_5GlcNAc_2$-peptide is not a substrate for Golgi α-mannosidase I (6) or ER α-mannosidase (4).

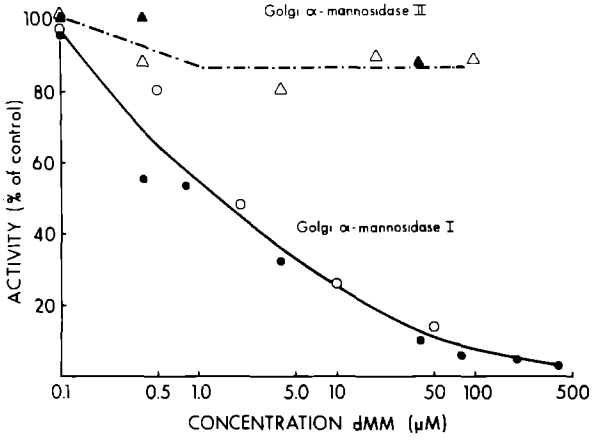

Figure 1. Effect of 1-Deoxymannojirimycin on Golgi α-Mannosidases I and II. The α-mannosidase I activity was assayed in Golgi membranes (○) and a Triton X-100 detergent extract of Golgi membranes (●) using $Man_9GlcNAc$ as a substrate in 10mM sodium phosphate pH 6.5, 5mM $MgCl_2$, and 0.1% Triton X-100 for 1 h. Activity is expressed as a percent of the activity in the absence of inhibitor which was 1.5 and 0.93 units/mg protein for the membranes and extract respectively. The α-mannosidase II activity was assayed in 40mM citrate-phosphate buffer pH 6.0, 0.1% Triton X-100, using as substrate either $GlcNAc-Man_5GlcNAc_2$-peptide (△) or pnp-Man (▲). The activity in the absence of inhibitor was 5.1 units/mg protein for the former assay and 23.6 units/mg protein for the latter assay.

insensitive to dMM as shown in Figure 1. Approximately 14% inhibition of activity is observed at 0.4μM dMM but further inhibition is not observed with increasing concentrations of dMM.

Rough endoplasmic reticulum membranes were prepared from rat liver by sucrose density gradient sedimentation and washed two times with buffer to remove any soluble α-mannosidase activity. The ER α-mannosidase was solubilized from the RER membranes with sodium deoxycholate and then tested for its sensitivity to dMM. The assays were carried out in the absence or presence of 10μM swainsonine to insure that only ER α-manosidase activity was being measured. Swainsonine has been shown to inhibit Golgi α-mannosidase II and lysosomal α-mannosidase (12) but not the ER α-mannosidase (4). Since Golgi α-mannosidase I does not hydrolyze pnp-Man, it is not measured in this assay. As seen in Table I, the ER α-mannosidase activity is unaffected by dMM in the presence or absence of swainsonine. In Table II, the lack of effect of dMM on the soluble α-mannosidase activity is demonstrated. The soluble α-mannosidase activity was purified 700 fold from a high speed supernatant of rat liver homogenate. This

TABLE I

The Effect of 1-Deoxymannojirimycin on ER α-Mannosidase

	[dMM]	[swainsonine]	Activity	% Inhibition by dMM
	μM	μM	units/ml	
A.	0	0	3.4	-
	0.5	0	3.4	0
	10.0	0	3.0	13
	50.0	0	3.3	2
B.	0	10	2.3	-
	0.5	10	2.2	6
	10.0	10	2.4	0
	50.0	10	2.2	5

A sodium deoxycholate detergent extract of rough ER was adjusted to 1mM $MnCl_2$ and assayed for α-mannosidase activity using 4mM pnp-Man in 50mM sodium cacodylate pH 6.5, 0.1% Triton X-100, 4mg/ml BSA and (A) no swainsonine or (B) 10μM swainsonine. The activity shown is the average of duplicate assays.

activity, originally described by Shoup and Touster (19), has all of the same characteristics as the ER α-mannosidase (4), and therefore we believe that the soluble activity is derived from the ER. The fact that dMM does not affect either the ER or soluble α-mannosidase activity is another similarity between these two activities. The soluble α-mannosidase activity present in the rough membrane wash fraction was also found to be insensitive to dMM when either pnp-Man or $Man_9GlcNAc$ was used as a substrate (data not shown).

The final α-mannosidase activity to be tested for its sensitivity to dMM was the lysosomal α-mannosidase. This activity was measured in the post-

TABLE II

The Effect of 1-Deoxymannojirimycin on the Soluble α-Mannosidase

A.	[dMM]	Activity (pnp-Man)	% Inhibition by dMM	B.	[dMM]	Activity ($Man_9GlcNAc$)	% Inhibition by dMM
	μM	units/ml			μM	units/ml	
	0	475	-		0	9.6	-
	0.1	480	0		0.1	11.1	0
	1.0	471	1		1.0	11.6	0
	10.0	505	0		10.0	11.2	0
	50.0	483	0		50.0	9.8	0
	100.0	511	0		100.0	9.9	0

The partially purified α-mannosidase activity towards two different substrates, (A) 2mM pnp-Man and (B) $Man_9GlcNAc$, was measured in 50mM sodium cacodylate pH 6.5, 0.1% Triton X-100, and 4mg/ml BSA in the presence of increasing dMM. The activities shown are averages of duplicate assays.

TABLE III

The Effect of 1-Deoxymannojirimycin on the Lysosomal α-Mannosidase

A. [dMM]	Activity (pnp-Man)	% Inhibition by dMM	B. [dMM]	Activity ($Man_9GlcNAc$)	% Inhibition by dMM
μM	units/ml		μM	units/ml	
0	76	-	0	1.02	-
0.1	74	3	0.1	0.97	5
0.5	78	0	0.5	1.20	0
2.0	74	3	2.0	0.98	2
10.0	77	0	10.0	0.90	12
50.0	72	5	50.0	0.70	32
100.0	72	6	100.0	0.80	22
200.0	61	19	200.0	0.60	41

A postnuclear supernatant was used to measure lysosomal α-mannosidase activity toward two substrates, (A) 4mM pnp-Man and (B) $Man_9GlcNAc$. The assay buffer was 50mM sodium acetate pH 4.5, 0.1% Triton X-100, and 4mg/ml BSA. The activities shown are average of duplicate assays.

nuclear supernatant fraction in the Golgi membrane preparation. Table III shows the results of this assay. At very high dMM concentrations, 200μM, the lysosomal α-mannosidase activity towards pnp-Man was inhibited 19%. In the $Man_9GlcNAc$ assay, 41% inhibition was observed at a dMM concentration of 200μM. This greater inhibition of activity toward $Man_9GlcNAc$ might be due to the fact that the $Man_9GlcNAc$ concentration is well below saturation of the enzyme. To test this possibility, the lysosomal α-mannosidase activity was measured at a series of pnp-Man concentration going as low as one-fiftieth of the K_m and no inhibition was observed with 200 μM dMM (data not shown). Thus, the reason for the greater inhibition in the $Man_9GlcNAc$ assay is unclear.

While the lysosomal α-mannosidase is sensitive at a 100 fold higher concentration, it appears that only Golgi α-mannosidase I is extremely sensitive to the mannose analogue, dMM. These results are consistent with the studies of Fuhrmann et al. (13) in which they found that incubation of hybridoma cells with 1mM dMM prevented the formation of complex-type oligosaccharides on IgM. The IgM oligosaccharides from the dMM-treated cells were released with endoglycosidase-H and fractionated on Biogel P-4. The majority of the high mannose-type oligosaccharides were $Man_9GlcNAc$, but at least half were processed to $Man_8GlcNAc$, $Man_7GlcNAc$, and $Man_6GlcNAc$. This limited trimming may be due

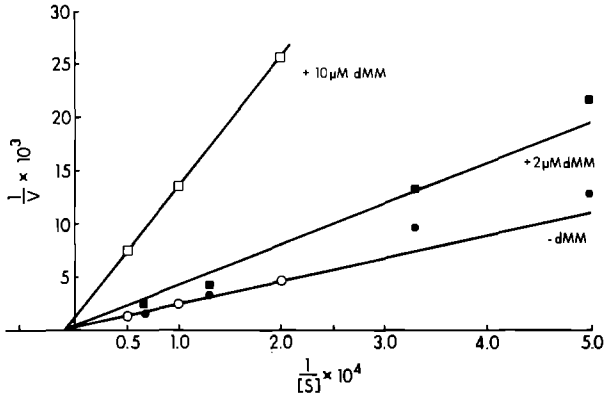

Figure 2. 1-Deoxymannojirimycin is a Non-competitive Inhibitor of Golgi α-Mannosidase I. Golgi α-mannosidase I activity was measured as a function of increasing substrate concentration using [2-^{3}H]mannose-labeled Man$_8$GlcNAc. Golgi membranes (○,□) or Triton X-100 extract of Golgi membranes (●,■) were assayed in the absence (○,●) or the presence of dMM at 2μM (■) or 10μM (□) concentration. Assays on the Triton X-100 extracts were carried out in duplicate. V is cpm of [2-^{3}H]mannose released, and S is the cpm of [2-^{3}H]-mannose-labeled Man$_8$GlcNAc added in each 20μl assay.

to ER α-mannosidase, or alternatively, to incomplete inhibition of Golgi α-mannosidase I.

Since dMM is a structural analogue of mannose, one might predict that dMM exerts its inhibitory effect by competing with the substrate (a high mannose-type oligosaccharide) for the active site of the enzyme. In order to test this idea, Golgi α-mannosidase I activity was measured at increasing Man$_8$-GlcNAc concentrations in the presence or absence of 2μM and 10μM dMM. Man$_8$-GlcNAc was used as a substrate rather than Man$_9$GlcNAc because Tabas and Kornfeld (5) have shown that Golgi α-mannosidase I prefers Man$_8$GlcNAc over Man$_9$-GlcNAc, and thus Man$_8$GlcNAc is a more suitable substrate. As seen in Fig. 2., 10μM dMM decreases the maximal velocity of Golgi α-mannosidase I six fold but does not significantly alter the apparent K_m of the enzyme for Man$_8$GlcNAc. Therefore, dMM appears to be acting as a non-competitive inhibitor of Golgi α-mannosidase I. The apparent K_i of dMM in this assay is 2μM. This inhibition constant was calculated using the values of V_{max} obtained at 0 and 10 μM dMM. When 2 μM dMM is present, the slope of the double reciprocal plot is, as predicted, twice the slope of the plot obtained in the absence of dMM. It must be pointed out that a real K_m or V_{max} cannot be calculated from this

data because the concentration of [2-^{3}H]Man$_8$GlcNAc is unknown. Thus, Figure 2 depicts the relative effect of dMM on Golgi α-mannosidase I activity toward Man$_8$GlcNAc.

1-Deoxymannojirimycin should prove to be a very useful inhibitor of α-mannosidase activity. First, including dMM in the assays will enable one to measure ER α-mannosidase activity toward Man$_8$GlcNAc without interference with Golgi α-mannosidase I activity in, for example, a crude membrane preparation. Secondly, dMM will be a useful for in vivo studies concerning oligosaccharide processing.

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REFERENCES

1. Hubbard, S.C. and Ivatt, R.J. (1981) Ann. Rev. Biochem. 55, 555-583.
2. Grinna, L.S. and Robbins, P.W. (1979) J. Biol. Chem. 254, 8814-8818.
3. Burns, D.M., and Touster, O. (1982) J. Biol. Chem. 257, 9991-10000.
4. Bischoff, J. and Kornfeld, R. (1983) J. Biol. Chem. 258, 7907-7910.
5. Tabas, I. and Kornfeld, S. (1979) J. Biol. Chem. 254, 11655-11663.
6. Tulsiani, D.R.P., Hubbard, S.C., Robbins, P.W., and Touster, O. (1982) J. Biol. Chem. 257, 3660-3668.
7. Tabas, I. and Kornfeld, S. (1978) J. Biol. Chem. 253, 7779-7786.
8. Harpaz, N., and Schachter, H. (1980) J. Biol. Chem. 255, 4894-4902.
9. Saunier, B., Kilker, R.D., Tkacz, J.S., Quaroni, A., and Herscovics, A. (1982) J. Biol. Chem. 257, 14155-14161.
10. Pan, Y.T., Hidetaka, H., Saul, R., Sanford, B.A., Molyneux, R.J., Elbein, A.D. (1983) Biochemistry 22, 3975-3984.
11. Datema, R., Romero, P.A., Legler, G., and Schwarz, R.T. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 6787-6791.
12. Tulsiani, D.R.P., Harris, T.M., and Touster, O. (1982) J. Biol. Chem. 257, 7936-7939.
13. Gross, V., Tran-Thi, T., Vosbeck, K., and Heinrich, P.C. (1983) J. Biol. Chem. 258, 4032-4036.
14. Fuhrmann, U., Bause, E., Legler, G., and Ploegh, H. (1984) Nature 307, 755-758.
15. Gabel, C.A. and Kornfeld, S. (1982) J. Biol. Chem. 257, 10605-10612.
16. Mellis, S.J. and Baenziger, J.U. (1981) Anal. Biochem. 114, 276-280.
17. Kreibach, G., Ulrich, B.L., and Sabatini, D.D. (1978) J. Cell Biol. 77, 464-487.
18. Leelevathi, D.E., Esters, L.W., Flingold, D.C. and Lombardi, B. (1970) Biochim. Biophys. Acta 211, 124-138.
19. Shoup, V.A. and Touster, O. (1976) J. Biol. Chem. 251, 3845-3852.
20. Lowry, O.H., Rosebrough, N.J., Fan, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 7936-7939.