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Amphomycin: A tool to study protein N-glycosylation

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Abstract. Radio-labelled amphomycin (^{3}H-amphomycin) forms a complex with dolichylmonophosphate in presence of Ca^{2+}. Complex formation has also been documented with retinylmonophosphate and perhydromonoeneretinylmonophosphate. Analysis of the space-filling model suggested both fatty acylated aspartic acid residue at the N-terminus of the lipopeptide and phosphate head group of dolichylmonophosphate are necessary for the complex formation. The binding ability of amphomycin is then utilized to localize dolichylmonophosphate in the microsomal membrane. Studies with microsomal membranes from hen oviduct suggested that dolichylmonophosphate is located in the cytoplasmic side of the membrane.

Keywords. Glycosylation inhibitor; protein N-glycosylation; dolichylmonophosphate; lipid-linked pathway.

Introduction

Many of the glycoproteins from eukaryotic cells contain oligosaccharide chains that are attached to protein *via* GlcNAc→ asparagine linkage (Kornfeld and Kornfeld, 1985). Detail structural analysis of these oligosaccharides indicated that they fall into 3 major categories termed high mannose, hybrid and complex. They all share the common core structure Man α1–3 (Man α1–6) Man *β*1–4 GlcNAc *β*1–4 GlcNAc-Asn, but differ in outer branches. The high mannose-type oligosaccharides typically have 2 or 6 additional mannose residues linked to the pentasaccharide core. The hybrid molecules have features of both high-mannose and complex-type oligosaccharides. Most hybrid molecules contain a 'bisecting' N-acetylglucosamine linked *β*1, 4 to the *β*-linked mannose residue, although there are some exceptions (Hunt *et al*., 1983; Varki and Kornfeld, 1983; Yamashita *et al.,* 1983). The complex-type structure contains two outer branches with the typical sialyl lactosamine sequence and shows two other commonly found substituents, namely a fucose in α1,6 linkage to the innermost N-acetylglucosamine residues and a 'bisecting' N-acetylglucosamine linked *β*1,4 to the *β*-linked mannose residue.

All types of asparagine-linked carbohydrate chains are initially synthesized as precursor oligosaccharides containing glucose, mannose and N,N'-diacetylchitobiose on a pyrophosphorylated lipid backbone called dolichol. Many aspects of this lipid-mediated process have been reviewed (Lucas and Waechter, 1976; Waechter and Lennarz, 1976; Hemming, 1977; Parodi and Leloir, 1979) but all the details in the synthesis of these oligosaccharides are still not known. One approach to examing the

Abbreviations used: Dol-P, Dolichylmonophosphate; Man-P-Dol, mannosylphosphoryl dolichol; Glc-P-Dol, glucosylphosphoryl dolichol; GlcNAc-PP-Dol, N-Acetylglucosaminylpyrophosphoryl dolichol; RP, Retinylmonophosphate; pRP, Perhydromonoeneretinylmonophosphate; MRP, Mannosylphosphory retinol; pMRP, Mannosylphosphorylperhydromonoene retinol.

details of these pathways is the use of inhibitors that block at various steps in the pathway. Since tunicamycin blocks the addition of the first N-acetylglucosamine (GlcNAc) residue in the dolichol-linked oligosaccharide by inhibiting the tansfer of GlcNAc-1-P to Retinylmonophosphate (Dol-P) (Tkacz and Lampen, 1975; Takatsuki *et al.,* 1975; Struck and Lennarz, 1977; Waechter and Harford, 1977; Ericson *et al*., 1977), the biosynthesis of the dolichol-linked oligosaccharides is blocked completely. Thus, tunicamycin becomes an useful tool for studying the biosynthesis, metabolic fate, and activity of the unglycosylated forms of glycoproteins normally containing N-glycosydically bound oligosaccharides. However, this antibiotic apparently has no effect on the synthesis of mannosylphosphoryl dolichol (Man-P-Dol) or glucosyl-phosphoryl dolichol (Glc-P-Dol) and therefore has limited warranty for studying intermediate stages in the assembly of dolichol-linked oligosaccharides.

The antibiotic amphomycin produced by *Streptomyces canas* (Hainemann *et al*., 1953) is a straight chain undecepeptide with either 3-isododecenoic or 3-anteisotri-decenoic acid residue attached to the N-terminal aspartic acid residue by an amide linkage (Bodanszky *et al.,* 1973). Earlier it has been show by Tanaka *et al.* (1977, 1979) that amphomycin inhibits the synthesis of peptidoglycan in gram-positive bacteria by blocking the transfer of phospho-N-acetyl-muramylpentapeptide from UMP to undecaprenylmonophosphate, the prokaryotic glycosyl carrier lipid. Considering the structural relationship between undecaprenylmonophosphate and Dol-P, it is reasonable to expect that the antibiotic would also affect glycosylation of the eukaryotic carrier lipid. Studies with isolated microsomal membrane prepa-rations from pig aorta and plants have, indeed, demonstrated that glycosylation reactions involving Dol-P are inhibited by amphomycin (Kang *et al.,* 1978a,b; Ericson *et al.,* 1978; Kang and Elbein, 1979). Later on Banerjee *et al.* (1981) have demonstrated that inhibition of Man-P-Dol, Glc-P-Dol and N-acetylglucosaminyl-pyrophosphoryl dolichol (GlcNAc-PP-Dol) synthesis in calf brain microsomal membranes is reversed by the addition of exogenous Dol-P but not by sugar nucleotides or divalent cations. Furthermore, amphomycin also shown to interfere with the extraction of exogenous prelabelled Dol–^{32}P but not [^{3}H]-Man-P-Dol or the major membrane phospholipids indicating, thereby an interaction between the lipopeptide and dolichylmonophosphate.

This paper presents evidence that amphomycin inhibits monosaccharide-lipid synthesis primarily due to its direct interaction with the glycosyl carrier lipid, Dol-P.

Materials and methods

Dolichylmonophosphate was purchased from Calbiochem. Dolichol, Triton X-100 were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Bio-Gel A-1·5 m (200–400 mesh), Bio-Gel P-2 (200–400 mesh) were obtained from Bio-Rad. [^{3} H] - KBH_4 (1·1 Ci/mmol) was supplied by ICN Pharmaceuticals Inc, Irvine, California, USA. Amphomycin (calcium salt) was a gift from Bristol Laboratories and Dr. M. Bodanszky, Case Western Reserve University. Retinylmonophosphate and perhydromonoeneretinyl-monophosphate were gift from Dr. L. M. DeLuca, National Cancer Institute. All other chemicals and reagents were analytical reagent grade. For all purposes amphomycin was dissolved in 0·1 N acetic acid, and the solution was adjusted to 0·05 M sodium acetate, pH 7·0 with 0·2 N NaOH.

Tritium labelling of amphomycin

Isotopic labelling of amphomycin was carried out at the free-NH_2 group of diaminobutyric acid residue. The method involves reductive methylation using formaldehyde and high specific activity [^{3}H] -potassium borohydride, essentially the same as described before (Kumarasamy and Symons, 1979) for proteins. In most cases 100 μg of amphomycin was taken in 500 μl of 0·2 M sodium borate, pH 9·5, cooled in ice and to this ice-cold solution was added 3 μl of 20 mM formaldehyde, followed after 30 s by 1 μl (160 μCi) of [^{3}H] -KBH_4 in 10 mM KOH . After 20 min, 2 μl of unlabelled 10 mM $NaBH_4$ was added and the reaction mixture left for a further 20 min at 0°C. The reaction was stopped by adding 200 μl of 0·4 M sodium phosphate, pH 5·8 and 100 mM glycine. The pH of the solution was then adjusted to 3·0 and amphomycin was extracted with n-butanol. The organic phase was washed with water and dried under N_2. Finally, the labelled lipopeptide antibiotic (26·7 mCi/mmol) was dissolved in 0·05 M sodium acetate, pH 7·0 and stored at – 20°C until use.

SG-81 Paper chromatography of [^{3}H]-amphomycin

Tritium-labelled as well as unlabelled amphomycin was chromatographed on EDTA-treated SG-81 paper (Steiner and Lester, 1972) and developed with (i) n-butanol-acetic acid-water (3:l:l); (ii) n-propanol-water (7:3) (Bodanszky *et al.,* 1973). Radioactivity was detected by spraying the paper with enhance spray (New England Nuclear, Boston, Massachusetts, USA) followed by exposing XAR-5 film at – 80°C (Bonner and Laskey, 1974) and or cutting 1 cm area and counting them in a liquid scintillation counter. For normal visualization, the paper was sprayed with 3% ninhydrin in acetone.

Measurment of radioactivity

All radioactive samples were counted in Hydrofluor (National Diagnostics).

Results

Purity of [^{3}H]-amphomycin

The purity of the labelled antibiotic was established by column chromatography on Bio-Gel P-2 as well as by SG-81 paper chromatography. As shown in figure 1a [^{3}H]-amphomycin is included in P-2 column (23 × 0·64 cm) and eluted out as single peak. Figure 1b shows that [^{3}H]-amphomycin migrates as single species with an R_f of 0·64. This migration pattern is identical to that observed before (Bodanszky *et al*., 1973). These criteria established the fact that the radiolabelled amphomycin is extremely pure.

Interaction of [^{3}H]-amphomycin with dolichylmonophosphate

In order to understand how Dol-P encountered the inhibition of Man-P-Dol synthesis by amphomycin, [^{3}H]-amphomycin was allowed to react at room

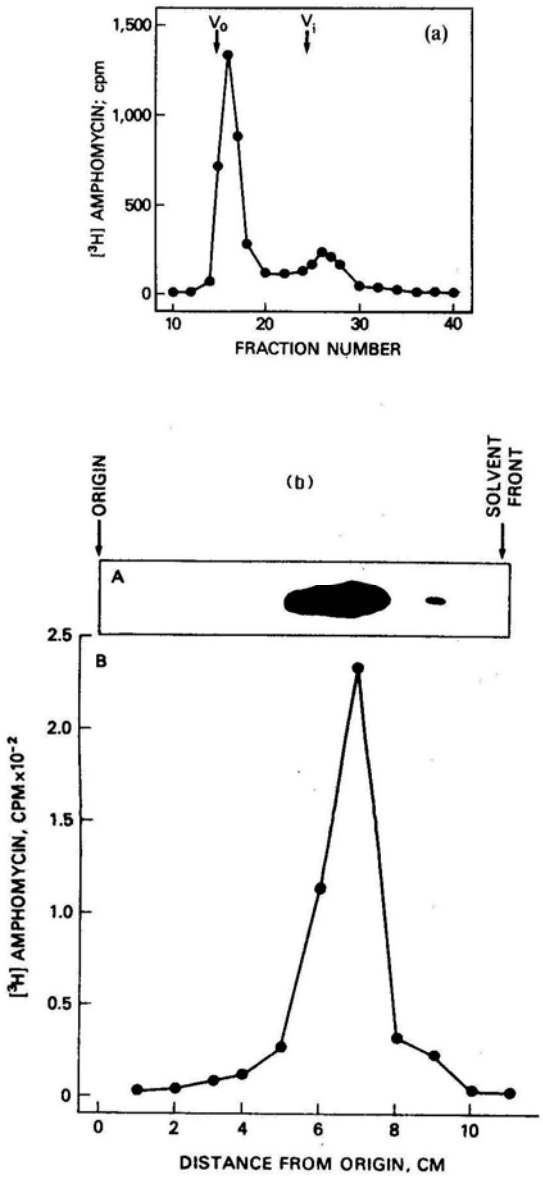

Figure 1. (a), Bio-Gel P-2 column chromatography of [^{3}H]-amphomycin. Amphomycin was labelled by reductive methylation and 7,500 cpm were applied to a Bio-Gel P-2 column (23 cm × 0·64 cm). The column was equilibrated and washed with 0·2 M NaCl. 0·5 ml fractions were collected and counted. (V_o), Blue dextran, (V_i), [^{14}C]-leucine.

(b), SG-81 paper chromatography of [^{3}H]-amphomycin. 10,000 cpm of [^{3}H]-amphomycin were spotted on SG-81 paper. The chromatogram was developed in n-butanol-acetic acid-water (3:1:1). The paper was then sprayed lightly with enhance spray and exposed XAR-5 film for two weeks (upper) at –80°C. The radioactivity was also followed by cutting 1 cm area from the original chromatogram and counting them in a liquid scintillation counter.

temperature with 50 μg of Dol-P in presence of 10 mM $CaCl_2$ and 0·05% Triton X-100. The reaction mixture was then analyzed on a Bio-Gel A-l·5 m column. The elution profile in figure 2 indicated that a major portion of the radioactivity was eluted in the void volume which suggested the formation of a soluble complex between the lipopeptide and the lipid.

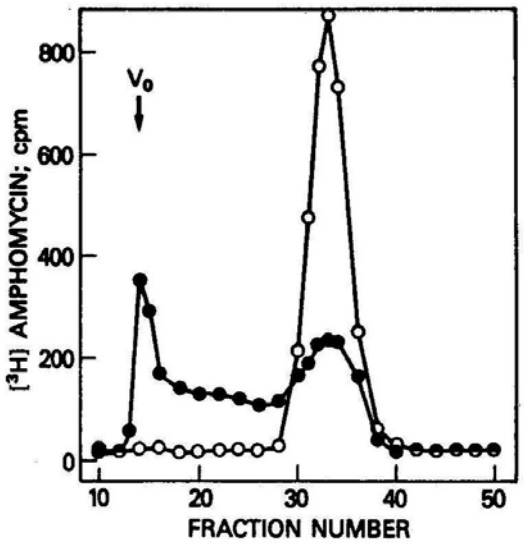

Figure 2. Bio-Gel A-1·5 m chromatography of [^{3}H]-amphomycin and Dol-P complex 50 μg of Dol-P was suspended by sonication in 20 mM Tris-HCl buffer, pH 7·0 containing 0·05% Triton X-100. It was then mixed with 5,000 cpm of [^{3}H]-amphomycin in the presence of 10 mM $CaCl_2$. The mixture was then applied to a Bio-Gel A-l·5 m column (25 cm × 0·64 cm). The column was equilibrated and washed with 20 mM Tris-HCl, pH 7·0 containing 10 mM $CaCl_2$, 0·05% Triton X-100. 0·5 ml fractions were collected and counted. V_o = Blue dextran. (O), [^{3}H]-amphomycin; (●), [^{3}H]-amphomycin complexed with Dol-P.

Complex formation of [^{3}H]-amphomycin with retinylphosphate and perhydromonoeneretinylphosphate

The possible connection between Dol-P and retinylmonophosphate (RP) systems were due to the facts that both Dol-P and chemically synthesized all-trans-β-retinylphosphate; all-trans-9-(4-methoxy-2, 3, 6-trimethylphenyl)-3, 7-dimethyl-2, 4, 6, 8-monotetraene- 1-yl phosphate; perhydromonoeneretinylphosphate; all-trans-β-retinyl-phosphate and 13-*cis*-β-retinylphosphate were acted as acceptors of [^{14}C]-mannose from GDP [^{14}C]-mannose (De Luca *et al.,* 1977). But when the mannosyldonor activity of both mannosylphosphorylretinol (MRP) and mannosyl-phosphorylperhydromonoeneretinol (pMRP) were studied, it was found that mannose could be transferred only from MRP to protein but not from pMRP (L. M. De Luca, unpublished results). Recently, it had been observed that mannosyl transfer reaction from GDP-mannose to retinylphosphate with rat liver microsomal membranes was inhibited by amphomycin (Shidoji and DeLuca, 1981). Similarity in the inhibitory activity of amphomycin for mannosyl transfer to either MRP or pMRP suggested to study the interaction, if any, between amphomycin and these

two compounds. When either RP orperhydromonoeneretinylmonophosphate (pRP) was reacted with [^{3}H]-arnphomycin under the conditions described for Dol-P and analyzed on a Bio-Gel A-1·5 m column, high-molecular weight complex was detected with both these componds. The results from such experiments are depicted in figure 3.

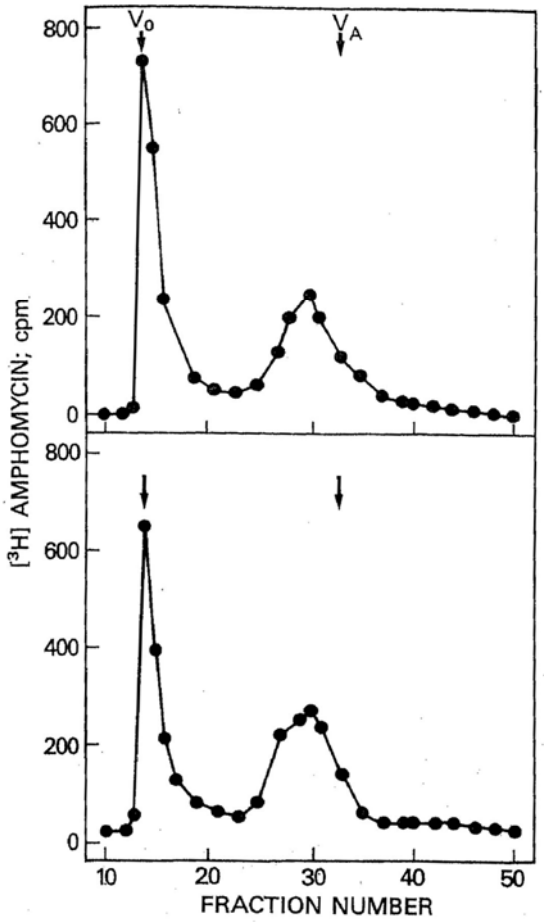

Figure 3. Interaction between [$_3$H]-amphomycin and retinylphosphate/perhydromono-eneretinylphosphate. 94 μg of retinylphosphate (upper panel) and 94 μg of perhydromono-eneretinylphosphate (lower panel) were suspended by sonication in equilibrating bufer and mixed with 4,000 cpm of [$_3$H] -amphomycin at room temperature. The mixtures were the applied to a Bio-Gel A-1·5 m column (25 cm × 0·64 cm) which was washed with 20 mM Tris-HCl, pH 7·0 containing 10 mM $CaCl_2$, 0·05% Triton X-100. 0·5 ml fractions were collected and counted. (V_o), Blue dextran; (V_A), [^{3}H]-amphomycin.

Localization of Dol-P in the microsomal membranes

In the recent past, a few attempts had been made either by protease treatment of rat liver microsomes and following the synthesis of Man-P-Dol, Glc-P-Dol, GlcNAc-PP-Dol as well as addition of glucose to oligosaccharide-lipid (Snider *et al.,* 1980) or by incorporating GlcNAc-PP-Dol into unilamellar liposomes (Hanover and Lennarz,

1978) to understand the transmembrane location of oligosaccharide-lipid synthesis and also the topological orientation of N,N′-diacetylchitobiosylpyrophosphoryl-dolichol in the membrane. But the localization and orientation of the glycosyl carrier-lipid, Dol-P in these membranes were not elucidated. Following the direct interaction between amphomycin and Dol-P, a competitive binding of [^{3}H]-amphomycin with hen oviduct microsomal membranes was performed. In this case membranes were pre-incubated for 5 min at 37°C in presence of 0–200 μg unlabelled amphomycin followed by [^{3}H] -amphomycin (5,000 cpm) for another 5 min. At the end of the second incubation, the supernatant was analyzed for radioactivity. Only 15% of the total radioactivity was recovered from the membranes pre-incubated in absence of unlabelled amphomycin. The gradual increase in radioactivity, however, was detected in the supernatant when membranes were incubated with increasing amount of amphomycin. Approximately, 50% of the total radioactivity was recovered from the membrane preparation when pre-incubated with 200 μg amphomycin (figure 4).

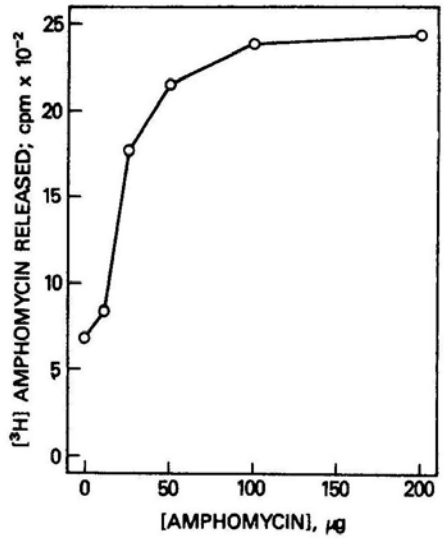

Figure 4. Competition between [^{3}H]-amphomycin and unlabelled amphomycin for binding to microsomal membranes from hen oviduct. 22·5 mg membrane protein was incubated in 50 mM Tris-HCl, pH 7·5 buffer containing 10 mM $CaCl_2$ and 0–200 μg unlabelled amphomycin at 37°C for 5 min in a total volume of 100 μl. At the end of pre-incubation period 5,000 cpm of [^{3}H]-amphomycin was added in each tube and reincubated for additional 5 min. At the end of the incubation period the tubes were centrifuged at 39,000 *g* for 10 min and the radioactivity was measured in the supernatant.

Discussion

We have shown earlier that amphomycin inhibited Man-P-Dol, Glc-P-Dol and GlcNAc-PP-Dol synthesis in calf brain membranes in a dose dependent manner (Banerjee *et al.,* 1981). It has also been suggested that the inhibitory effect of the lipopeptide antibiotic is probably due to a direct interaction between the lipopeptide

and glycosyl carrier-lipid, Dol-P. The present communication describes studies on the interaction between the lipopeptide and polyisoprenylmonophosphate. As a consequence of this interaction, Dol-P in the microsomal membrane has also been investigated.

Tritium labelling of amphomycin proved to be an important tool in studying the lipid-lipopeptide interaction. SG–81 paper chromatography as well as Bio-Gel P-2 column chromatography demonstrated the purity of the labelled product. No difference in migration rate between the unlabelled amphomycin (R_f 0·64) and [^{3}H]-amphomycin has been observed in n-butanol-acetic acid-water (3: 1: 1) system However, it generated a faster moving product (R_f 0·73) when treated with 0·25 N acetic acid at 100°C for 3 h, a condition that has been shown to liberate the fatty acylated aspartic acid residue at the N-terminus (Bodanszky *et al*., 1973).

When [^{3}H]-amphomycin was allowed to interact with Dol-P and monitored over Bio-Gel A-1·5 m column, it has been observed that amphomycin forms a soluble complex with Dol-P. It also forms a complex with retinylphosphate as well as with perhydromonoeneretinyl phosphate. The lipid-lipopeptide interaction is found to be dependent upon the presence of Ca^{2+} in the reaction mixture, because inclusion of

Figure 5. Site of interaction between amphomycin and Dol-P. A space filling model for amphomycin (upper) as well as for Dol-P (lower) have been constructed. The hydrophobic domain of Dol-P is for the anchorage to the membrane and the hydrophilic site acts as an acceptor and also interacts with the hydrophobic domain of the lipopeptide antibiotic amphomycin.

10-fold molar excess of EDTA immediately dissociated the complex (data not shown).

The Ca^{2+} dependent complex formation between Dol-P and amphomycin raises further question about its potential use to locate Dol-P in biologically active membranes. Simple kinetic measurments (figure 4) is in favour of Dol-P being present in the cytoplasmic site of the microsomal membranes. This observation is supported by the facts that (i) in absence of unlabelled amphomycin the binding of [^{3}H] -amphomycin to the oviduct microsomes is almost quantitative and (ii) increasingly less binding in presence of excess unlabelled amphomycin. The quantitative reversal of [^{3}H]-amphomycin binding to the oviduct membranes however, could not be detected even at the highest concentration of amphomycin used here. This may partially account for a non-specific interaction with other membrane phospholipids or 50% of the membranes formed wrong-side out vesicles The probable site of interaction between these two molecules is explained in the space filling model (figure 5) but how Ca^{2+} plays a central role in holding the two molecules together needs to be investigated.

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