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# The stabilisation of purified, reconstituted P-glycoprotein by freeze drying with disaccharides ☆

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ABSTRACT

The drug efflux pump P-glycoprotein (P-gp) (ABCB1) confers multidrug resistance, a major cause of failure in the chemotherapy of tumours, exacerbated by a shortage of potent and selective inhibitors. A high throughput assay using purified P-gp to screen and characterise potential inhibitors would greatly accelerate their development. However, long-term stability of purified reconstituted ABCB1 can only be reliably achieved with storage at −80 °C. For example, at 20 °C, the activity of ABCB1 was abrogated with a half-life of <1 day. The aim of this investigation was to stabilise purified, reconstituted ABCB1 to enable storage at higher temperatures and thereby enable design of a high throughput assay system. The ABCB1 purification procedure was optimised to allow successful freeze drying by substitution of glycerol with the disaccharides trehalose or maltose. Addition of disaccharides resulted in ATPase activity being retained immediately following lyophilisation with no significant difference between the two disaccharides. However, during storage trehalose preserved ATPase activity for several months regardless of the temperature (e.g. 60% retention at 150 days), whereas ATPase activity in maltose purified P-gp was affected by both storage time and temperature. The data provide an effective mechanism for the production of resilient purified, reconstituted ABCB1.



Malignant cells are seen to employ a number of strategies to evade the cytotoxic effects of drugs used in chemotherapy. The generic term for these strategies is multidrug resistance (MDR) and is a significant clinical barrier to effective chemotherapy. Multidrug efflux pumps belonging to the ATP binding cassette (ABC) family of proteins such as P-glycoprotein (P-gp), multidrug resistance proteins 1–5 and breast cancer resistance protein confer the MDR phenotype to some cancers [19,42]. P-gp has been the focus of study following the discovery over 30 years ago that it mediated the efflux of chemotherapy drugs [21] as it has also been shown to affect the absorption, distribution and elimination of a wide range of pharmaceutical agents [22,45].

During these 30 years there has been a major effort to produce clinical inhibition of ABCB1 as a mechanism to restore sensitivity to chemotherapy. A large number of inhibitors have been developed from (i) existing drugs, (ii) chemical modifications of existing drugs, (iii) combinatorial chemistry and (iv) natural sources [26,28]. Whilst a large number of drugs are successful in vitro, the translation to clinical success has been poor. Reasons include, lack of potency, low selectivity, unwanted drug interactions and poor pharmacokinetic properties [19]. Combinatorial chemistry has been used to create one of the most promising inhibitors, tariquidar (XR9576). Tariquidar shows great selectivity, duration of inhibition and potency of interaction with P-gp and restored the efficacy of anticancer drugs in solid tumour models [49], yet even this drug has failed to progress through clinical trials [34].

There is, therefore, a real need to screen greater numbers of potential inhibitors against P-gp, whether generated by combinatorial chemistry or rational, structure based drug design. A genuine specific, high throughput screen would be hugely advantageous in this search. Current P-gp screens include the use of P-gp expressing cell lines (Caco-2, Madine–Derby canine kidney) which mimic the gastrointestinal tract and are used for bi-directional flux assays [17]. The information is not specific to P-gp-drug interactions as these cell layers contain many drug transporters, including several related ABC proteins [46]. The fact that P-gp is an ATPase has been exploited to measure drug efflux in both cell membrane fragments and pure P-gp containing proteoliposomes [2,3,40,43]. However, the membrane fragments contain many ATP dependent drug efflux pumps and P-gp must be inhibited in a separate experiment to yield specific data. Proteoliposomal systems utilising pure P-gp do not suffer from a lack of specificity and as an ATPase assay have a microtitre plate layout. These would seem to be a good can-

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didate for the development of a high throughput screen. However, the instability of purified membrane proteins is a well established phenomenon and samples must be used immediately after purification or stored at −80 °C. This requirement precludes the development of a high throughput system that may have commercial application.

Stabilisation of labile proteins during freeze drying has been well documented, commonly involving the use of excipients such as mono and disaccharides [1,6,8,7,23,24]. The interaction of excipients with membranes and specifically liposomes during freeze drying has also been studied in some detail [11,14,13,20,30,48]. However, there has to date been very little investigation into the stabilisation of membrane bound proteins and no reported success in freeze drying a proteoliposomal system. The present investigation provides a method to generate a freeze drying protocol for the generation of stable ABCB1 preparations.

## Materials and methods

### *Materials*

Cholesterol, disodium adenosine triphosphate ($Na_2ATP$), nicardipine and buffer salts were obtained from Sigma (Poole, UK). DC detergent compatible protein assay kit and SM-2 Biobeads from BioRad (Hemel Hemstead, UK). Octyl-β-d-glucoside (OG), leupeptin hemisulfate, pepstatin, benzamide HCl and nickel-nitrilotriacetic acid (Ni-NTA) resin from Calbiochem (Nottingham, UK). *Escherichia coli* total lipid extract was purchased from Avanti Polar Lipids (Alabama, USA). [$^3$H]-phosphatidylcholine (84 Ci/mmol) was from Amersham Biosciences (UK) the silver staining kits for SDS PAGE were from ICN (Thame, UK) and PageBlue$^{TM}$ gel staining system was from Fermentas Life Sciences (Maryland, USA).

### *Purification and reconstitution of P-gp from insect cell membranes*

Trichoplusia ni (High Five) cells were infected with recombinant baculovirus and used for expression of hexahistidine tagged ($His_6$) P-gp [47]. Cell membranes were isolated by centrifugation and ABCB1 was purified by immobilised metal affinity chromatography (IMAC) as previously described [47,38,44]. Briefly 50 mg total membrane proteins were solubilised in buffer 1 (20 mM Tris pH 6.8, 150 mM NaCl, 1.5 mM $MgCl_2$ and 2% (w/v) octyl-β-d-glucoside) supplemented with 20% (v/v) glycerol and 0.4% (w/v) of a 4:1 mixture of *E. coli* lipid and cholesterol. Solubilised proteins were added to 0.5 ml Ni-NTA resin and washed with buffer containing increasing concentrations of imidazole before elution at 120 mM imidazole. P-gp containing fractions were then reconstituted into proteoliposomes using detergent adsorption to SM-2 Bio-Beads and the efficiency was assessed by sucrose density gradient centrifugation as described [47,38,44]. Protein concentration was determined by densitometric analysis of SDS PAGE stained with Coomassie blue. Buffer 1, used in purification, was modified to examine the properties of disaccharides in freeze-drying experiments by substituting glycerol for trehalose or maltose, as required.

### *Measurement of P-gp ATPase activity*

Hydrolysis of ATP was assessed by colorimetric assay based on the measurement of inorganic phosphate (Pi) [9]. ATPase activity was measured over varying concentrations of ATP (0–2 mM) in the absence (basal activity) and presence (drug stimulated activity) of 30 μM nicardipine. The effect of different nicardipine concentrations (0–100 μM) was also measured in the presence of 2 mM ATP. P-gp containing proteoliposomes were incubated for 20 min at 37 °C before the reaction was quenched, color developed and measured. To assess their effect on function, trehalose or maltose were added to ATPase buffer (50 mM Tris pH 7.4, 150 mM $NH_4Cl$, 5 mM $MgSO_4$, 0.02% (w/v) $NaN_3$) in the range 0–300 mM. Following freeze drying experiments samples were rehydrated with room temperature deionised water before ATPase activity was measured.

### *Freeze drying*

Freeze drying was carried out using a CHRIST Alpha 2-4 (MartinChrist, Germany) with the condenser temperature set to −80 °C. Product was frozen and dried in 2 ml freeze-drying vials with 13 mm freeze drying stoppers (Fisher Scientific, UK). Following purification and reconstitution 400 μl aliquots were frozen in the vials with stoppers on in a −80 °C freezer. After initial optimisation, the drying cycle used was 0.011 mBar for 72 h with vials sealed under vacuum. Shelf temperature during secondary drying stabilised at 8.6 ± 0.17 °C though this was outside of direct control due to limitations with the freeze drying apparatus.

### *Determination of residual water*

Residual water content of freeze dried products was determined by coulometric Karl Fischer titration using an AF7 Coulometric Karl Fischer (QCL Ltd., UK). Residual water was calculated as a percentage of the total weight of the dry product.

### *Stability testing*

Following freeze drying, samples were sealed under vacuum and stored at 4 °C, 20 °C or 37 °C for up to 240 days. At various time intervals the ATPase activity was assessed and the thermal properties investigated by DSC.

### *Differential scanning calorimetry (DSC)*

The thermal properties of the freeze dried product were investigated at various time points and storage temperature by DSC performed on a Polymer Laboratories Thermal Science Division DSC Gold (UK). Samples (3 mg protein/lipid/disaccharide mixture) were prepared in aluminum pans and heated at 10 °C/min from −100 °C to 200 °C with 15–20 ml/min nitrogen purge gas. The glass transition temperature ($T_g$) was taken to be the mid point between the onset and end of the glass transition as this is in agreement with the assertion that $T_g$ should be considered as the temperature of half vitrification on cooling [50].

### *Analysis of data*

Analysis of ATPase data was by nonlinear regression performed by GraphPad Prism 4.0 (GraphPad Software Inc., USA). ATPase activity with varying ATP concentration was fitted with the Michaelis–Menten equation whilst activity with varying nicardipine concentration was fitted with the general dose–response equation [16]. Statistical analysis was done via by two way analysis of variance (ANOVA) and paired *t*-test using GraphPad Prism 4.0.

## Results

### *Purification and reconstitution of P-gp proteoliposomes*

A starting amount of 50 mg total membrane protein from High-5 insect cells generated a yield of 115 ± 41 μg ABCB1 purified to greater than 85%. Following reconstitution, ABCB1 displayed a basal ATPase activity of $V_{max}$ = 0.5 ± 0.1 μmol Pi/min/mg with an