

## Selective labelling of cell-surface polyamine-binding proteins on leukaemic and solid-tumour cell types using a new polyamine photoprobe

Donna M. FELSCHOW\*<sup>†1</sup>, Zenghui MI\*<sup>1</sup>, J. STANEK<sup>‡</sup>, J. FREI<sup>‡</sup> and Carl W. PORTER\*<sup>2</sup>

\*Grace Cancer Drug Center, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263, U.S.A.; †Johns Hopkins School of Medicine, 600 N.Wolfe St., Oncology 3-110, Baltimore, MD 21287, U.S.A., and ‡Research Laboratories, Novartis Pharmaceuticals Inc., CH-4002 Basel, Switzerland

Polyamine transport is an active process which contributes to the regulation and maintenance of intracellular polyamine pools. Although the biochemical properties of polyamine transport in mammalian cells have been extensively studied, attempts to isolate and characterize the actual protein(s) have met with limited success. As one approach, photoaffinity labelling of cell surface proteins using a polyamine-conjugated photoprobe may lead to the identification of polyamine-binding proteins (pbps) associated with the transport apparatus and/or other regulatory responses. In a previous study [Felschow, MacDiarmid, Bardos, Wu, Woster and Porter (1995) *J. Biol. Chem.* **270**, 28705–28711], we demonstrated that the photoprobes N<sup>1</sup>-ASA-spermidine and N<sup>1</sup>-ASA-norspermine [where the ASA (azidosalicylamidoethyl) group represents the photoreactive moiety] competed effectively with polyamines for transport and selectively labelled two major pbps at 118 and 50 kDa on the surface of murine and human leukaemia cells. In the present study, a new and more potent

polyamine-conjugated photoprobe, N<sup>1</sup>-ASA-spermine, has been synthesized and used to develop a method based on detergent lysis for identifying putative cell-surface pbps on solid-tumour cell types. Transport kinetic assays showed that the new photoprobe competed with spermidine uptake with an apparent  $K_i$  of 1  $\mu$ M, a value 20–50-fold lower than those of earlier probes. In L1210 cells, the new probe identified pbp50 and pbp118 thus reaffirming their identity as pbps. Two new bands were also detected. In A549 human lung adenocarcinoma cells, N<sup>1</sup>-ASA-spermine identified pbps at 39, 62, 73 and 130 kDa, the latter believed to be a size variant of pbp118. The presence of pbp130/118 in two very different cell types suggests the generality of the protein among mammalian cell types as well as its importance for further study. The high affinity of the photoprobe for the polyamine-transport system strongly suggests that at least some of the identified pbps may be associated with that function.

### INTRODUCTION

The polyamine requirement during cell proliferation is typically met and sustained by three separate but interrelated systems, polyamine biosynthesis, catabolism and transport, which are all sensitively regulated by intracellular polyamine pools [1,2]. The proteins responsible for polyamine transport have not yet been identified and characterized in mammalian cell systems. Thus critical interrelationships involved in polyamine homeostasis and its attendant regulatory responses cannot be fully understood or therapeutically exploited.

Early attempts to purify the transporter protein by affinity chromatography relied on the interaction of positively charged polyamines with putative binding sites located on the cell surface [3]. A difficulty in this approach, however, has been the labile interactions of polyamines with the large number of negatively charged cell-surface proteins. As a prelude to cloning the mammalian polyamine transporter, Byers et al. [4] used interspecies transfection methods to demonstrate the transfer of uptake activity from a transport-competent cell to a transport-deficient cell, a finding that clearly supports the protein nature of at least one component of the transport apparatus. Greater success has been achieved in cloning genes encoding proteins of bacterial polyamine-transport systems, for which Igarashi and

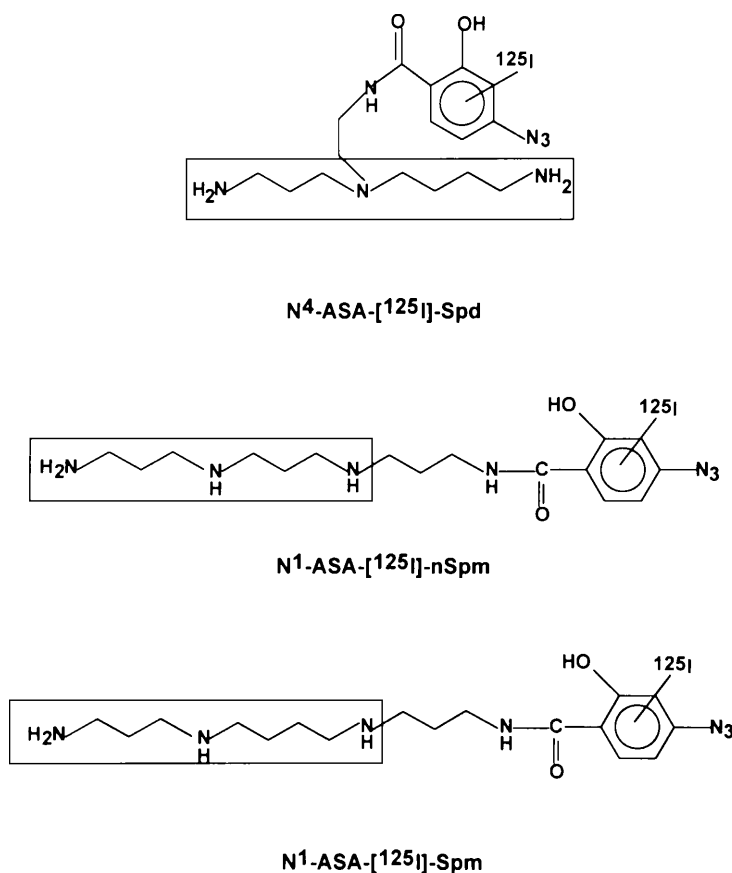
co-workers [5–10] have identified a total of eight relevant genes (designated *potA* to *potD* and *potF* to *potI*) with functions varying from ligand binding to ATPase activity. If the complexity of the bacterial transport system is relevant to the mammalian transporter, deletion or mutation of any one of these proteins could be sufficient to interrupt transport function.

The utility of photoaffinity labelling for examining plasma-membrane proteins is well established. The approach relies on the principles proposed by Ji [11] whereby photoreactive agents can be used to covalently modify plasma-membrane sites to detect receptors of low abundance and circumvent the difficulties of low-affinity purification. Polyamine-conjugated photoprobes have been used to map binding sites of polyamines on nucleosome core particles as well as the effect of polyamines on the helical twist of DNA [12,13]. Leroy et al. [14] characterized polyamine-binding sites on the  $\beta$ -subunit of casein kinase 2 by photoaffinity labelling. The methodology was first applied to polyamine transport by Minchin et al. [15], who used a spermine (Spm) photoprobe to inactivate putrescine, but not spermidine (Spd), transport into B16 melanoma cells. More recently, photoaffinity labelling has been used to investigate polyamine-binding sites associated with the *N*-methyl-D-aspartate (NMDA) receptors [16]. Where these studies used the procedure to interfere with protein function, our approach has been to identify polyamine-

Abbreviations used: Boc, *t*-butyloxycarbonyl; DESpm, N<sup>1</sup>,N<sup>12</sup>-diethylspermine [also known as BESPM, N<sup>1</sup>,N<sup>12</sup>-bis(ethyl)spermine]; N<sup>1</sup>-ASA-nSpm, N<sup>1</sup>-azidosalicylamidoethylnorspermine; N<sup>1</sup>-ASA-Spm, N<sup>1</sup>-azidosalicylamidoethylspermine; N<sup>1</sup>-ASA-Spd, N<sup>1</sup>-azidosalicylamidoethylspermidine; NHS-ASA, *N*-hydroxysuccinimidy-4-azidosalicylic acid; pbp, polyamine-binding protein; Spd, spermidine; Spm, spermine; NMDA, *N*-methyl-D-aspartate.

<sup>1</sup> Shared first authorship.

<sup>2</sup> To whom correspondence should be addressed.



**Figure 1** Structure of previously described polyamine-conjugated photoprobes *N*<sup>4</sup>-ASA-Spd, *N*<sup>1</sup>-ASA-nSpm [3] and the newly synthesized photoprobe, *N*<sup>1</sup>-ASA-Spm

binding proteins (pbps) by combining photoaffinity labelling with gel separation and autoradiography. We recently reported on the successful application of two structurally different polyamine photoprobes to identify pbps [3]. By labelling intact cells under non-penetrating conditions, we were able to focus the procedure to the cell surface and thus enhance the probability of interaction with transporter protein(s). In so doing, two prominently labelled proteins designated pbp118 and pbp50 (an indication of their estimated molecular mass in kDa) were identified on the surface of L1210 and U937 cells [3]. Although evidence for an association of these proteins with the transporter system was obtained, their functional significance and the generality of their presence on other cell types requires further study.

In this report, we describe the synthesis and characterization of a new polyamine conjugate, *N*<sup>1</sup>-azidosalicylamidoethylspermine (*N*<sup>1</sup>-ASA-Spm) (Figure 1), which exhibits far superior properties to previous photoprobes in terms of its ability to interact with the polyamine transporter. By using the new probe in combination with a detergent-based method of membrane isolation, we have developed a method that permits the identification of pbps on the surface of solid-tumour cell types.

## MATERIALS AND METHODS

### Materials

The polyamine analogue used for competition studies, *N*<sup>1</sup>,*N*<sup>12</sup>-diethylspermine (DESpm), was synthesized and kindly provided by Dr. R. Bergeron (University of Florida, Gainesville, FL,

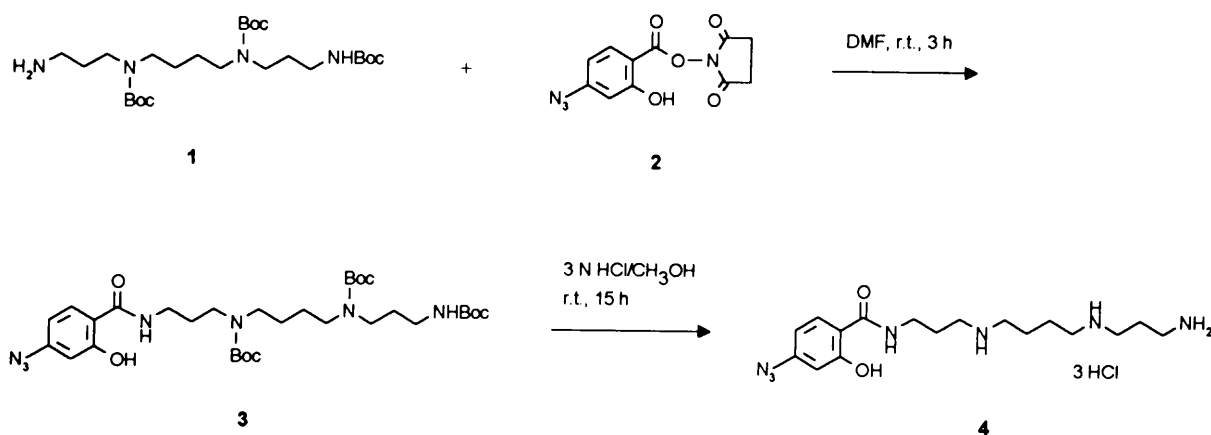
U.S.A.). The polyamines, Spd and Spm, and methylglyoxal bis(guanyldrazone) were purchased from Sigma (St. Louis, MO, U.S.A.). Tritiated Spd and the non-reducing form of Na<sup>125</sup>I used for iodinating the photoprobes were purchased from DuPont-NEN (Boston, MA, U.S.A.). Photoprobe *N*<sup>4</sup>-azidosalicylamidoethylspermidine (*N*<sup>4</sup>-ASA-Spd) was synthesized by Joan MacDiarmid (State University of New York at Buffalo), and photoprobe *N*<sup>1</sup>-azidosalicylamidoethylnorspermine (*N*<sup>1</sup>-ASA-nSpm) by Dr. P. Woster and Dr. R. Wu (Wayne State University, Detroit, MI, U.S.A.) as previously described [3].

### Cell culture

L1210 murine lymphocytic leukaemia cells and the polyamine-transport-deficient subline (designated L1210/MG) were kindly provided by Dr. O. Heby (Umea, Sweden) [17] and grown as described elsewhere [3]. The A549 human lung carcinoma cells were obtained from Dr. R. A. Casero (Johns Hopkins University) and grown in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco-BRL) and 1:100 dilution of penicillin/streptomycin (Gibco-BRL). Both cell cultures were maintained in exponential growth phase and were routinely tested for mycoplasma using a kit purchased from GenProbe (San Diego, CA, U.S.A.).

### Synthesis of photoprobes

The synthetic route used to prepare the photoprobe precursor 4 (*N*<sup>1</sup>-ASA-Spm) is illustrated in Scheme 1. All chemical reactions



**Scheme 1** Synthetic scheme used to prepare  $N^1$ -ASA-Spm

Boc,  $\text{COOC}(\text{CH}_3)_3$ ; DMF, dimethylformamide; r.t., room temperature.

were performed under an atmosphere of dry  $\text{N}_2$  and low light conditions. Compounds **1** and **2** have been described previously (**1**[18], **2**[19]). Reaction of the *t*-butyloxycarbonyl (Boc)-protected spermine derivative **1** (tri-Boc-Spm) with the photoreactive donor **2** [*N*-hydroxysuccinimidyl-4-azidosalicylic acid (NHS-ASA)] yielded the intermediate **3** ( $N^1$ -ASA-tri-Boc-Spm). Removal of the *N*-Boc-protecting groups from compound **3** with 3 M HCl/methanol afforded the target compound **4** as the trihydrochloride salt.

$N^1$ -ASA-tri-Boc-Spm[1-(4-azido-2-hydroxybenzoyl)-5,10,14-tris[(1,1-dimethylethoxy)carbonyl]-1,5,10,14-tetra-azatetradecane (**3**)] was prepared as follows. To a stirred ice-cooled solution of 5.78 g (11.50 mmol) of 1,5,10,14-tetra-azatetradecane (**1**) in 50 ml of DMF was added dropwise a solution of 3.50 g (12.04 mmol) of 1-[(4-azido-2-hydroxybenzoyl)oxy]pyrrolidine-2,5-dione (**2**) (95%) in 30 ml of DMF over 15 min. The mixture was stirred for 3 h at ambient temperature and then concentrated to dryness *in vacuo* at 40 °C. The crude reaction product was purified by flash chromatography on Merck silica-gel 60, 230–400 mesh [eluted with *t*-butyl methyl ether/hexane (7:3, v/v)] to give 6.64 g (yield 87.0%) of pure  $N^1$ -ASA-tri-Boc-Spm (**3**) as a resinous solid. The product was characterized by  $^1\text{H-NMR}$  spectroscopy on a Varian Gemini-200 spectrometer.

$N^1$ -ASA-Spm [1-(4-azido-2-hydroxybenzoyl)-1,5,10,14-tetra-azatetradecane trihydrochloride (**4**)] was prepared as follows. A mixture of 6.54 g (9.85 mmol) of 1-(4-azido-2-hydroxybenzoyl)-5,10,14-tris[(1,1-dimethylethoxy)carbonyl]-1,5,10,14-tetra-azatetradecane (**3**) and 60 ml of 3 M HCl/methanol was stirred at room temperature for 15 h. The reaction mixture was filtered and the filter cake washed with diethyl ether. The resulting crystalline solid was dried *in vacuo* for 6 h at 110 °C to give 4.23 g (yield 90.8%) of pure  $N^1$ -ASA-Spm (**4**). The product was characterized by melting point (250–253 °C), elemental analysis and  $^1\text{H-NMR}$  spectroscopy.

### $[^3\text{H}]\text{Spd}$ competition analysis

Spermidine-transport kinetics in L1210 cells were analysed by the procedure of Kramer et al. [20], and those for the monolayer A549 cells by the method of Kramer et al. [21]. Apparent inhibitor constants were generated using 100  $\mu\text{M}$  competitor. Photoprobe competition assays were performed in the dark

under a red safety filtered light to avoid photoactivation. Michaelis–Menten parameters for both cell lines were defined in terms of transport velocity  $V_{\text{max}}$ , binding affinity  $K_t$  and apparent inhibitor constant  $K_i$ .

### Photoaffinity labelling of intact cells

Radioiodination of the polyamine-conjugated photoprobes and photoaffinity labelling of L1210 cells were carried out as previously described [3]. The photoaffinity labelling of A549 cells was modified to accommodate cells growing as a monolayer. In brief, A549 cells were grown to semiconfluency in tissue culture plates. Cells were washed twice with PBS and the plates were placed on ice. In the dark and under a red safety filtered light, radioiodinated polyamine-conjugated photoprobe was added to a concentration of 35  $\mu\text{M}$  and carefully spread across the plate by tilting. Plates were incubated for 5 min and then exposed to UV light for 3 min (Mineralight, Gabriel, CA, U.S.A.). Both the preincubation and UV cross-linking procedure were performed at 4 °C to minimize probe internalization [22]. After UV irradiation, dithiothreitol was added to each of the plates to a concentration of 100  $\mu\text{M}$  to scavenge any unchanged probe [23]. The photoprobe solution was removed and the plates repeatedly washed with cold PBS.

### Membrane Isolation

Two methods of plasma-membrane isolation were utilized, sucrose fractionation and detergent lysis. The former was carried out with labelled L1210 or A549 cells by the method of Thom et al. [24] modified as previously reported [3]. With detergent lysis [25] as modified by Saxon et al. [26], labelled L1210 or A549 cells were lysed for 10 min using a digitonin buffer containing 20 mM Tris/HCl, pH 7.2, 2 mM EGTA, 2 mM EDTA, 0.5 mg/ml digitonin (Fisher Scientific, Fair Lawn, NJ, U.S.A.) and a mixture of protease inhibitors [10  $\mu\text{g}/\text{ml}$  leupeptin and aprotinin and 10  $\mu\text{M}$  PMSF (Sigma)]. The lysate was centrifuged at 100000 *g* for 30 min and the pellet redissolved in 0.5% Triton X-100/PBS buffer by vigorous pipetting. Precipitate was removed by microcentrifugation and the supernatant used for protein separation by gel electrophoresis. Sample protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA, U.S.A.).

### Photoprobe effects on [<sup>3</sup>H]Spd accumulation

For [<sup>3</sup>H]Spd-accumulation studies, A549 cells were grown to semiconfluency in six-well plates, cross-linked with 100  $\mu$ M N<sup>1</sup>-ASA-Spm for 2 min. Cells were washed with cold sterile PBS, and then prewarmed RPMI 1640 containing 2  $\mu$ M [<sup>3</sup>H]Spd (136  $\mu$ Ci/ $\mu$ mol) was added. As a control for specificity of effect, accumulation of [<sup>3</sup>H]leucine (1  $\mu$ Ci/ $\mu$ mol) was also measured. Triplicate wells were placed in a 37 °C bath or on ice for 10 min. Plates were then put on ice and chased with cold PBS containing 50  $\mu$ M Spd. After removal of the chase medium, cells were repeatedly washed with PBS. Using 1.0 M NaOH, cells were solubilized and then sonicated with two 7 s bursts. HCl (1.0 M) was added for neutralization and an aliquot of the mixture was taken to determine the amount of accumulated radioactivity by using a Beckman scintillation counter model LS1800. Each sample was analysed for protein concentration by the Bradford method following the manufacturer's instructions for the micro-assay.

### Protein separation by SDS/PAGE

Membrane proteins isolated as described above by sucrose fractionation or detergent lysis were separated by SDS/PAGE by the procedure of Laemmli [27] as used previously [3]. Gels were fixed and stained using 0.25% Coomassie Blue in 50% methanol and 7% acetic acid. The stained gels were then dried and exposed to Kodak X-OMAT-AR film with intensifying screens (DuPont-NEN) for 4–21 days.

### RESULTS AND DISCUSSION

In earlier studies [12–14,16], photoaffinity labelling was used to identify and map polyamine-binding sites on chromatin, casein kinase and the NMDA receptor. Our original assumption that cell-surface proteins with polyamine-binding properties such as the polyamine transporter might be susceptible to photoaffinity labelling was modelled after the work of Freisheim and co-workers [28,29], who used the technology to label and identify folate-binding proteins on the surface of L1210 cells. In our initial application of this approach, we found that photoaffinity labelling with either N<sup>4</sup>-ASA-Spd or N<sup>1</sup>-ASA-nSpm can be used to selectively label a small number of cell-surface proteins presumed to be putative pbps [3]. The specificity of the most prominently labelled proteins in L1210 cells as pbps was supported by (a) a similarity in labelling with either photoprobe, (b) the absence of labelling with the unconjugated photoprobe NHS-ASA and (c) the ability of exogenous polyamines to interfere competitively with labelling by the photoprobes.

In an attempt to extend earlier studies to adherent cells and to improve the probability for labelling proteins associated with the polyamine transporter, we have designed and synthesized a new photoprobe, based on our earlier observation that the N<sup>1</sup>-ASA-nSpm conjugate showed considerably greater affinity for the polyamine transporter than N<sup>4</sup>-ASA-Spd [3]. It should be noted that because the photosensitive substituent ASA is attached to the terminal amine of Spm via an acyl bond, the remaining binding motif based on charge is that of Spd (Figure 1). The ability of the N<sup>1</sup>-ASA-Spm photoprobe to interact with the polyamine transporter relative to the other photoprobes was assessed by competitive inhibition analysis of [<sup>3</sup>H]Spd uptake into murine L1210 leukaemia cells. Under non-activating light conditions, the apparent inhibition constant ( $K_i$ ) for the N<sup>1</sup>-ASA-Spm was approx. 1.0  $\mu$ M as compared with 23  $\mu$ M for the N<sup>1</sup>-ASA-nSpm conjugate, 52  $\mu$ M for the N<sup>4</sup>-ASA-Spd conjugate and 71  $\mu$ M for the known competitor of polyamine transport

**Table 1 Comparison of Spd uptake and photoprobe competition in L1210 and A549 cells**

The data represent mean values from experiments run in duplicate or triplicate with < 15% variation. All competitors were tested at 100  $\mu$ M except for DESpm which was tested at 10  $\mu$ M. Certain data for the L1210 cells are taken from Felschow et al. [3] for comparison purposes. MGBG, methylglyoxal bis(guanylhydrazine).

Cell line	Substrate or competing probe	$V_{max}$ (pmol/min per 10 <sup>7</sup> cells)	Apparent $K_i$ ( $\mu$ M)	Apparent $K_i$ ( $\mu$ M)
L1210***	[ <sup>3</sup> H]Spd	53	1.0	—
	MGBG			71
	DESpm			3.0
	N <sup>4</sup> -ASA-Spd			52
	N <sup>1</sup> -ASA-nSpm			23
A549	[ <sup>3</sup> H]Spd	44	0.4	—
	N <sup>1</sup> -ASA-Spm			0.9

methylglyoxal bis(guanylhydrazine) (Table 1). Thus the binding constant ( $K_i$ ) of the new probe is comparable with that for [<sup>3</sup>H]Spd (i.e. in the range 1  $\mu$ M) in both murine leukaemic and human carcinoma cell lines, suggesting that the photoprobe at least has the potential to interact with polyamine transporter with affinity comparable with that of the natural ligand. More importantly, the apparent  $K_i$  was 20–50-fold lower than that for either of the photoprobes used in earlier studies [3]. The basis for this improved binding affinity is not clear since the two photoprobes and their binding motifs (Spd and nSpd) differ by only a single intra-amine carbon. Its applied importance, however, is more obvious since the more efficient photoprobe has enabled us to develop methodology for labelling cell-surface proteins in adherent cell lines.

The possible relationship between the photoprobes and polyamine transport was further examined by comparing the accumulation of <sup>125</sup>I-labelled N<sup>1</sup>-ASA-Spm and N<sup>4</sup>-ASA-Spd in L1210 cells and polyamine-transport-deficient L1210 (L1210/MG) cells [17]. As shown in Table 2, parental L1210 cells accumulated both photoprobes whereas, by comparison, the transport-deficient L1210/MG cells took up much less. The results suggest dependence on the polyamine transporter for entry into cells. Relative to N<sup>4</sup>-ASA-<sup>125</sup>I-Spd, the new photoprobe N<sup>1</sup>-ASA-<sup>125</sup>I-Spm seemed to accumulate to higher levels in the parental L1210 cells and lower levels in the transport-deficient cells, although these differences did not prove to be statistically significant. Further evidence for the interaction of N<sup>1</sup>-ASA-Spm with the

**Table 2 Photoprobe accumulation in L1210 and L1210/MG cells under non-activating conditions**

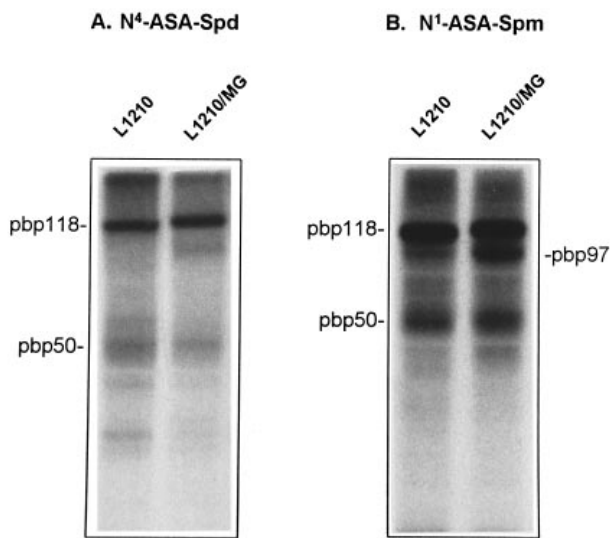
Cells were incubated in PBS containing 2.5  $\mu$ M <sup>125</sup>I-labelled photoprobe for 30 min at 37 °C in the dark. They were then washed with medium containing 25  $\mu$ M unlabelled photoprobe, lysed and radioactivity was determined. Results are means  $\pm$  S.D. ( $N = 3$ ).

[ <sup>125</sup> I]-Photoprobe	Cellular accumulation (nmol/10 <sup>7</sup> cells)	
	L1210	L1210/MG
N <sup>4</sup> -ASA-Spd	80 $\pm$ 19	28 $\pm$ 13
N <sup>1</sup> -ASA-Spm	112 $\pm$ 20	15 $\pm$ 4

**Table 3** Effects of membrane protein cross-linking by N<sup>1</sup>-ASA-Spm on uptake and cell viability in A549 human lung adenocarcinoma cells

Cells were incubated for 5 min in photoprobe before exposure to UV light for 3 min. Uptake data are presented as means  $\pm$  S.D. during a 10 min incubation ( $n = 3$ ).

Pretreatment	Uptake (% of control)		Cell viability (%)
	Spd	Leucine	
100 $\mu$ M probe	64 $\pm$ 4	105 $\pm$ 3	98
UV light only	77 $\pm$ 9	86 $\pm$ 18	97
Probe plus UV	14 $\pm$ 2	81 $\pm$ 3	94

**Figure 2** Autoradiographic comparison of photoaffinity labelling of plasma-membrane proteins from L1210 and L1210/MG cells labelled with N<sup>4</sup>-ASA-<sup>125</sup>I-Spd (A) or N<sup>1</sup>-ASA-<sup>125</sup>I-Spm (B)

Cells were labelled with probe at a concentration of 35  $\mu$ M. Protein prepared by sucrose fractionation was loaded at 50  $\mu$ g on an SDS/12% polyacrylamide gel, and SDS/PAGE was followed by autoradiographic exposure for 15 days.

polyamine transporter was obtained by measuring the effect of membrane cross-linking with the photoprobe on polyamine transport. Because of their ease of manipulation in the dark, adherent A549 cells were used for these studies. Before [<sup>3</sup>H]Spd uptake determinations, cells were cross-linked with 100  $\mu$ M non-radiolabelled N<sup>1</sup>-ASA-Spm for 2 min. It was observed that cell-surface cross-linking with N<sup>1</sup>-ASA-Spm effectively reduced [<sup>3</sup>H]Spd accumulation by more than 85% while lowering that of [<sup>3</sup>H]leucine by only about 20% and having a minor effect on cell viability (Table 3). By comparison, exposure of cells to either UV light in the absence of photoprobe or photoprobe in the absence of UV light had only a small effect on transport of [<sup>3</sup>H]Spd. Similar results were obtained with the new probe in L1210 cells where, by comparison, N<sup>1</sup>-ASA-Spm lowered [<sup>3</sup>H]Spd uptake by 70% (results not shown).

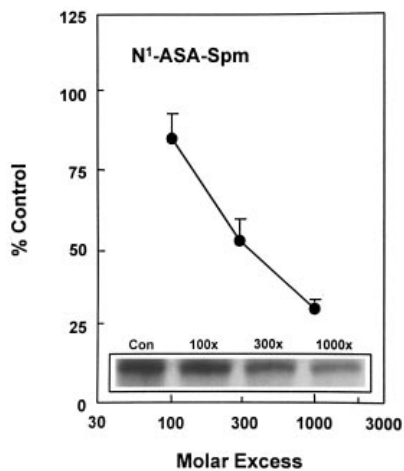
In order to validate the new photoprobe relative to earlier studies [3], cell-surface labelling was compared with that obtained using the photoprobe N<sup>4</sup>-ASA-<sup>125</sup>I-Spd. Photoaffinity tagging of both the parental and transport-deficient L1210 cells was carried out. As shown in Figure 2, photolabelling by N<sup>1</sup>-ASA-Spm and N<sup>4</sup>-ASA-Spd revealed similar but not identical profiles of radio-

labelled proteins. Our subsequent analysis focused on prominently labelled protein bands. Proteins previously identified by the N<sup>4</sup>-ASA-Spd photoprobe, pbp118 and pbp50, were clearly labelled with N<sup>1</sup>-ASA-Spm. In addition, N<sup>1</sup>-ASA-Spm revealed a diffusely labelled protein in the range of 97 kDa. The faint 35 kDa band seen with N<sup>4</sup>-ASA-Spd labelling probably represents a non-specifically labelled protein since we have previously shown that unconjugated ASA interacts with a protein of approximately the same size [3]. As further indication that the two probes were labelling identical proteins, a 10-fold excess of unlabelled N<sup>1</sup>-ASA-Spm probe was included during labelling with the radioiodinated N<sup>4</sup>-ASA-Spd probe. Under these conditions, photocross-linking of all proteins by N<sup>4</sup>-ASA-Spd was effectively blocked (results not shown).

As reported previously [3], there was no loss of any labelled bands in the transport-deficient L1210/MG cells. Instead, pbp97 appeared to be much more abundant than in parent cells especially with N<sup>1</sup>-ASA-Spm labelling and, on the basis of gel mobility, pbp118 consistently displayed a slightly higher molecular mass (Figure 2). Thus the association between certain photolabelled proteins and polyamine transport could not be clearly confirmed by comparison studies of L1210 transport-competent and -deficient cells. It should be noted, however, that, on the basis of findings from bacterial systems [6,7,8–10], the polyamine-transport apparatus in mammalian systems probably exists as a complex of proteins. It is possible therefore that any of the proteins involved in the putative transport complex may be defective or absent. Another interpretation of these findings is that the cellular function of the photolabelled pbps may be unrelated to transport. Polyamines have been implicated in the regulation of several different cell-surface proteins including the NMDA receptor [3,30] and inward rectifying K<sup>+</sup> channels [31–33]. Polyamine-binding sites of uncertain significance have been previously detected on the surface of cells by visualizing polyamine-conjugated latex particles by scanning electron microscopy [34].

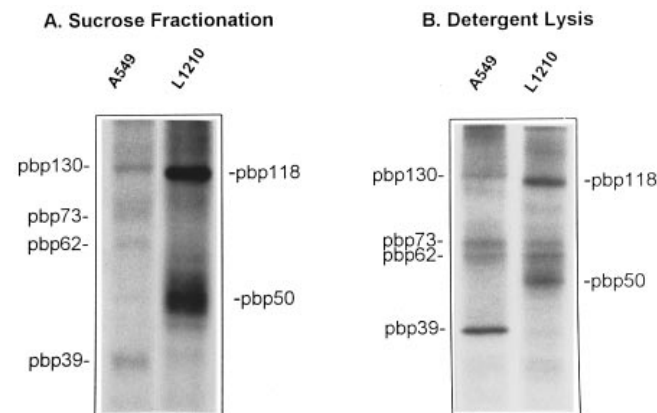
The identity of pbp118 as a pbp in L1210 cells was thus reaffirmed by concomitant labelling with two structurally distinct polyamine photoprobes. As further indication of that identity, competition studies with Spm were carried out during labelling with photoprobe. L1210 cells were treated with an excess of the natural polyamine Spm before and during photoaffinity labelling with the N<sup>1</sup>-ASA-Spm photoprobe. In the presence of increasing amounts of Spm, labelling of the 118 kDa protein decreased in a dose-dependent manner (Figure 3). At a ratio of 300-fold Spm to photoprobe, labelling of pbp118 was reduced by about 50%. Felschow et al. [3] and Leroy et al. [14] have previously noted the high concentrations required for competition during photoaffinity labelling. Both groups attribute it to the fact that ionic competitors, such as the natural polyamines, would be expected to antagonize the irreversible binding of the photoprobe during the initial recognition phase but not once photoactivation has occurred. Thus the relatively high concentrations of competitor are considered necessary.

Previously, we reported the presence of pbps on the surface of murine L1210 cells and human U937 cells both of which are of lymphoid origin [13]. The following series of experiments examined whether similar cell-surface pbps can be detected by photoaffinity labelling on the surface of a solid-tumour cell line. Feasibility was first established by comparing L1210 cells with A549 large cell lung carcinoma cells using the above methodology of membrane isolation. By this procedure, radioiodinated N<sup>1</sup>-ASA-Spm photoprobe was added directly to A549 cells growing as semiconfluent monolayer cultures, and this was followed by plasma-membrane protein isolation by sucrose fractionation



**Figure 3** Competitive interference by Spm with photoaffinity labelling of pbp118 of L1210 cells using  $N^1$ -ASA-Spm

Data presented in the graph were obtained by quantification of pbp118 in the autoradiogram (inset). Cells were preincubated for 5 min in Spm at concentrations up to 1000 times greater than the radiolabelled photoprobe (35  $\mu$ M) and then incubated for 3 min in the presence of photoprobe under labelling conditions.



**Figure 4** Autoradiographic comparison of photoaffinity labelling of plasma-membrane proteins from L1210 and A549 cells labelled with  $N^1$ -ASA- $^{125}$ I-Spm and prepared by sucrose fractionation (A) or detergent lysis (B).

[24]. As shown in Figure 4(A), the photoaffinity-labelling pattern of A549 cells revealed the presence of a protein of somewhat higher molecular mass (130 kDa) in place of pbp118 and a less abundant pbp50. In addition, prominent protein bands were also apparent at 39, 62 and 73 kDa in the A549 cells but not in L1210 cells.

Sucrose fractionation of plasma membranes requires large numbers of labelled monolayer cells ( $2 \times 10^8$  cells), which results in the consumption of excessive amounts of photoprobe, a factor that could be limiting in peptide-mapping studies. To circumvent these problems, an alternative labelling and membrane-isolation procedure was adapted from that of Pelech et al. [25] as modified by Saxon et al. [26]. It relies on direct lysis of the labelled cells on the tissue culture plate followed by membrane isolation based on detergent (digitonin) solubility. This straightforward method requires approx. 200-fold fewer cells ( $\sim 10^6$ ) and consumes proportionally less photoprobe. As shown in Figure 4, mem-

brane-labelling patterns developed by this methodology resembled those generated by sucrose fractionation in both L1210 and A549 cells. As noted above with sucrose fractionation, the pbp118 seen in L1210 cells was not seen in A549 cells which instead displayed pbp130. Since the most prominent pbp118 seen in L1210 cells was not present, the two proteins may be analogous, with the size difference being due to cell type, species or both. The possibility that they represent size variants of the same protein suggests a generality of distribution of this protein among cell types. A precedent for this is available in our earlier study in which the equivalent of L1210 pbp118 in human lymphoma U937 cells was found to be slightly smaller (i.e. approx. 112 kDa) [3].

The detergent-lysis methodology revealed two new proteins, pbp73 and pbp62, in both cell types. By sucrose fractionation, these same proteins were much less obvious in A549 cells and only faintly apparent in the L1210 cells, suggesting that they may be associated plasma-membrane proteins. One very prominent protein, pbp39, was consistently observed in A549 cells but not in L1210 cells. Since it was seen with both methodologies, it may represent a protein unique to monolayer cells and hence is possibly involved in substrate adhesion. Because plasma-membrane preparation via the new methodology is less rigorous than with the original approach, it is possible that some proteins present only in the detergent-lysis preparations may not be of plasma-membrane origin. We emphasize, however, that labelling was carried out under conditions (i.e. 4 °C) designed to preclude photoprobe interaction with cytoplasmic proteins [22].

A major goal of the present study was to extend the technology of photolabelling cell-surface pbps to adherent cell types so that the findings with murine and human leukaemia cells [3] might be extended to solid tumour cell types. The identification of a photoaffinity probe 20–50-fold more potent in binding affinity than our previously described photoprobes was critical to this endeavour. A second limiting factor in this effort was plasma-membrane preparation by sucrose fractionation which required large numbers of radiolabelled cells. The detergent (digitonin) method of lysing and preparation of membranes resolved this difficulty. The much more efficient probe and labelling procedure can now be used to generate sufficient quantities of labelled tumour cells for protein characterization. Combining this approach with peptide mapping [35], we have attempted to identify pbp39 from A549 cells. Microsequence information of peptide fragments (Dr. J. Leszyk, Worcester Foundation for Biomedical Research, Worcester, MA, U.S.A.) revealed homology of the pbp (based on 18 amino acid units) with glyceraldehyde-3-phosphate dehydrogenase (38 kDa). Although a significant portion of the enzyme is membrane-associated [36,37] and capable of functions unrelated to regulation of glycolysis [38], failure of an enzyme-specific antibody [37] to precipitate the labelled protein suggests that the finding may be due to co-migration of unrelated peptides. This continuing study nonetheless illustrates the potential of photoaffinity labelling for determining the molecular nature of cell-surface pbps.

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