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Removal of nonspecific binding proteins from cell and tissue extracts using 2-aminobenzimidazole-tethered affinity resin

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Received March 2, 2011, accepted April 15, 2011

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Pharmazie 66: 662–665 (2011) *doi: 10.1691/ph.2011.1027*

Cellular drug target identification through affinity chromatography is often hindered by the quantity of nonspecific binders, such as cytoskeletal and heat shock proteins. Thus, we prepared a 2-aminobenzimidazole-tethered depletion resin designed for removal of these proteins, and tested it on human lung carcinoma cell and rat tissue extracts. Column-bound proteins were identified by two-dimensional gel electrophoresis and MS. Among others, tubulins, actins and heat shock proteins were successfully depleted. Due to the reduction of these highly abundant proteins detection of potential drug targets is considerably facilitated in the pre-purified samples.

1. Introduction

Target validation is a critical step in drug development for understanding the mechanism of action and potential toxic effects of the candidate therapeutic agent. Affinity chromatography is a suitable technique for the identification of drug-interacting proteins (Darvas et al. 2004), but the nonspecific binding of abundant proteins to small molecule-immobilized matrices make the detection of these low-abundance proteins in complex biological samples fairly challenging.

Various approaches have been tried to overcome this problem. Competitive elution of specific target proteins from affinity columns with an excess of free compounds is generally used. In a further approach the specific binding proteins were determined by subtracting the identified proteins retained on an inactive compound-immobilized affinity column from that in an active compound-immobilized affinity column (Kosaka et al. 2005). In serial affinity chromatography a mixture of proteins is sequentially treated several times with affinity resins bearing ligand and the binding proteins on each resin are then comparatively analyzed (Yamamoto et al. 2006). Surface (von Rechenberg et al. 2005; Shimizu et al. 2000) and spacer structure (Tamura et al. 2003) optimization methods provide additional options for reducing interactions between nonspecific binders and the solid support.

Another approach – particularly used in plasma proteomic studies – is the previous removal/reduction of the abundant (nonspecific) proteins from the analyzed sample via enrichment or depletion methods. ProteoMiner™ (Bio-Rad, Hercules, CA, USA) enrichment technology (Bandow 2010) is based on the treatment of complex protein samples with a large, highly diverse library of hexapeptides bound to chromatographic supports, resulting in concentration of low-abundance proteins via reduction of the dynamic range in the sample. This method was optimized for plasma and serum samples, however, it is possibly adaptable for other sample types as well. Because of the high salt and low pH elution buffers, the resulting protein solution is though compatible with SELDI applications, but cannot be used for down-stream processes requiring proteins with native conformation, such as enzymatic screening or affinity chromatography. Immunoaffinity depletion columns are composed of beads coated with specific antibodies for removal of highly abundant serum proteins (Szafranski et al. 2004; Chromy et al. 2004), and result intact, native-conformation proteins in the flow-through. Depletion columns may be appropriate options for getting rid of well-identified nonspecific binders in affinity chromatographic assays as well, and instead of antibodies, even specific small molecules could be used as capture ligands.

For instance, highly abundant cytoskeletal proteins such as tubulin and actin often interfere with drug target validation. It is established that benzimidazoles bind to the tubulin in nematodes (Lacey 1988) and in mammalian brain (Friedman and Platzer 1978), hence a benzimidazole-coupled resin seems to be useful for pre-purification of cell and tissue extracts.

Thus, we present here a 2-aminobenzimidazole-tethered resin designed for removal of tubulin and abundant tubulin-interacting proteins from crude protein extracts, and hereby enriching potential target proteins in a cell or tissue sample.

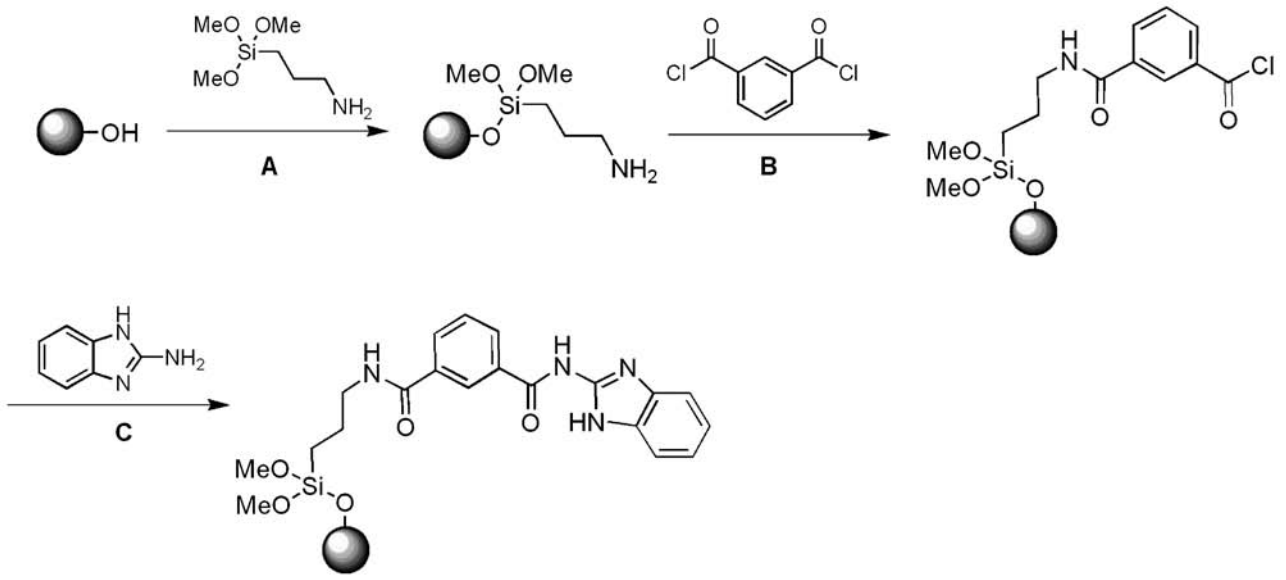
Previously, several chemical modifications of glass surfaces were developed to capture small molecules. However, most of the technology was applied for fabrication of chemical microarrays (Darvas et al. 2004; Hackler jr. et al. 2003). For the preparation of the 2-aminobenzimidazole-tethered depletion resin the surface of controlled pore glass (CPG) particles were modified using a method which is adaptable for additional affinity chromatography applications.

2. Investigations, results and discussion

Our main objective was to develop a pre-purification affinity resin for the removal of nonspecific binding proteins,

Table 1: Carbon content and calculated surface coverage of products resulted in the course of the preparation of the 2-aminobenzimidazole affinity resin

Product	Carbon content (w/w %)	Number of carbon atoms/anchored group	Surface coverage (μ mol/m^2)
Amino-silanized CPG	0.20	5	0.66
Isophtalic-CPG	0.45	8	0.52
2-aminobenzimidazole-CPG	0.59	7	0.33



Scheme: Preparation of the 2-aminobenzimidazole-tethered affinity resin. CPG particles were treated with 3-aminopropyl-trimethoxysilane (A), the amino-silanized CPGs were treated with dichloro-isophtalic acid (B), the resulting isophtalic-CPGs were treated with 2-aminobenzimidazole (C).

which often interfere with cellular drug target identification by affinity chromatography. Therefore, a 2-aminobenzimidazole-tethered depletion resin was prepared (Scheme): CPG particles were treated with 3-aminopropyl-trimethoxysilane, and 2-aminobenzimidazole was coupled to the amino-silanized CPG through isophtalic acid dichloride.

Surface coverage of the 2-aminobenzimidazole-CPG was calculated from the carbon content of each product resulted in the course of the preparation of the affinity resin (Table 1). From the results we can conclude that half of the active groups (0.33 μmol/m^2) of the amino-silanized CPG particles was successfully derivatized with 2-aminobenzimidazole.

In order to evaluate the efficiency of the 2-aminobenzimidazole resin, it was used to deplete crude cell and tissue protein extracts, respectively. Protein profiles of crude sample and flow-through fractions were compared by two-dimensional gel electrophoresis. By comparing the presence/intensity of spots between the crude sample and the flow-through fraction, several spots were found to be missing/reduced in the flow-through fraction (Fig.). These spots were excised from the crude sample gel and identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

20 different proteins from 12 spots were identified in the case of the human cell sample (Table 2). Beyond tubulins, mainly other cytoskeletal components (actins), members of the 14–3–3 family and heat shock proteins were observed among the 2-aminobenzimidazole-bound proteins. 14 spots from the rat brain sample were excised and subjected to mass spectrometry (Table 3). The bulk of the 16 resulted proteins correspond to those detected in the human cell sample.

Some of the identified hits were previously described as tubulin-binding proteins, namely: hsp70 (Sanchez et al. 1994; Gache et al. 2005); hsp90 (Garnier et al. 1998; Gache et al. 2005); hsp70/hsp90-organizing protein and grp78 (Gache et al. 2005); GAPDH (Kumagai and Sakai 1983). These are suspected to be co-entrapped with tubulin, other identified proteins are attached to the matrix via unrevealed interactions.

In both depleted samples an increase in the number of the detected spots as well as an increase in the intensity of some spots (altogether 21 in the cell extract and 20 in the rat brain sample) were observed on the flow-through fraction gels in comparison with the crude sample gels. These new or more intensive protein spots are due to the depletion of some nonspecific binders, which enables the detection of more low-abundance proteins compared to the non-depleted samples. Consequently, application of the 2-aminobenzimidazole resin on crude cell or tissue protein samples can facilitate the identification of cellular drug targets.

3. Experimental

3.1. Preparation of 2-aminobenzimidazole affinity resin

20 g native CPG (3Prime, Aston, PA, USA) was introduced into a 500 ml conical flask, and shaken for 1 h with 3% (v/v) 3-aminopro-

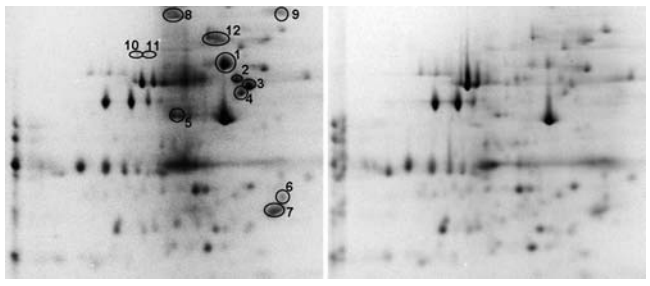


Fig.: Two-dimensional gel images of non-depleted A549 cell extracts (left) and that of depleted by 2-aminobenzimidazole-tethered affinity resin (right). Circles indicate protein spots, which are missing/reduced in depleted sample and were identified by mass spectrometry. The numbers correspond to the hits listed in Table 2.

Table 2: Depleted protein hits from human cell sample

No.	Protein name	Accession no.	MW (Da)	pl
1	Chaperonin	gi|31542947	61187	5.70
1	beta-Actin	gi|4501885	42052	5.29
2	alpha-Tubulin	gi|32015	50503	4.95
3	beta-Tubulin	gi|338695	50240	4.75
3	Mitochondrial ATP synthase	gi|89574029	48083	4.95
4	Mitochondrial ATP synthase	gi|89574029	48083	4.95
4	Protein disulfide isomerase-related protein 5	gi|1710248	46512	4.95
5	beta-Actin	gi|4501885	42052	5.29
6	14-3-3 Protein epsilon	gi|5803225	29326	4.63
6	14-3-3 Protein zeta/delta	gi|4507953	27899	4.73
6	14-3-3 Protein theta	gi|5803227	28032	4.68
7	14-3-3 Protein zeta/delta	gi|4507953	27899	4.73
7	14-3-3 Protein theta	gi|5803227	28032	4.68
7	14-3-3 Protein gamma	gi|9507245	28456	4.80
8	Heat shock protein hsp90 alpha 2	gi|61656603	98622	5.09
9	Heat shock protein hsp90 beta 1	gi|4507677	92696	4.76
10	Stress-induced-phosphoprotein 1 (hsp70/hsp90-organizing protein)	gi|5803181	63227	6.40
11	Aldehyde dehydrogenase 1A1	gi|21361176	55454	6.30
11	Stress-induced-phosphoprotein 1 (hsp70/hsp90-organizing protein)	gi|5803181	63227	6.40
12	Heat shock 70kDa protein 9	gi|12653415	73967	6.03
12	hsp70–2	gi|4529892	70267	5.48
12	Heat shock 70kDa protein 8 isoform 1	gi|5729877	71082	5.37
12	Heat shock-induced protein	gi|188492	70775	5.76
12	Heat shock protein 60	gi|77702086	61346	5.70
12	Actin, gamma 1 propeptide	gi|4501887	42108	5.31
12	Chaperonin	gi|31542947	61187	5.70

pyltrimethoxysilane in 200 ml 95% (v/v) methanol. The resulting amino-silanized CPG resin was then washed with methanol and water, then heated at 100 °C for 15 min using a block heater. Then amino-silanized CPG was shaken for 2 h with 15% (w/v) dichloro-isophtalic acid in dichloromethane (DCM) (100 ml), then washed with DCM and methanol and dried under vacuum. The resulting isophtalic-CPG was shaken for 2 h with 5% (w/v) 2-aminobenzimidazole in DCM (100 ml), then washed and dried as previously. Remaining activated groups were blocked by treating with ethanolamine (5% (v/v) in DCM, 100 ml) for 2 h, then 2-amino-benzimidazole-CPG was washed and dried as previously.

3.2. Determination of the carbon content and calculation of the surface coverage

The carbon content of each product, resulted in the course of the preparation of the affinity resin, was determined using a Vario Macro CNS elemental analyzer (Elementar, Hanau, Germany), and was used for calculation of the surface coverage of these products according to the extended Berendsen-de Galan equation (Sandoval 1999). Specific surface area of the native CPG (51 m^2/g) was given by the manufacturer.

3.3. Protein extraction from A549 cells

Human lung carcinoma A549 cells ($\sim 2 \times 10^8$) were washed with phosphate buffered saline (PBS), collected by centrifugation at 1000 rpm for 5 min, and resuspended in 1.5 volume of Solution A (50 mM Hepes pH 8.0, 10 mM KCl, 1 mM ethyleneglycoltetraacetic acid (EGTA), 1 mM dithiothreitol, Complete^{TM} Protease Inhibitor Cocktail Tablet from Roche Diagnostics, Mannheim, Germany) per volume of cell pellet on ice for 10 min. The suspension was mixed with 0.5% (v/v) Triton X-100. After a rapid centrifugation at 6000 rpm the supernatant was collected.

Table 3: Depleted protein hits from rat brain sample

Protein name	Accession no.	MW (Da)	pl
Heat shock 70kD protein 5 (grp78)	gi|25742763	72473	5.07
Heat shock protein 8	gi|13242237	71055	5.37
Chaperonin 60	gi|1778213	61029	5.78
ATP synthase, H + transporting, mitochondrial F1 complex, beta subunit	gi|54792127	56318	5.19
ATP synthase beta subunit	gi|1374715	51171	4.92
Actin, cytoplasmic 2 (gamma-actin)	gi|109507063	42109	5.31
beta-Actin	gi|119959830	31955	5.24
G protein beta 1 subunit	gi|984553	38167	5.47
Guanine nucleotide binding protein beta 2	gi|71089939	35722	5.91
14-3-3 zeta Isoform	gi|1051270	27955	4.73
14-3-3 Protein eta	gi|6981710	28365	4.81
14-3-3 Protein gamma	gi|9507245	28456	4.80
Dihydropyrimidinase-like 2	gi|40254595	62638	5.95
ATP Synthase alpha subunit precursor	gi|203055	58904	9.22
ATP Synthase, H+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1	gi|40538742	59831	9.22
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	gi|8393418	36090	8.14

3.4. Protein extraction from rat brain

Brains from 5 male Wistar rats were homogenized in Buffer A (10 mM Hepes pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA and Complete™ Protease Inhibitor Cocktail Tablet from Roche) with a Braun Teflon-glass homogenizer (10,000 rpm on ice). The homogenate was centrifuged at 45,000 *x* g for 30 min. The pellet was resuspended in 10 ml of Buffer A containing 1% Triton X-100, and was incubated for 30 min at 4 °C with permanent shaking. The suspension was centrifuged at 45,000 *x* g for 30 min. After the supernatant was separated, it was centrifuged at 147,000 *x* g for 30 min again.

3.5. Depletion of highly abundant proteins

The affinity resin (1 ml) was packed into a small polypropylene (Qiagen, Düsseldorf, Germany) column and equilibrated with PBS. Crude cell and tissue protein samples were diluted by PBS to 8 mg/ml, loaded onto the 2-aminobenzimidazole column, and allowed to pass through by gravity. After sample application, the column was washed with 10 ml PBS and 0.5 M NaCl in 10 ml PBS. Flow-through samples were pooled and termed as "flow-through fraction".

3.6. Two-dimensional gel electrophoresis

Fractionated samples equivalent to 60 μg protein were precipitated with the ReadyPrep™ 2-D Cleanup Kit (Bio-Rad) and then dissolved in 150 μl sample rehydration buffer (7 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 50 mM dithiothreitol and a trace of bromophenol blue). Dissolved samples were diffused into a ReadyStrip™ IPG strip (pH 3–10, 7 cm) (Bio-Rad) and resolved on a Protean IEF Cell (Bio-Rad) followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The gels were stained with colloidal Silver Blue, then imaged with the VersaDoc MP 4000 Imaging System (Bio-Rad) and analyzed by the PDQuest Advanced 8.0.1 2D Gel Analysis Software (Bio-Rad).

3.7. Mass spectrometry analysis

The protein content of the gel plugs was in-gel digested with side-chain protected trypsin as described at http://msf.ucsf.edu/ingel.html. The resulting tryptic digests were analyzed by matrix-assisted laser desorption/ionization time-of-flight peptide mass fingerprinting or by LC-MS/MS using an Eldex nanoHPLC on-line coupled to an LCQ-Fleet ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) in triple play operation mode.

Acknowledgements: We thank Iván Kanizsai, Ph.D. for drawing of the chemical structures. This work was supported by GOP-1.1.1-08/1-2008-0001, "Gábor Baross" DA07-DA_TECH_07-2008-00039, Ányos Jedlik "AVINOMID" and National Technology Program "GLINOLID" (National Research and Technology Office, Hungary).

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