

## PRELIMINARY STUDIES ON APPLICATION OF LIBRARY OF ARTIFICIAL RECEPTORS FOR DIFFERENTIATION OF METABOLITES IN URINE OF HEALTHY AND CANCER BEARING MICE

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**Abstract:** A library of artificial receptors formed by the self-organization of *N*-lipidated peptides attached to the cellulose via *m*-aminophenylamino-1,3,5-triazine was used for differentiation of metabolites in urine of healthy and cancer bearing mice. The interactions of urine metabolites with the receptors were visualized by using competitive adsorption-desorption of an appropriate reporter dye. Analysis of the binding pattern (fingerprint) of urine metabolites from healthy and from cancer suffering mice showed that there were several structures among 120-elements molecular receptors which were able to differentiate bonded ligands depending on the healthy state. For all three tested types of cancers two structures: Lipid-Pro-Ala-NH-C<sub>6</sub>H<sub>4</sub>-NH-DMT-cellulose and Lipid-Arg-Pro-NH-C<sub>6</sub>H<sub>4</sub>-NH-DMT-cellulose were selected as diagnostic.

**Keywords:** cancer marker, medical diagnostic, molecular receptor

Biomedical research is constantly challenged to clarify the relationship between health, disease and metabolism influenced by nutrition, pharmaceuticals and others. Biological fluids, such as urine, sweat, saliva, blood, contain a great number and variable metabolites that may offer valuable information on the metabolism of an organism, and consequently about its health status (1). In parallel with genomics, transcriptomics and proteomics, metabolomics will amplify our understanding of the pathophysiological processes involved and should be useful in an identification of potential biomarkers to develop new therapeutic strategies (2).

Indeed, metabolic fingerprinting, also referred to as metabolomics, metabonomics, metanomics (3) or associated terms, is a technique that allows identification and quantification of metabolites (low molecular weight molecules e.g., lipids, amino acids, and sugars) in biological fluids and is useful in determining the phenotype of disease (4).

From a clinical point of view, the study of metabolic changes that occur in response to divergent physiological processes are helpful in the study of the mechanisms underlying the development of various diseases. In terms of personalized medicine,

metabonomic approaches can provide the tool to predict the action of drugs in a particular individual based on the predose urinary metabolite profile (5). Analysis of complex metabolome is not a trivial task. There are several analytical strategies useful in analysis of metabolome (6): NMR (7) FT-IR (8), MS connected with separation techniques: HPLC, GC and CE. Only the combination of these analytical techniques offers analysis of complete metabolome. However, technological advances have increased the number of metabolites that can be quantified simultaneously (9).

Metabolic fingerprinting does not identify the whole set of metabolites but compare patterns or fingerprints of metabolites that change in response to a disease state, pharmacological therapies or environmental alterations. This approach is useful as a diagnostic tool to evaluate the disease state by comparing healthy controls and disease subjects, or to assay the success of a particular treatment (prognosis/recovery) (10). However, in the case of understanding the mechanisms underlying a disease, qualitative and quantitative analyses are necessary (11). Metabolite profiling focuses on the analysis of a group of metabolites related to a specific metabolic

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pathway (12). Here we present preliminary studies on application of library of *N*-lipidated peptides immobilized on cellulose in fingerprinting of urine metabolites of healthy and tumor bearing mice.

## MATERIALS AND METHODS

### Immobilization of 2,4-dichloro-6-methoxy-1,3,5-triazine on Whatman 7 cellulose filter paper

One hundred thirty five (120 + 15) Whatman 7 cellulose filter plates (10 × 10 cm) were washed with hexane (200 mL) and ethyl acetate (200 mL). After drying in a vacuum desiccator, plates were treated with 1 M NaOH (400 mL) for 15 min. In the next step, alkaline solution was removed and plates were air dried for 10 min and then immersed in a suspension of 1 M solution of 2,4-dichloro-6-methoxy-1,3,5-triazine (DCMT) in THF (400 mL) and solid NaHCO<sub>3</sub> (42 g, 0.5 mol). Suspension with immersed cellulose plates was gently shaken for 45 min, then plates were washed successively with THF (250 mL), 50% solution THF in water (250 mL) and acetone (250 mL). DCMT functionalized plates were dried to constant weight in a vacuum desiccator. Loading of 2-chloro-4-methoxy-1,3,5-triazine was calculated on the basis of the elemental analysis content of nitrogen and chlorine.

Elemental analysis: found: N 3.64%, Cl 2.66%; calculated from nitrogen content: 2.60 mmol N/1 g equivalent to  $31.9 \times 10^{-6}$  mol N/cm<sup>2</sup>, corresponding to  $31.9 \times 10^{-6}$  mol triazine/cm<sup>2</sup>; calculated from chlorine content: 0.75 mmol Cl/1 g equivalent to  $9.2 \times 10^{-6}$  mol Cl/cm<sup>2</sup>, corresponding to  $9.2 \times 10^{-6}$  mol triazine/cm<sup>2</sup>.

### Functionalization of 2-chloro-1,3,5-triazine derivative immobilized on the cellulose support with 1,3-phenylenediamine

One hundred thirty (120 + 10) Whatman 7 cellulose plates modified with DCMT (10 × 10 cm) were immersed in 1 M solution of 1,3-phenylenediamine in THF (400 mL) and gently shaken for 15 h at room temperature. Solution was removed, plates were washed with CH<sub>2</sub>Cl<sub>2</sub> (250 mL), THF (250 mL), DMF (250 mL) and CH<sub>2</sub>Cl<sub>2</sub> (250 mL) and dried to constant weight in a vacuum desiccator.

### Synthesis of library of *N*-lipidated dipeptides

#### *Incorporation of N-Fmoc-protected amino acids (1<sup>st</sup> amino acids) on the surface of DMT-NH-C<sub>6</sub>H<sub>4</sub>-NH<sub>2</sub> functionalized cellulose sheets*

Synthesis of N-Fmoc-Ala-NH-C<sub>6</sub>H<sub>4</sub>-NH-DMT-cellulose sheet {1}. General procedure

Forty two (40 + 2) DMT-NH-C<sub>6</sub>H<sub>4</sub>-NH<sub>2</sub> functionalized cellulose plates, labeled respectively with

graphite pencil as 1, were immersed in solution of 4-(4,6-dimethoxy-[1,3,5]-triazin-2-yl)-4-methylmorpholinium toluene-4-sulfonate (DMT/NMM/TsO) (4.13 g, 10 mmol) in DMF (40 mL), Fmoc-Ala-OH (3.29 g, 10 mmol) and NMM (2.5 mL, 23 mmol) and gentle shaking was continued for 12 h. The excess solution was removed and the modified cellulose plates were washed with DMF (3 × 50 mL) and methylene chloride (3 × 50 mL).

#### *Synthesis of N-Fmoc-Trp(Boc)-NH-C<sub>6</sub>H<sub>4</sub>-NH-DMT-cellulose sheet {2}*

Forty two (40 + 2) DMT-NH-C<sub>6</sub>H<sub>4</sub>-NH<sub>2</sub> functionalized cellulose sheets labeled as 2 were treated with DMF (40 mL) solution containing Fmoc-Trp(Boc)-OH (5.27 g, 10 mmol), DMT/NMM/TsO (4.13 g, 10 mmol) and NMM (2.5 mL, 23 mmol).

#### *Synthesis of N-Fmoc-Pro-NH-C<sub>6</sub>H<sub>4</sub>-NH-DMT-cellulose sheet {3}*

Forty two (40 + 2) DMT-NH-C<sub>6</sub>H<sub>4</sub>-NH<sub>2</sub> functionalized cellulose sheets labeled as 3 were treated with DMF (40 mL) solution containing Fmoc-Pro-OH (3.38 g, 10 mmol), DMT/NMM/TsO (4.13 g, 10 mmol) and NMM (2.5 mL, 23 mmol).

#### *Deprotection of fluorenylmethyloxycarbonyl (Fmoc) group from amino acids incorporated on the surface of functionalized cellulose sheets {1-3}*

Cellulose sheets {1-3} were treated with 25% solution of piperidine in DMF (200 mL) for 15 min. After removal of excess piperidine solution, {1-3} were washed with DMF (3 × 100 mL) and methylene chloride (1 × 100 mL).

#### *Incorporation of the second N-Fmoc-protected amino acids on the surface of H<sub>2</sub>N-amino acid-NH-C<sub>6</sub>H<sub>4</sub>-NH-DMT-cellulose sheets. General procedure*

To solution of DMT/NMM/TsO (4.13 g, 10 mmol) in DMF (40 mL), Fmoc-protected amino acids (10 mmol) and NMM (2.5 mL, 23 mmol) were added. After obtaining homogeneous reaction mixture, H<sub>2</sub>N-amino acid-NH-C<sub>6</sub>H<sub>4</sub>-NH-DMT-cellulose sheets labeled with graphite pencil were immersed and gently shaken for 12 h. The excess solution was removed and the cellulose sheets were washed with DMF (3 × 50 mL) and methylene chloride (3 × 50 mL).

#### *Incorporation of Fmoc-Ala-OH*

Six (3 × 2) modified cellulose sheets {1, 2, 3} were treated with solution containing Fmoc-Ala-OH (3.29 g, 10 mmol), DMT/NMM/TsO (4.13 g, 10

mmol) and NMM (2.5 mL, 23 mmol) and labeled as {1.1}, {2.1} and {3.1}.

#### Incorporation of Fmoc-Phe-OH

Six (3 × 2) modified cellulose sheets {1, 2, 3} were treated with solution containing Fmoc-Phe-OH (3.87 g, 10 mmol), DMT/NMM/TsO<sup>-</sup> (4.13 g, 10 mmol) and NMM (2.5 mL, 23 mmol) and labeled as {1.2}, {2.2} and {3.2}.

#### Incorporation of Fmoc-Leu-OH

Six (3 × 2) modified cellulose sheets {1, 2, 3} were treated with solution containing Fmoc-Leu-OH (3.53 g, 10 mmol), DMT/NMM/TsO<sup>-</sup> (4.13 g, 10 mmol) and NMM (2.5 mL, 23 mmol) and labeled as {1.3}, {2.3}, {3.3}.

#### Incorporation of Fmoc-Ile-OH

Six (3 × 2) modified cellulose sheets {1, 2, 3} were treated with solution containing Fmoc-Ile-OH (3.53 g, 10 mmol), DMT/NMM/TsO<sup>-</sup> (4.13 g, 10 mmol) and NMM (2.5 mL, 23 mmol) and labeled as {1.4}, {2.4}, {3.4}.

#### Incorporation of Fmoc-Val-OH

Six (3 × 2) modified cellulose sheets {1, 2, 3} were treated with solution containing Fmoc-Val-OH (3.39 g, 10 mmol), DMT/NMM/TsO<sup>-</sup> (4.13 g, 10 mmol) and NMM (2.5 mL, 23 mmol) and labeled as {1.5}, {2.5}, {3.5}.

#### Incorporation of Fmoc-Met-OH

Six (3 × 2) modified cellulose sheets {1, 2, 3} were treated with solution containing Fmoc-Met-OH (3.71 g, 10 mmol), DMT/NMM/TsO<sup>-</sup> (4.13 g, 10 mmol) and NMM (2.5 mL, 23 mmol) and labeled as {1.6}, {2.6}, {3.6}.

#### Incorporation of Fmoc-Cys(Trt)-OH

Six (3 × 2) modified cellulose sheets {1, 2, 3} were treated with solution containing Fmoc-Cys(Trt)-OH (5.86 g, 10 mmol), DMT/NMM/TsO<sup>-</sup> (4.13 g, 10 mmol) and NMM (2.5 mL, 23 mmol) and labeled as {1.7}, {2.7}, {3.7}.

#### Incorporation of Fmoc-Ser(tBu)-OH

Six (3 × 2) modified cellulose sheets {1, 2, 3} were treated with solution containing Fmoc-Ser(tBu)-OH (3.83 g, 10 mmol), DMT/NMM/TsO<sup>-</sup> (4.13 g, 10 mmol) and NMM (2.5 mL, 23 mmol) and labeled as {1.8}, {2.8}, {3.8}.

#### Incorporation of Fmoc-Tyr(tBu)-OH

Six (3 × 2) modified cellulose sheets {1, 2, 3} were treated with solution containing Fmoc-

Tyr(tBu)-OH (3.29 g, 10 mmol), DMT/NMM/TsO<sup>-</sup> (4.13 g, 10 mmol) and NMM (2.5 mL, 23 mmol) and labeled as {1.9}, {2.9}, {3.9}.

#### Incorporation of Fmoc-Thr(tBu)-OH

Six (3 × 2) modified cellulose sheets {1, 2, 3} were treated with solution containing Fmoc-Thr(tBu)-OH (3.37 g, 10 mmol), DMT/NMM/TsO<sup>-</sup> (4.13 g, 10 mmol) and NMM (2.5 mL, 23 mmol) and labeled as {1.10}, {2.10}, {3.10}.

#### Incorporation of Fmoc-Glu(OtBu)-OH

Six (3 × 2) modified cellulose sheets {1, 2, 3} were treated with solution containing Fmoc-Glu(OtBu)-OH (4.25 g, 10 mmol), DMT/NMM/TsO<sup>-</sup> (4.13 g, 10 mmol) and NMM (2.5 mL, 23 mmol) and labeled as {1.11}, {2.11}, {3.11}.

#### Incorporation of Fmoc-Asp(OtBu)-OH

Six (3 × 2) modified cellulose sheets {1, 2, 3} were treated with solution containing Fmoc-Asp(OtBu)-OH (4.11 g, 10 mmol), DMT/NMM/TsO<sup>-</sup> (4.13 g, 10 mmol) and NMM (2.5 mL, 23 mmol) and labeled as {1.12}, {2.12}, {3.12}.

#### Incorporation of Fmoc-Gln(Trt)-OH

Six (3 × 2) modified cellulose sheets {1, 2, 3} were treated with solution containing Fmoc-Gln(Trt)-OH (6.11 g, 10 mmol), DMT/NMM/TsO<sup>-</sup> (4.13 g, 10 mmol) and NMM (2.5 mL, 23 mmol) and labeled as {1.13}, {2.13}, {3.13}.

#### Incorporation of Fmoc-Asn(Trt)-OH

Six (3 × 2) modified cellulose sheets {1, 2, 3} were treated with solution containing Fmoc-Asn(Trt)-OH (5.97 g, 10 mmol), DMT/NMM/TsO<sup>-</sup> (4.13 g, 10 mmol) and NMM (2.5 mL, 23 mmol) and labeled as {1.14}, {2.14}, {3.14}.

#### Incorporation of Fmoc-His(Trt)-OH

Six (3 × 2) modified cellulose sheets {1, 2, 3} were treated with solution containing Fmoc-His(Trt)-OH (6.20 g, 10 mmol), DMT/NMM/TsO<sup>-</sup> (4.13 g, 10 mmol) and NMM (2.5 mL, 23 mmol) and labeled as {1.15}, {2.15}, {3.15}.

#### Incorporation of Fmoc-Lys(Boc)-OH

Six (3 × 2) modified cellulose sheets {1, 2, 3} were treated with solution containing Fmoc-Lys(Boc)-OH (4.69 g, 10 mmol), DMT/NMM/TsO<sup>-</sup> (4.13 g, 10 mmol) and NMM (2.5 mL, 23 mmol) and labeled as {1.16}, {2.16}, {3.16}.

**Incorporation of Fmoc-Pro-OH**

Six (3 × 2) modified cellulose sheets {**1**, **2**, **3**} were treated with solution containing Fmoc-Pro-OH (3.37 g, 10 mmol), DMT/NMM/TsO<sup>-</sup> (4.13 g, 10 mmol) and NMM (2.5 mL, 23 mmol) and labeled as {**1.17**}, {**2.17**}, {**3.17**}.

**Incorporation of Fmoc-Trp(Boc)-OH**

Six (3 × 2) modified cellulose sheets {**1**, **2**, **3**} were treated with solution containing Fmoc-Trp(Boc)-OH (5.67 g, 10 mmol), DMT/NMM/TsO<sup>-</sup> (4.13 g, 10 mmol) and NMM (2.5 mL, 23 mmol) and labeled as {**1.18**}, {**2.18**}, {**3.18**}.

**Incorporation of Fmoc-Arg(Pbf)-OH**

Six (3 × 2) modified cellulose sheets {**1**, **2**, **3**} were treated with solution containing Fmoc-Arg(Pbf)-OH (6.49 g, 10 mmol), DMT/NMM/TsO<sup>-</sup> (4.13 g, 10 mmol) and NMM (2.5 mL, 23 mmol) and labeled as {**1.19**}, {**2.19**}, {**3.19**}.

**Incorporation of Fmoc-Gly-OH**

Six (3 × 2) modified cellulose sheets {**1**, **2**, **3**} were treated with solution containing Fmoc-Gly-OH (2.97 g, 10 mmol), DMT/NMM/TsO<sup>-</sup> (4.13 g, 10 mmol) and NMM (2.5 mL, 23 mmol) and labeled as {**1.20**}, {**2.20**}, {**3.20**}.

**Deprotection of fluorenylmethoxycarbonyl (Fmoc) group from amino acids incorporated on the surface of functionalized cellulose sheets {1.1-3.20}**

Functionalized cellulose sheets {**1.1-3.20**} were treated with 25% solution of piperidine in DMF (200 mL) for 15 min. After removal of excess piperidine solution, the cellulose sheets {**1.1-3.20**} were washed with DMF (3 × 100 mL) then methylene chloride (1 × 100 mL).

**Incorporation of lipid fragment. Synthesis of triazine esters of fatty acids. General procedure**

To a vigorously stirred and cooled to 0°C solution of DMM/NMM/TsO<sup>-</sup> (6.20 g, 15 mmol) in methylene chloride (50 mL) *N*-methylmorpholine (NMM) (0.85 mL, 7.5 mmol) and fatty acid (15 mmol) were added. As a fatty acids were used 12-hydroxy-(*cis*)-9-octadecenoic acid (ricinoleic acid) (4.48 g, 15 mmol) or stearic acid (4.27 g, 15 mmol). The mixture was intensively stirred and cooled for additional 2 h. In the next step, the filtrate was concentrated to half volume by rotary evaporation. The residue was used for treating the H<sub>2</sub>N-dipeptide-NH-C<sub>6</sub>H<sub>4</sub>-NH-DMT-cellulose sheets. After 12 h of coupling with triazine ester of fatty acids, modified

cellulose sheets were washed successively with DMF (4 × 100 mL), CH<sub>2</sub>Cl<sub>2</sub> (1 × 100 mL), MeOH (1 × 100 mL) and CH<sub>2</sub>Cl<sub>2</sub> (1 × 100 mL).

**Aminolysis of triazine ester of 12-hydroxy-(*cis*)-9-octadecenoic acid (ricinoleic acid)**

Modified cellulose sheets labeled {**1.1-3.20**} were treated with solution of triazine ester of 12-hydroxy-(*cis*)-9-octadecenoic acid (ricinoleic acid). Final functionalized cellulose supports were labeled as {**1.1.A**} – {**1.20.A**}, {**2.1.A**} – {**2.20.A**} and {**3.1.A**} – {**3.20.A**}.

**Aminolysis of triazine ester of stearic acid**

Modified cellulose sheets labeled {**1.1-3.20**} were treated with solution of triazine ester of stearic acid. Final functionalized cellulose supports were labeled as {**1.1.B**} – {**1.20.B**}, {**2.1.B**} – {**2.20.B**} and {**3.1.B**} – {**3.20.B**}.

**Deprotection of protecting groups in the side chains of dipeptides immobilized on cellulose supports**

All modified cellulose sheets (120 sheets) were treated with mixture consisting of 50% (v/v) trifluoroacetic acid in methylene chloride (250 mL) with 3% (v/v) water and 2% (v/v) triisopropylsilane for 3 h. In the next step, modified cellulose sheets were washed with methylene chloride (2 × 200 mL), ethanol (2 × 200 mL) and individually dried in a vacuum desiccator.

Ten (9 + 1) sets of 6 mm diameter discs were cut from every one labeled plate and labeled with graphite pencil as plate label. Then, the discs were buffered with phosphate buffer pH 7 for 25 min. After removing solution of buffer, sets of discs were washed twice with water and with mixture MeOH : H<sub>2</sub>O (1 : 1, v:v) for 15 min. Finally, the discs were dried to constant weight in the vacuum desiccator.

**Selection of conditions for binding of urine to binding pockets of library *N*-lipidated peptides immobilized on cellulose****Method A**

Set of discs was treated with solution of urine diluted with (1 : 1). After 15 min, the excess of the solution was removed and discs were washed three times with 0.9% NaCl solution. The library was dried in vacuum desiccator and then treated with solution of the reporter dye and scanned to investigate further mathematical analysis.

**Method B**

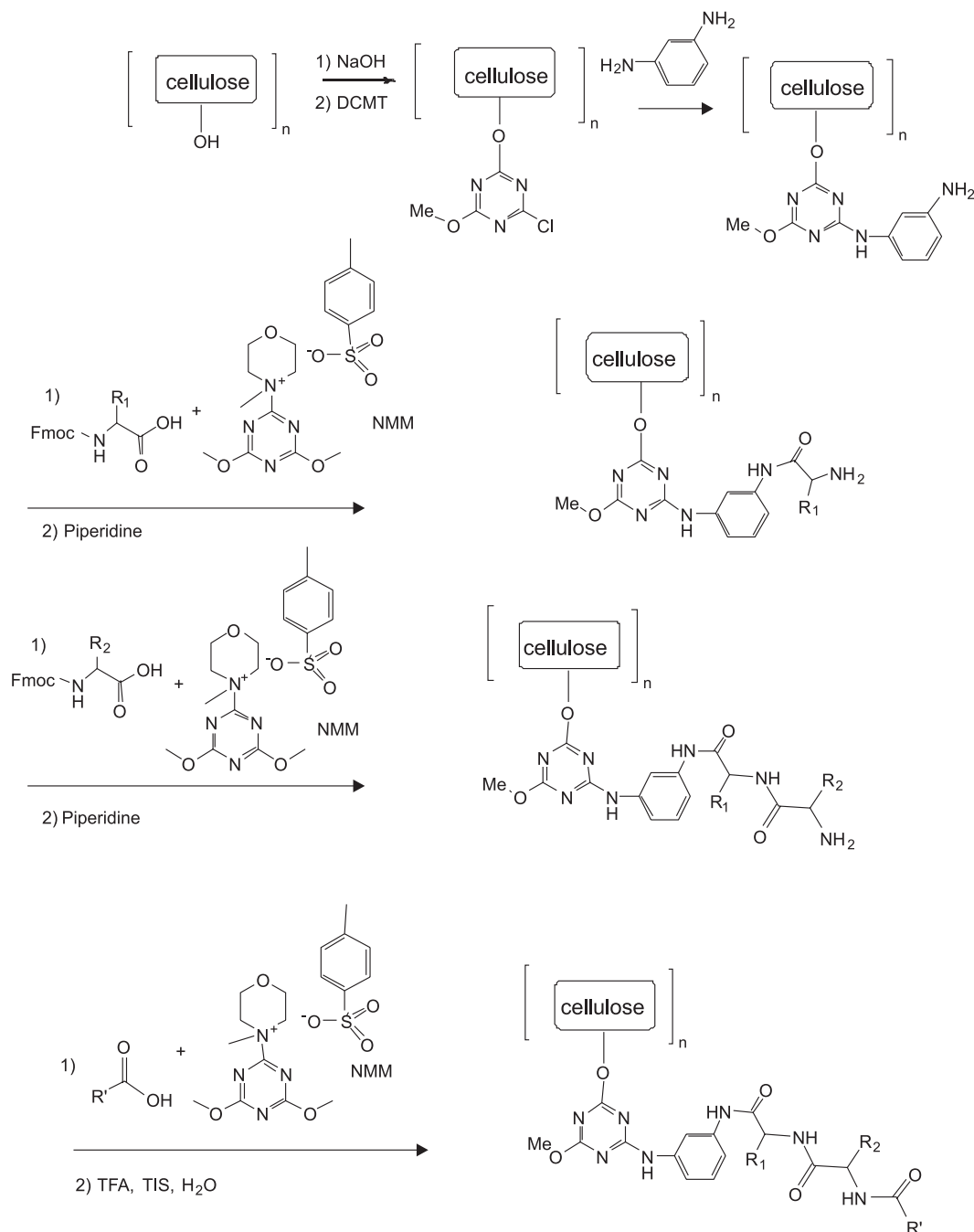
Set of discs was treated with urine solution under identical conditions as in the case of method

A. However, after docking the urine components, cellulose discs were drained to constant weight in vacuum desiccator and stored in the refrigerator for 3 days. Then, discs were washed three times with 0.9% NaCl solution to remove the excess of the analyte. The library was dried in vacuum desiccator and then treated with solution of the reporter dye and

scanned to investigate further mathematical analysis.

#### Method C

Set of discs was treated with urine solution under identical conditions as in the case of method A, but after docking the urine components, wet



Scheme 1. The synthesis of library of *N*-lipidated peptides attached on the cellulose

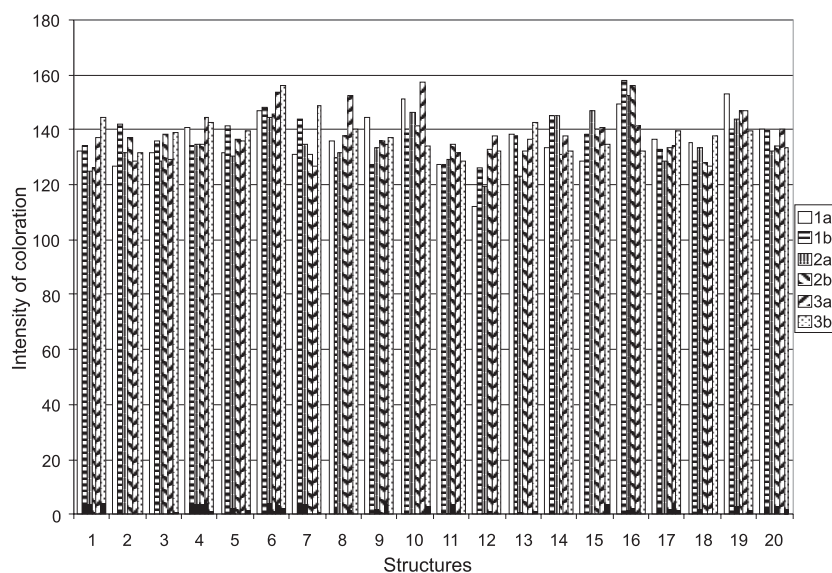


Figure 1. The binding profile of Brilliant Black by library of *N*-lipidated peptides {**1.1A** – **3.20B**}. The numbers 1–20 code the second residue of amino acids of *N*-lipidated peptides. Numbers (1, 2, 3) code the first residue of amino acids of *N*-lipidated peptides and letters (a, b) code *N*-acyl residue. For every group of bars, signs refers to the same group of sub-structures

(without drying) cellulose discs were stored in the refrigerator for 3 days. In the next step, the discs were washed three times with 0.9% NaCl solution to remove the excess of the analyte. Then, the discs were washed three times with 0.9% NaCl solution to remove the excess of the analyte. The library was dried in vacuum desiccator and then treated with solution of the reporter dye and scanned to investigate further mathematical analysis.

## RESULTS

The library of *N*-lipidated peptides was synthesized in a stepwise procedure involving immobilization of a 2,4-dichloro-6-methoxy-1,3,5-triazine (DCMT) scaffold on cellulose plate (13) followed by reaction with appropriate *meta*-phenylenediamine. Peptide fragment was attached under typical solid phase peptide synthesis (SPPS) using a quaternary *N*-triazinylammonium salt as coupling reagent (14) according to Fmoc/tBu strategy, after removing Fmoc group from immobilized dipeptides. Appropriate carboxylic acids, previously activated by means of a triazine coupling reagent (see Scheme 1), were attached. In order to obtain the same properties of every one element of library on the one cellulose plate it was prepared only one structure. Then, the library was cloned by cutting discs 6 mm in diameter an individually labeling every disc with the label of the respective library plate.

It has been expected that library of artificial receptors is able to bind ligands from mouse urine with efficiency comparable with those observed previously in the case of colored (15) or colorless (16) ligands. For monitoring the competitive adsorption-desorption mechanism of binding colorless ligands, a Brilliant Black as a reporter dyeing agent was used. Brilliant Black was selected as reporter dye, due to its high and very uniform affinity to binding (17) all elements of the library of *N*-lipidated peptides (see Fig. 1).

We expected that libraries of *N*-lipidated peptides immobilized on cellulose allow selective binding of metabolites from urine and will allow tracking changes in urine metabolites in the demand of health.

In view of the instability of urine, low concentrations of metabolites and the presence of structurally diversified mixture of chemical entities, in this preliminary study the peptide fragments of the binding pockets of the library components were prepared from all 20 coded amino acids. In spite of instability of urine, preliminary testing was performed to find optimal experimental condition of binding process.

In these studies, three different binding conditions of urine components by the library of *N*-lipidated peptides were tested (see Fig. 2). Selection of the conditions was aimed to check the influence of time and storage conditions of set discs. Under the

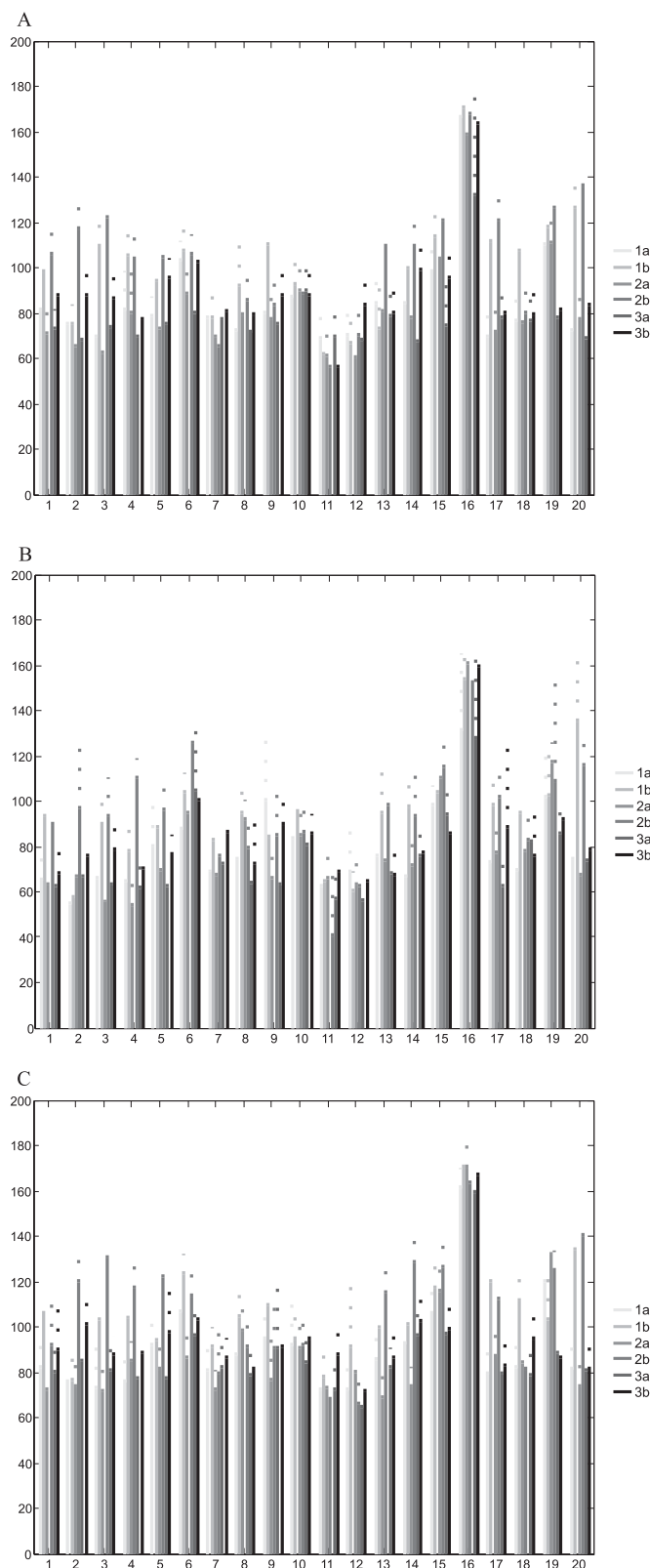


Figure 2. Intensity of coloration with standard deviations obtained for the library *N*-lipidated peptides treated urine by methods A–C. Arithmetic means (solid line) with standard deviations (dashed line). All experiments binding urine metabolites by the library of *N*-lipidated peptides were repeated five times. The numbers 1–20 code the second residue of amino acids of *N*-lipidated peptides. Numbers (1, 2, 3), in figure legend, code first residue of amino acids of *N*-lipidated peptides and letters (a, b) code *N*-acyl residue

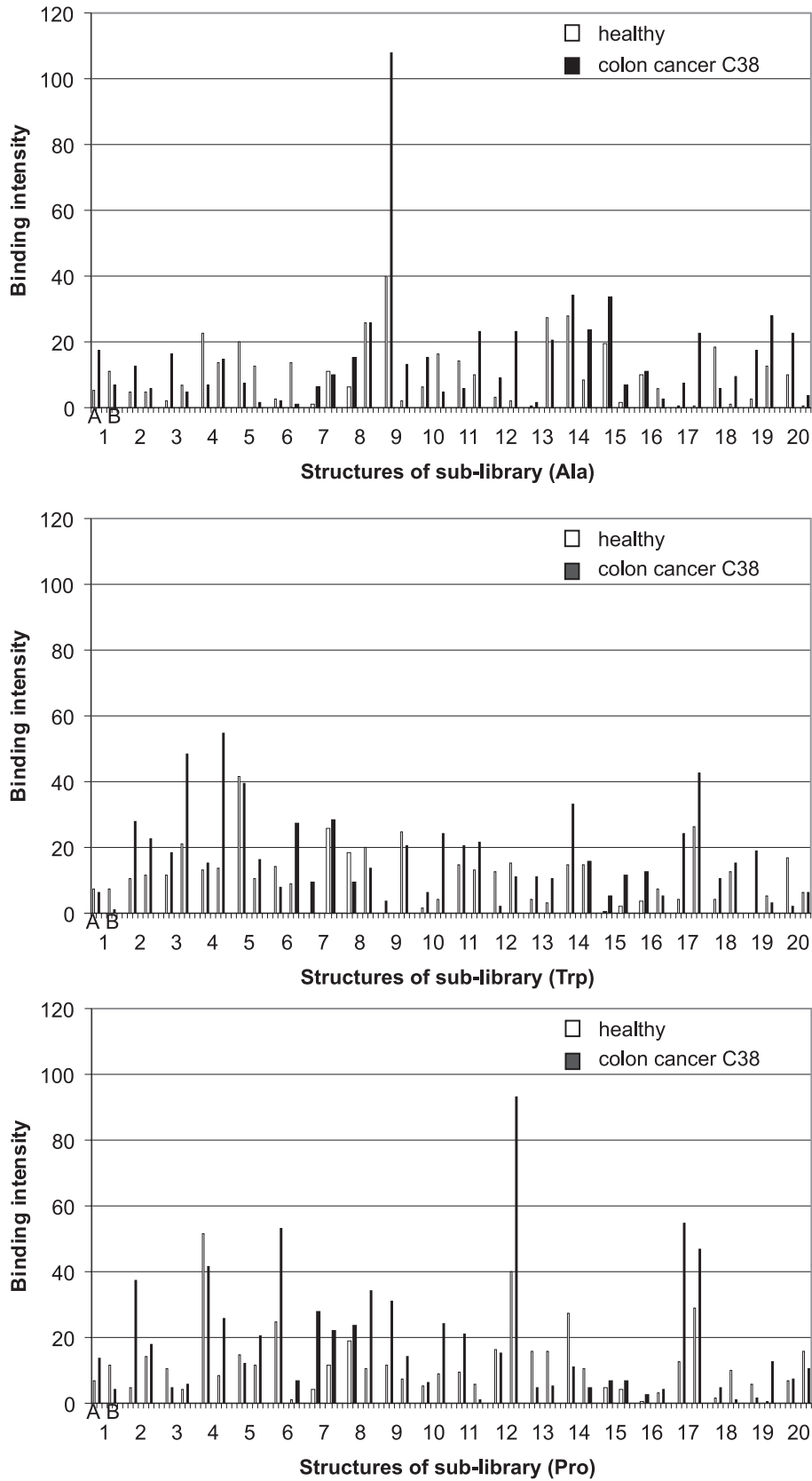


Figure 3. The binding profile of C57B1/6 mouse urine components for sub-libraries of *N*-lipidated peptides



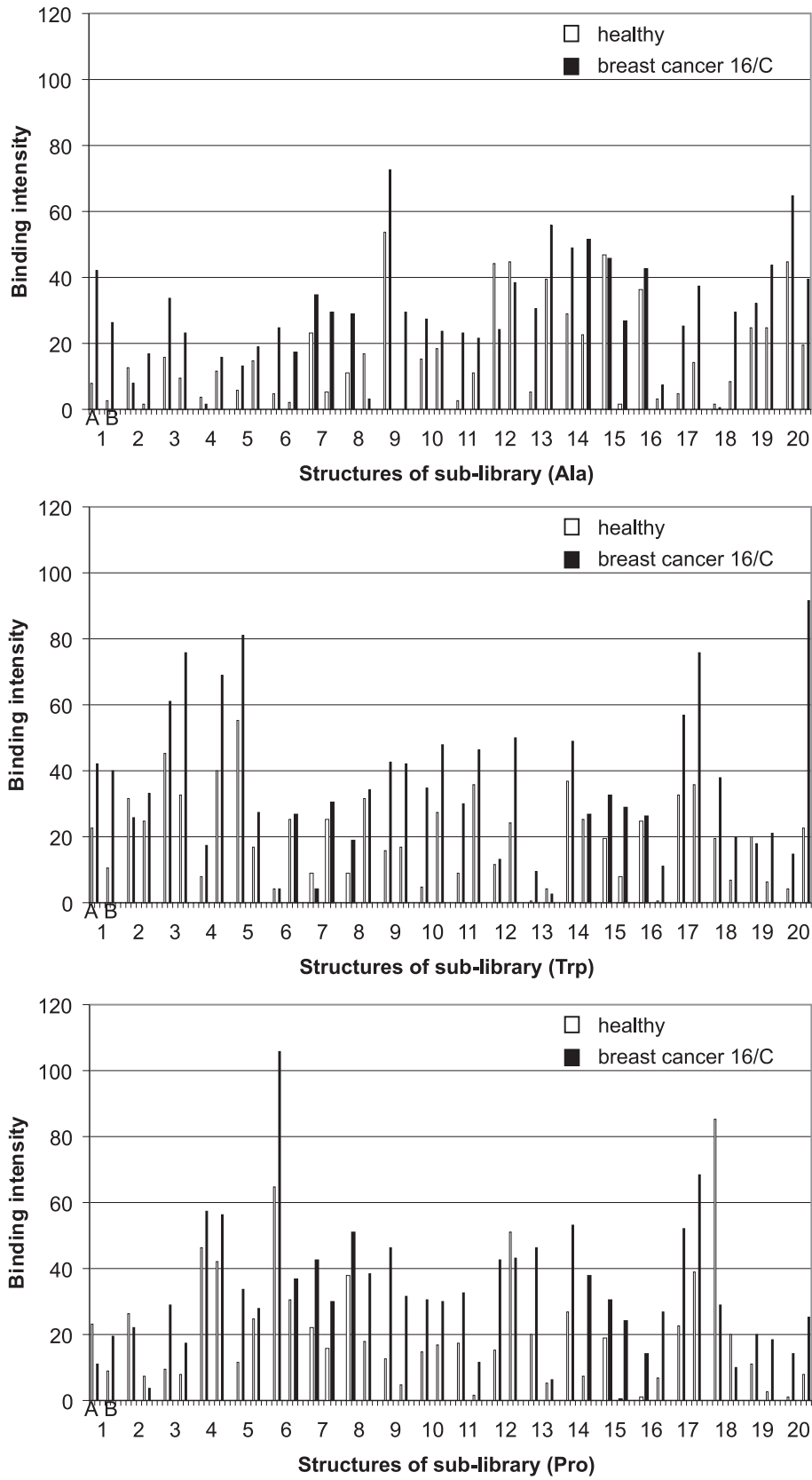


Figure 4. The binding profile of C3H mouse urine components for sub-libraries of *N*-lipidated peptides

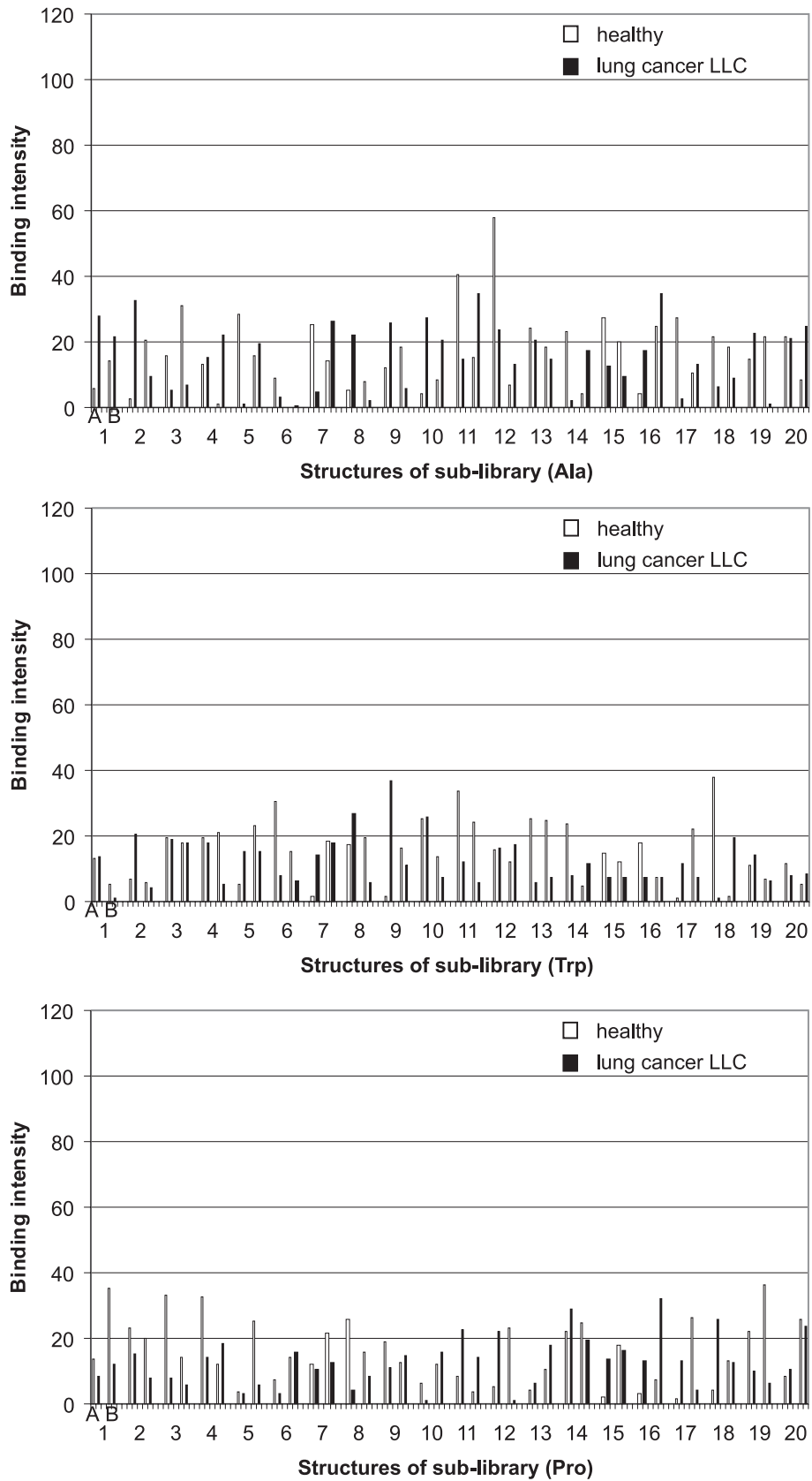


Figure 5. The binding profile of BDF1 mouse urine components for sub-libraries of *N*-lipidated peptides

Table 1. Elements of *N*-lipidated peptides library differentiating the urine of healthy mice and C57B1/6 mice with implanted colon cancer C38.

{1} (sub-Ala)	{2} (sub-Trp)	{3} (sub-Pro)
<b>1.3A</b> – ~AlaLeu-ricinoyl	<b>2.4B</b> – ~TrpIle-stearoyl	<b>3.2A</b> – ~ProPhe-ricinoyl
<b>1.4A</b> – ~AlaIle-ricinoyl	<b>2.6B</b> – ~TrpMet-stearoyl	<b>3.4B</b> – ~ProIle-stearoyl
<b>1.6B</b> – ~AlaMet-stearoyl	<b>2.10B</b> – ~TrpThr-stearoyl	<b>3.7A</b> – ~ProCys-ricinoyl
<b>1.9A</b> – ~AlaTyr-ricinoyl	<b>2.17A</b> – ~TrpPro-ricinoyl	<b>3.8B</b> – ~ProSer-stearoyl
<b>1.12B</b> – ~AlaAsp-stearoyl	<b>2.20A</b> – ~TrpGly-ricinoyl	<b>3.9A</b> – ~ProTyr-ricinoyl
<b>1.17B</b> – ~AlaPro-stearoyl		<b>3.12B</b> – ~ProAsp-stearoyl
		<b>3.19B</b> – ~ProArg-stearoyl

Table 2. Structures of elements of *N*-lipidated peptides library differentiating the urine of healthy mice and C3H mice with implanted breast cancer 16/C.

{1} (sub-Ala)	{2} (sub-Trp)	{3} (sub-Pro)
<b>1.1A</b> – ~AlaAla-ricinoyl	<b>2.1A</b> – ~TrpAla-ricinoyl	<b>3.3A</b> – ~ProLeu-ricinoyl
<b>1.1B</b> – ~AlaAla-stearoyl	<b>2.1B</b> – ~TrpAla-stearoyl	<b>3.9A</b> – ~ProTyr-ricinoyl
<b>1.2B</b> – ~AlaPhe-stearoyl	<b>2.3B</b> – ~TrpLeu-stearoyl	<b>3.9B</b> – ~ProTyr-stearoyl
<b>1.6A</b> – ~AlaMet-ricinoyl	<b>2.9A</b> – ~TrpTyr-ricinoyl	<b>3.14B</b> – ~ProAsn-stearoyl
<b>1.6B</b> – ~AlaMet-stearoyl	<b>2.9B</b> – ~TrpTyr-stearoyl	<b>3.18A</b> – ~ProTrp-ricinoyl
<b>1.7B</b> – ~AlaCys-stearoyl	<b>2.10A</b> – ~TrpThr-ricinoyl	<b>3.19B</b> – ~ProArg-stearoyl
<b>1.11A</b> – ~AlaGlu-ricinoyl	<b>2.15B</b> – ~TrpHis-stearoyl	<b>3.20A</b> – ~ProGly-ricinoyl
<b>1.13B</b> – ~AlaGln-stearoyl	<b>2.19B</b> – ~TrpArg-stearoyl	
<b>1.15B</b> – ~AlaHis-stearoyl	<b>2.20B</b> – ~TrpGly-stearoyl	
<b>1.17A</b> – ~AlaPro-ricinoyl		
<b>1.18B</b> – ~AlaTrp-stearoyl		

Table 3. Structures of elements of *N*-lipidated peptides library differentiating the urine of healthy mice and BDF1 mice with implanted lung cancer LLC.

{1} (sub-Ala)	{2} (sub-Trp)	{3} (sub-Pro)
<b>1.1A</b> – ~AlaAla-ricinoyl	<b>2.4B</b> – ~TrpIle-stearoyl	<b>3.1B</b> – ~ProAla-stearoyl
<b>1.2A</b> – ~AlaPhe-ricinoyl	<b>2.6A</b> – ~TrpMet-stearoyl	<b>3.3A</b> – ~ProLeu-ricinoyl
<b>1.3B</b> – ~AlaLeu-stearoyl	<b>2.7A</b> – ~TrpCys-ricinoyl	<b>3.4A</b> – ~ProIle-ricinoyl
<b>1.4B</b> – ~AlaIle-stearoyl	<b>2.9A</b> – ~TrpTyr-ricinoyl	<b>3.5B</b> – ~ProVal-stearoyl
<b>1.5A</b> – ~AlaVal-ricinoyl	<b>2.11B</b> – ~TrpGlu-stearoyl	<b>3.8A</b> – ~ProSer-ricinoyl
<b>1.7A</b> – ~AlaCys-ricinoyl	<b>2.18A</b> – ~TrpTrp-ricinoyl	<b>3.12B</b> – ~ProAsp-stearoyl
<b>1.9B</b> – ~AlaTyr-stearoyl	<b>2.18B</b> – ~TrpTrp-stearoyl	<b>3.16B</b> – ~ProLys-stearoyl
<b>1.10A</b> – ~AlaThr-ricinoyl		<b>3.17B</b> – ~ProPro-stearoyl
<b>1.14A</b> – ~AlaAsn-ricinoyl		<b>3.18A</b> – ~ProTrp-ricinoyl
<b>1.17A</b> – ~AlaPro-ricinoyl		<b>3.19A</b> – ~ProArg-ricinoyl
<b>1.18A</b> – ~AlaTrp-ricinoyl		

conditions of method A, set of discs after binding urine metabolites into the pockets was directly used to reporter dye binding. Under the conditions of method B, set of disks after binding urine metabolites was dried to constant weight and then stored in refrigerator for 3 days. In method C, set of discs after treatment with urine, without drying to a constant weight, was stored in the refrigerator for 3 days.

A comparison of graphs showed that the assay method and particularly conditions of storage of discs sets affects the binding profile. The highest

spreads in the obtained results were observed in the case of method C, which indicates that the storage of wet set disks is undesirable. It turned out also that even in the case of storage of dry disks sets in the refrigerator (method B), it was observed scattering of results. These observations were taken into account during further experiments using murine urine. Thus, in all further tests method A was applied.

In experiments evaluating the utility of artificial receptors libraries for noninvasive medical diagnostics, urine derived from healthy mice and

mice with different types of cancer was used. In this studies, three different types of cancers were tested. The urine was collected from mice, when tumor volume reached  $\sim 700 \text{ mm}^3$ . For each 120-elements molecular receptors library were obtained different binding profile (fingerprint) of mouse urine metabolites.

In the first test, a binding profile of urine from C57BL/6 healthy mice was compared with profile obtained using urine from C57BL/6 mouse suffering from colon cancer C38 (see Fig. 3).

In the next set of experiments, a binding profile obtained by treatment the library with urine of C3H healthy mouse was compared respectively with binding profile obtained with urine from C3H mouse bearing mouse mammary gland cancer 16/C (see Fig. 4).

The last studies involved binding metabolites from BDF1 (C57BL/6xDBA2) healthy mouse urine and urine from BDF1 mouse bearing lung cancer LLC (see Fig. 5).

Based on the graphs (Figs. 3–5), several characteristic changes in binding profile can be identified.

## DISCUSSION AND CONCLUSIONS

A comparison of the binding profiles of healthy mice C57BL/6, C3H and BDF1 urine components (Figs. 3–5) shown that even in the case of standardized laboratory animals the results are varied. This is due to the fact that the composition of urine varies and depends on many factors including the species, diet and many others.

Comparative analysis of the binding profiles of urinary metabolites of healthy mice and mice with cancer enabled the selection of elements of the *N*-lipidated peptides library, where intensity of binding were mostly diversified, reflecting differentiation dependent upon the health.

Among the 120-element library of *N*-lipidated peptides immobilized on cellulose, there were found 18 structures differentiating the urine of healthy mice and C57BL/6 mice implanted with colon cancer C38 (see Table 1).

Analysis of three sub-libraries {1.1–20.A-B}, {2.1–20.A-B} and {3.1–20.A-B} (splitting up due to the structure of the amino acid connected with *m*-phenylenediamine residue) shown the strongest difference in coloration of field (4 Ile residue attached to the first amino acid) is common to all sub-libraries. It should be emphasized that in this case the structure of the attached lipid does not affect the ability to discriminate between binding the urine of

healthy mice and C57BL/6 mice bearing colon cancer C38. From the analysis emerges another dependency, namely, the impact of the “size” of the first amino acid residue.

In the case of sub-libraries comprising an alanine residue (small amino acid) in the first position and sub-libraries containing a proline residue (relatively small amino acid) were selected two additional common elements, namely, the field {1.9} and {3.9} (with Tyr residue), and {1.12} and {3.12} (with Asp residue). In the case of the last two library members, full compliance is observed in the structure of the lipid.

Analysis of the ability of binding of urinary metabolites from healthy C3H mouse and C3H mice bearing mammary gland cancer 16/C showed no presence of shared structures (see Table 2).

However, has been found the common correlation between the sub-libraries. For sub-library {1} with an alanine residue in the first position and the sub-library {2} containing a tryptophan residue it has been revealed the presence of up to three common structures: with Ala residue in the second position and with both lipid residues (A and B) and with His residue in the second position with the lipid B. In the case of sub-libraries {2} with tryptophan residue and sub-library {3} (proline in the first position) it has been found four common structures: {2.3} and {3.3} (with Leu residue), {2.9} and {3.9} (with Tyr residue), {2.19} and {3.19} (with Arg residue) and {2.20} and {3.20} (with Gly residue) (see Table 2). In the last case it has not been observed an influence of the lipid structure on binding selectivity.

Analysis of binding profile of urine BDF1 mice and BDF1 mice with implanted LLC lung carcinoma discovered the presence of two common elements for all three sub-libraries, e.g., with Ile residue and with Trp residue in the second position (see Table 3).

Furthermore, it has been found an additional correlation between the elements of two sub-libraries: {1} (sub-Ala) and {2} (sub-Trp). It has been selected an additional three common elements: with Cys, Tyr and Trp residue at the second position.

The analysis selected two structures: Lipid-Pro-Ala-NH-C<sub>6</sub>H<sub>4</sub>-NH-DMT-cellulose and Lipid-Arg-Pro-NH-C<sub>6</sub>H<sub>4</sub>-NH-DMT-cellulose diagnostic for all three tested types of cancers. In this case, the influence of the lipid on selectivity of receptors was found to be negligible compared to the peptide structure.

### Acknowledgment

This work was supported by Ministry of Science and Education Poland N N 405 669540.

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