Biomarker Discovery by Imaging Mass Spectrometry

TRANSTHYRETIN IS A BIOMARKER FOR GENTAMICIN-INDUCED NEPHROTOXICITY IN RAT*

Hélène Meistermann‡, Jeremy L. Norris§, Hans-Rudolf Aerni§, Dale S. Cornett§, Arno Friedlein‡, Annette R. Erskine§, Angélique Augustin‡, Maria Cristina De Vera Mudry‡, Stefan Ruepp‡, Laura Suter‡, Hanno Langen‡, Richard M. Caprioli§, and Axel Ducret‡¶

Adverse drug effects are often associated with pathological changes in tissue. An accurate depiction of the undesired affected area, possibly supported by mechanistic data, is important to classify the effects with regard to relevance for human patients. MALDI imaging MS represents a new analytical tool to directly provide the spatial distribution and the relative abundance of proteins in tissue. Here we evaluate this technique to investigate potential toxicity biomarkers in kidneys of rats that were administered gentamicin, a well known nephrotoxicant. Differential analysis of the mass spectrum profiles revealed a spectral feature at 12,959 Da that strongly correlates with histopathology alterations of the kidney. We unambiguously identified this spectral feature as transthyretin (Ser28–Gln146) using an innovative combination of tissue microextraction and fractionation by reverse-phase liquid chromatography followed by a top-down tandem mass spectrometric approach. Our findings clearly demonstrate the emerging role of imaging MS in the discovery of toxicity biomarkers and in obtaining mechanistic insights concerning toxicity mechanisms. Molecular & Cellular Proteomics 5:1876–1886, 2006.

Proteomic strategies have been exploited to generate differential protein expression that can be correlated with environmental disease or toxicant exposure. In particular, improvements in two-dimensional gel electrophoresis and liquid chromatography coupled to mass spectrometry and the use of other emerging techniques have demonstrated the usefulness of a proteomic approach in toxicology (1–3). The early detection of disease markers and the rapid screening of experimental compounds either for toxicity or protective efficacy are of particular interest in this field (4).

Imaging mass spectrometry (IMS)1 is a technique for the direct analysis of peptides and proteins from thin tissue sections using conventional MALDI-TOF mass spectrometers while preserving the abundance and spatial distribution of each analyte (5). The usefulness of this technique has been demonstrated in experimental models and in clinical settings (6, 7) as in the accurate and sensitive classification of non-small cell lung cancer in tumor biopsies (8).

In this study we further demonstrated the potential applications of an IMS strategy in the study of a well known nephrotoxicant, the aminoglycoside antibiotic gentamicin (9), in the rat kidney. Aminoglycoside antibiotics are widely used, but nephrotoxicity (10) is a clear risk and occurs in 10–20% (11) of treated patients. Gentamicin-induced nephrotoxicity is seldom fatal and is usually reversible but often results in long hospital stays. Thus, there is a great interest in finding potential markers for the toxicity event and to further elucidate the toxicity mechanism.

We first established our IMS strategy by determining the differential protein expression within the main areas of the rat kidney (cortex, medulla, and papilla) to ensure that we could accurately differentiate the main substructures of this organ. We then investigated whether the kidney lesions secondary to gentamicin treatment could be visualized using IMS. We studied kidney sections from control and drug-treated rats using both a profiling strategy (a few localized measurements per sample, several replicates per animals) and an imaging strategy (systematic, regiospecific measurement on the whole kidney section, one replicate per animal) to evaluate the best approach to depict the histopathology. As we were able to pinpoint several potential markers for gentamicin kidney toxicity, we set up a protein identification strategy combining a protein liquid microextraction step followed by fractionation by reverse-phase (RP) HPLC and sequencing by top-down tandem mass spectrometry. In this study we demonstrated that the fragment (Ser28–Gln146) of transthyretin (pre-albumin), a 13-kDa transporter plasma protein, accumulates in large amounts in the cortex of the kidney following gentamicin

From the ‡Pharmaceuticals Division, F. Hoffmann-La Roche Ltd., Grenzacherstrasse 124, CH-4070 Basel, Switzerland and the §Mass Spectrometry Research Center, Vanderbilt University, Nashville, Tennessee 37232
Received, December 7, 2005, and in revised form, May 11, 2006
Published, MCP Papers in Press, May 16, 2006, DOI 10.1074/mcp.M500399-MCP200

1 The abbreviations used are: IMS, imaging mass spectrometry; RP, reverse-phase; S/N, signal-to-noise ratio.
treatment. We propose therefore that transthyretin might have value as a marker for nephrotoxicity in the monitoring of gentamicin administration.

**EXPERIMENTAL PROCEDURES**

**Animal Treatment**—Permission for animal studies was obtained from the Swiss regulatory agencies, and all study protocols were in compliance with animal welfare guidelines. Male Wistar rats of 12 weeks of age (300 g ± 20%) were obtained from Inkotri. Animals were treated for 7 consecutive days with 100 mg/kg/day gentamicin (Sigma; dissolved in saline) or vehicle by subcutaneous injections and sacrificed by CO2 inhalation 24 h after the last application.

**Sample Preparation**—Kidneys were dissected out, snap frozen in liquid nitrogen, and stored at −80 °C prior to further processing. Sections of 12-μm thickness were obtained on a cryostat (LEICA CM 3000, Leica Microsystems) at −18 °C and deposited on indium tin oxide-coated conductive glass slides (Delta Technologies). The sample preparation was performed according to a published procedure (12). Tissue sections were fixed by immersion in ethanol baths and allowed to dry for 30 min under vacuum. Matrix deposition was performed either manually with a pipette or automatically with a spotter. Manually 2 × 200 nl of freshly prepared 20 mg/ml sinapinic acid (Fluka) dissolved in 50% acetonitrile, 0.1% trifluoroacetic acid were sequentially deposited onto the tissue section. For automatic spotting, ground sinapinic acid (0.5–3-μm particles) was obtained by grinding with a ceramic mortar. Seeding of the tissue was achieved by manual deposition of ground matrix material onto the tissue, and excess crystals were removed with a blow of dry air. A rectangular array of matrix droplets was deposited using a prototype Acoustic Reagent Multispotter (ARM) (13). Matrix spots of 200–220-μm diameter were printed on demand with a 10-Hz drop ejection rate and an array pitch of 250 μm. The matrix solution was 25 mg/ml sinapinic acid prepared in 50% acetonitrile, 0.2% trifluoroacetic acid. Two print iterations with 13 droplets each were necessary to obtain dense crystal spots on the tissue.

**Mass Spectrometry**—MALDI MS spectra of the manually spotted tissue sections were acquired on an UltraFlex MALDI-TOF/TOF instrument (Bruker Daltonics) with a standard 337-nm N2 laser operating at 50 Hz and a laser spot size of 50 μm. The instrument was operated in positive linear mode (2–30 kDa) at constant laser power. A total of 8 × 100 laser shots were accumulated from each deposited matrix droplet. MALDI MS spectra of the robotic spotted tissue sections were obtained on an Applied Biosystems DE STR instrument in linear positive mode. The instrument was operated in delayed extraction mode with a 337-nm N2 laser operating at 20 Hz. A total of 200 spectra were summed from each matrix spot by rastering the spot with the laser. The data were base line-subtracted and rendered into an ordered array of spectra (image) using a modified Analyze 7.5 (Mayo Clinic) format. The Biomap software package (Novartis) was used for image visualization and to extract selected ion images from the data.

**Data Analysis**—Base-line subtraction, normalization, peak detection, and spectral alignment were performed using the ProTS-Data Software package (Efeckta Technologies, Inc.). A list of 13 common peaks was selected to be internal alignment points, and these common peaks were used to align the spectra according to m/z using a quadratic calibration function. The criteria used to select common peaks were that the peak must occur in greater than 80% of the spectra to be aligned, the peak must not have interfering or overlapping peaks, and the peaks must have a standard deviation of the observed centroid values less than 7 mass units.

The peak lists were binned according to their m/z values using an in-house program developed at Vanderbilt University. The exported MALDI-TOF MS peaks were aligned across samples by use of a genetic algorithm parallel search strategy (8). Briefly peaks were binned together such that the number of peaks in a bin from different samples is maximized while the number of peaks in a bin from the same sample is minimized. A series of mass windows or peak bins were generated that separated similar peaks across multiple spectra. The spectral features were ranked according to the extent of the observed difference to determine relevant biomarkers for gentamicin-induced kidney toxicity using a combination of three different criteria: weight value (14, 15), average signal-to-noise ratio (S/N), and t test.

**Protein Identification**—Protein liquid microextraction was achieved by directly pipetting up and down for 5 s ± 1 μl of extraction solvent (50% acetonitrile, 0.1% trifluoroacetic acid) on the region of interest of the tissue section. After collection of −20 μl (the pool of 40 microextractions over three sections), the sample was dried and resolubilized in water/acetonitrile (95:5), 0.1% trifluoroacetic acid. The peptide mixture was fractionated onto a 1-mm inner diameter polymeric column (catalog number 219TP5110, Vydac) using a linear acetonitrile gradient delivered at 50 μl/min. Eluting fractions were directly collected into a 96-well microtiter plate at a rate of 30 s/fraction. The elution position of the peaks of interest was assessed by spotting 1 μl of each fraction with 1 μl of 1.5 g/liter sinapinic acid solution in water/acetonitrile (TFA (90:100:0.1) onto a 384 AnchorChip MALDI target (Bruker Daltonics). The mass profiles were recorded by MALDI MS using the same acquisition parameters as for tissue imaging. For sequencing, the HPLC fractions of interest were pooled, dried in a SpeedVac, and resolubilized in water/acetonitrile (1:1) containing 1% formic acid. About 1 ml of the resolubilized fraction was transferred directly into a nanoelectrospray capillary needle. Mass spectra were acquired on a QSTAR Pulsar i quadrupole TOF tandem mass spectrometer (Applied Biosystems/MDS-Sciex) equipped with a nanoelectrospray ion source (Proxeon). The proteins of interest were identified by tandem mass spectrometry using a scan-down approach (16, 17). One or several multiply charged parent masses were selected to be fragmented for sequence analysis in the QSTAR instrument. Raw spectra analysis and peak list generation were performed using the instrument-provided Analyst/Bioanalyst QS software, Version 1.4 (Applied Biosystems/MDS-Sciex). After manual inspection of the tandem mass spectrum for determination of a sequence tag, protein identification was performed with the Mascot sequence query search program (Matrix Science, Mascot Version 2.1.03) using the Swiss-Prot database (release 46.6) filtered for the taxonomy “Rattus” (5,327 sequences) and a tolerance of 1.0 Da for fragment and precursor masses. MS/MS score evaluation was performed as follows. We routinely performed a first database search using the singly charged fragment ions manually determined to form a sequence tag, and we considered all protein sequences for which a MOWSE score higher than 33 (indicating significant homology as displayed by the Mascot search engine) could be obtained. The hit had to be further confirmed by taking into account all observed sequence ions in the spectrum, in particular the multiply charged fragment ions typically present around the parent mass. Only protein sequences with a MOWSE score higher than 46 (indicating extensive homology or identity, expectancy p < 0.05) obtained through a second database search including all pieces of evidence were considered in this work. Methionine oxidation (as variable modification) and N-terminal acetylation (as fixed modification) were only considered if there was no unmodified protein candidate satisfying the conditions described above.

**Western Blot**—Cytosolic fractions from whole kidney tissue extract were obtained by homogenization of a piece of control and treated kidney cortex followed by successive centrifugation steps at 680 × g, 10,000 × g, and 100,000 × g. For Western blot analysis, 8 μg of a cortex homogenate or an HPLC fraction of interest (dried in a SpeedVac and resuspended in SDS buffer) were applied onto a 4–20%
Tris-glycine SDS-polyacrylamide gel electrophoresis system (Invitrogen). Gels were subsequently transferred onto a polyvinylidene fluoride membrane (Polyscreen, PerkinElmer Life Sciences). Blots were blocked in 5% milk in PBS containing 0.1% Tween 20 for 1 h and incubated overnight at room temperature with anti-sheep transthyretin (Abcam, catalog number ab9015) diluted 1:5,000. Detection was performed by enhanced chemiluminescence (Amersham Biosciences) after a 1-h incubation with horseradish peroxidase-conjugated anti-sheep IgG (Silenus).

**Immunohistochemistry**—Immunohistochemistry was performed using a standard peroxidase-based staining method. Paraffin-embedded tissue sections (6 μm) were deparaffinized with xylene, and...
endogenous peroxidase activity was quenched with 3% H₂O₂ in methanol for 1 h. Blocking was performed with an avidin-biotin blocking kit (Vector Laboratories, catalog number SP-2001) and with 2.5% horse serum albumin (from Vectastain kit, see below) for 1 h in each solution. Successive incubations with the primary transthyretin antibody (Abcam, catalog number ab9015, at a dilution of 1:1,000), the secondary antibody, streptavidin, and the chromogen (3,3′-diaminobenzidine-nickel, Peroxidase Substrate DAB Kit SK-4100, Vector Laboratories) were performed according to the kit manufacturer’s instructions (Vector Laboratories, RTU Vectastain Universal Quick Kit PK-7800). Contrasts were increased by counterstaining the sections with cresyl violet.

RESULTS

Differential Protein Expression in the Kidney Cortex, Medulla, and Papilla—The potential of an IMS strategy to detect toxicity-related differences in protein expression was explored by exploiting the structural differences of the kidney and by analyzing peptides and proteins specific to each of those regions. The cortex, medulla, and papilla of kidneys from control rats were analyzed by two different analytical strategies (Fig. 1): a profiling strategy in which spots of matrix were manually deposited on selected areas of the tissue sections and an imaging strategy in which whole sections were arrayed with a robotic matrix spotter.

Spectra generated from manual spotting clearly differentiated the cortex from the papilla (Fig. 1, f and g). In each spectrum, around 250 peaks could be detected from m/z 3,500 to m/z 25,000 with the majority of the peaks between m/z 5,000 and m/z 13,000. The peak at m/z 5,486 was clearly one of the major spectral features of the cortex, whereas the peak at m/z 9,940 was especially abundant in the papilla. In contrast, the analysis of a whole kidney section by an imaging strategy clearly differentiated the cortex and the papilla from the medulla, an intermediate region of the kidney that cannot be easily spotted by hand due to its relatively small size. Fig. 1, h–j, shows a representative example of ion images from regionally discriminating peaks. The cortex was clearly delineated by the peak centered around m/z 17,514, the papilla was demarcated by the peak at m/z 9,940 (also highlighted in Fig. 2. Protein identification strategy. The protein extraction was performed by directly pipetting up and down solvent on the surface of the section. This process was repeated several times to obtain enough material for sequencing purpose. The pool was then dried, resolubilized, and separated by RP HPLC. Fractions were collected and first spotted on a MALDI target to select the fraction of interest containing the marker highlighted by IMS. The fraction was finally dried and resolubilized for sequence analysis by nano-ESI tandem mass spectrometry.
the manual spotting), and the medulla was marked by the peak at m/z 6,542.

Protein Identification Strategy—A systematic identification of the spectral features (peaks) analyzed by IMS provides valuable information, further validating the accuracy of the technique and the biological significance of the candidate biomarkers.

The strategy presented here is based on an approach mimicking the imaging process (Fig. 2). Proteins and peptides were directly eluted from tissue by microextraction, fractionated by RP HPLC, and surveyed by MALDI MS. After manual spectral correlation, fractions of interest were collected and analyzed by top-down sequencing. Using this strategy we attempted to identify the major components of each kidney substructure as observed in IMS. The major peak in the cortex at m/z 5,486 was identified as cytochrome c oxidase polypeptide VIIc (Ser17–Lys63). Several additional cortex polypeptides were also characterized, and these were mostly ubiquitous proteins such as cytochrome c oxidase polypeptide Vic-2.
Biomarker Discovery by Imaging Mass Spectrometry

Fig. 4. Differential imaging analysis of a control versus a treated rat kidney section. a, average spectrum (cortex) and intensity distribution of m/z 12,959 on a control and a treated kidney section. b, average spectrum (cortex) and intensity imaging of m/z 8,242 on a control and a treated kidney section. The color intensity stands for the ion count in the corresponding mass spectrum.

(Ac-Ser\(^1\)-Lys\(^75\); m/z 8,365.9, m/z 8,381.9 with one oxidation, m/z 8,397.8 with two oxidations), ATP synthase coupling factor 6 (Asn\(^33\)-Ser\(^108\); m/z 8,927.4), ubiquitin (Met\(^1\)-Arg\(^74\); m/z 8,450.8; Met\(^1\)-Gly\(^76\); m/z 8,564.9), β\(_2\)-microglobulin (Ile\(^21\)-Met\(^119\); m/z 11,633.9), 10-kDa heat shock protein (Ac-Ala\(^1\)-Asp\(^101\); m/z 10,812.4), and ubiquinol-cytochrome c reductase complex (Ac-Ala\(^1\)-Lys\(^110\); m/z 13,469.4) (data not shown). In contrast, the major peak in the papilla at m/z 9,940 could not be identified. Although our analytical procedure was rather straightforward, ~50% of the polypeptides analyzed could not be identified. For some, we could not generate an informative tandem mass spectrum due to low signal intensity or because we could not find a suitable parent mass for fragmentation. In other cases, however, we were able to obtain high quality tandem mass spectra that could not be correlated to a polypeptide sequence in the database, probably due to an unknown amino acid modification or other post-translational modification.
Application to Gentamicin Nephrotoxicity—Gentamicin, a well-known nephrotoxicant, has been associated with proximal tubular cell damage in the cortex due to the reduction of protein synthesis and altered phospholipid metabolism (18). Histopathological examination revealed marked degeneration and regeneration of proximal tubules in rats treated with 100 mg/kg/day gentamicin for 7 days (Fig. 3). We attempted to correlate this finding by IMS as follows. We ran a pilot study comprising three kidneys from rats treated with 100 mg/kg/day gentamicin for 7 days and three kidneys from control rats. Three sections were generated from each kidney, and each section was manually profiled using five spots in the cortex and in the medulla/papilla area in controls and was significantly down-regulated in the whole treated kidney, offering a first line of evidence that the site of action of gentamicin could also include the more distal tubular structures.

The differential features highlighted in the profiling mode were confirmed through an imaging approach using one kidney section of each group (Fig. 1d). Data analysis from the imaging experiment was in most cases consistent with results obtained from the profiling approach. For example, the peaks at m/z 12,959 (Fig. 4a) and its doubly charged species at m/z 6,479 (data not shown) were significantly detected in the cortex of the treated kidney section but not in the control section. Furthermore the imaging approach highlighted a number of additional discriminating spectral features that were not detected in the profiling dataset. For example, a peak at m/z 8,242 (Fig. 4b) was detected both in the medulla and in the cortex of the control kidney section, but it was significantly down-regulated only in the cortex of the gentamicin-treated section. A similar pattern was also observed for several other peaks, such as m/z 8,127, m/z 8,457, and m/z 8,369 (data not shown).

We focused our efforts in identifying the peak of m/z 12,959 that were clearly highlighted by both the imaging and the profiling approaches to be discriminating for gentamicin nephrotoxicity. Pools of ~120 protein liquid microextractions from the renal cortex of treated and control sections were fractionated in three HPLC runs to gain enough material for sequencing purposes. The peak of m/z 12,959 was only found

**Fig. 5.** Electrospray tandem mass spectrum analysis of the fraction containing the peak m/z 12,959. A, survey mass spectrum of the HPLC fraction containing the marker of interest. The arrows point to the three multiply charged ion signals of the protein of average mass 12,959 Da. The large background peaks result from the co-eluting hemoglobin α1 protein (average mass, 15,197 Da). B, tandem mass spectrum of the [M + 14H]14+ precursor ion (m/z 926.63). A first database search, which was performed with the singly charged fragment ions labeled y1 to y7 (manually determined to represent an ion series), yielded a top score of 42 (expect, 0.057; significant homology) for transthyretin (Ser28–Gln146). The hit was further confirmed by the observed ion series at m/z 926–955 and at m/z 1013–1042, which corresponded to two multiply charged b-ion series, [(b102)11 to (b102)11+] and [(b102)2+ to (b102)2+], respectively. A second database search taking into account both pieces of evidence yielded a score of 58 (expect, 0.008; extensive homology) for transthyretin (Ser28–Gln146)
in fraction 39 of the treated sample by survey MALDI MS, and this fraction was then analyzed by top-down tandem mass spectrometry. We identified the peak of m/z 12,959 as transthyretin (Swiss-Prot:TTHY_RAT; Ser 28–Gln146; protonated calculated mass, 12,959.5 Da) (Fig. 5), a blood transporter protein characterized as a biomarker of nutritional status (20–22). This finding was first confirmed by Western blot analysis using an antibody highly specific for this protein. A band was clearly detected around 13 kDa both in situ (lane G) and in the HPLC fraction (lane G39). The band migrating at around 26 kDa in lane G is assumed to represent the dimeric form. B, survey MALDI MS spectra. The protein mixture directly microextracted from the kidney section (upper two panels) and HPLC fraction 39 (lower two panels) were analyzed by MALDI MS. A peak at 12,959 Da (transthyretin Ser28–Gln146) is only detected in the gentamicin-treated kidney samples.

**DISCUSSION**

The cortex and medulla/papilla represent two distinct structural and functional areas of the kidney that are clearly delineated by IMS, substantiating that regiospecific patterns of protein expression can directly be observed at the proteome level using this strategy. In the present study, we attempted to identify directly at the tissue level structural and functional changes in an organ that has been affected by a toxicant. It was important to use a well established biological model whose outcome is confirmed by an independent methodology. Gentamicin-induced nephrotoxicity is extensively described in the literature (9, 11, 18), and gentamicin-induced nephrotoxicity in rat was confirmed in this study by histopathology.

We applied a mixed profiling and imaging strategy followed by targeted protein identification by protein microextraction and top-down tandem mass spectrometry to study the lesions secondary to gentamicin administration in the rat kidney. The uptake of gentamicin (cationic at a physiological pH) is thought to be carried out either passively by diffusion with acidic phospholipids of the brush-border membrane from the proximal tubular cells or through receptor-mediated endocytosis, leading to lysosomal accumulation and ultimately resulting in renal toxicity (10). We demonstrated that the nephropathy secondary to treatment with aminoglycoside antibiotics might be characterized by the accumulation in the cortex of affected kidneys of transthyretin Ser28–Gln146. Transthyretin is a plasma carrier of both retinol-binding protein and thyroxine (22) and has been described as a biomarker for nutritional status in various diseases (20, 21) such as pancre-
atitis (23) and end stage renal disease (24). A truncated form of transthyretin lacking the first NH$_2$-terminal 10 amino acids has also been proposed for the detection of early stage ovarian cancer (25). More relevant to our study, transthyretin has also been described as a ligand of megalin (26), an endocytic receptor expressed at the apical membrane of renal proximal tubules that has been reported to also mediate gentamicin uptake (27, 28). Among a number of hypotheses that could explain the accumulation of transthyretin in proximal tubules, several lines of evidence indicate that megalin could preferentially bind gentamicin and prevent the efficient reabsorption of transthyretin into the bloodstream (26–29). Other examples of competition with aminoglycosides for megalin have been reported. Calcium binding to megalin is inhibited in vitro by aminoglycosides (29), and Cui et al. (30) reported that gentamicin almost completely inhibits the uptake of bovine serum albumin by purified megalin in proximal tubular cells.

Further investigations to refine a model for transthyretin accumulation upon gentamicin treatment could include the quantitative evaluation of transthyretin by IMS and immunohistochemistry from animals treated at different doses and durations to confirm its dose-dependent accumulation at the target tissue. Our first analysis by immunohistochemistry confirmed the elevated levels of transthyretin in the cortex of kidney sections from gentamicin-treated rats as observed by IMS. However, a direct correlation between the intensity of the mass spectrometric signal and transthyretin abundance in the tissue remains to be demonstrated.

In addition, larger studies including several types of known nephrotoxicants could demonstrate whether transthyretin holds as a more general biomarker for nephrotoxicity and whether the detected changes of protein patterns precede...
renal dysfunction and histopathological alteration. If so, IMS could represent a powerful complementary approach to histopathology in discovering early protein targets of the toxicant and would facilitate the classification of new compounds with known toxicants according to common pharmacological and toxicological features. Such predictive models exist based on gene expression data (31) but not on proteomic platforms so far.

The present study clearly highlights the advantages and limitations of the present IMS approaches. A profiling strategy by manually spotting matrix on predefined areas of interest has the advantages of speed, robustness, and good reproducibility. However, this approach is biased by design as it requires manual intervention, and only a fragmentary analysis of the tissue at low spatial resolution can be obtained. Alternatively an imaging strategy by automatic spotting of matrix on the tissue in an array format results in a comprehensive structural analysis at a higher spatial resolution. Although we were able to uncover some treatment-related differences using a profiling strategy, a clear advantage of the imaging strategy is to go further into structural or morphological detail. In this study, a differential comparison of the much richer imaging dataset was essential to ascertain the regiospecificity of the peaks selected for protein identification in the profiling dataset. Further reducing the raster width or optimizing a reproducible homogeneous matrix coating deposition would be an absolute requirement if the functional anatomy of the organ, such as the proximal tubule or glomerulus of the kidney, is to be assessed in a differential study. However, an imaging strategy will heavily depend on the development of software packages for the rapid and accurate differential analysis of vastly more complex datasets than those generated in this study.

A critical step of an IMS-based biomarker discovery approach remains the unambiguous identification of a potential protein target of interest. In this study, we applied an innovative protein identification strategy that closely preserves the biological content observed by IMS. In particular, a local and specific liquid microextraction allows the conservation of the protein mixture composition observed by IMS, thus avoiding the dilution effect of a classical lysis protocol. Proteins of interest can be further purified using standard fractionation methods, and the biologically relevant isoforms can then be unambiguously determined using top-down sequencing by tandem mass spectrometry. Protein identification strategies, such as described in this study, are essential to enable the study and the validation of candidate biomarkers using orthogonal methods in a large number of samples using, for example, Western or ELISA-based assays.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: F. Hoffmann-La Roche Ltd., Roche Center for Medical Genomics, Bldg. 93/44, CH-4070 Basel, Switzerland. Tel.: 41-61-688-9739; Fax: 41-61-688-1448; E-mail: axel.ducret@roche.com.

REFERENCES

1. Bandara, L. R., and Kennedy, S. (2002) Toxicoproteomics—a new preclinical tool. Drug Discov. Today 7, 411–418

2. Bandara, L. R., Kelly, M. D., Lock, E. A., and Kennedy, S. (2003) A correlation between a proteomic evaluation and conventional measurements in the assessment of renal proximal tubular toxicity. Toxicol. Sci. 73, 195–206

3. Wittmann, F. A., and Li, J. (2004) Proteomics and nephrotoxicity. Proteomics Nephrol. 141, 104–123

4. Petricoin, E. F., Rajapakse, V., Herman, E., Arekani, A. M., Ross, S., Johann, D., Knapton, A., Zhang, J., Hitt, B. A., Conrads, T. P., Veenastra, T. D., Liotta, L. A., and Sistare, F. D. (2004) Toxicoproteomics: serum proteomic pattern diagnostics for early detection of drug induced toxicities and cardioprotection. Toxicol. Pathol. 32, 122–130

5. Chaurand, P., Fouchécourt, S., DaGue, B. B., Xu, B. J., Reyzer, M. L., Orgebin-Crist, M.-C., and Caprioli, R. M. (2003) Profiling and imaging proteins in the mouse epididymis by imaging mass spectrometry. Proteomics 3, 2221–2239

6. Pierson, J., Norris, J. L., Aerni, H.-R., Svenningson, P., Caprioli, R. M., and Andrin, P. E. (2004) Molecular profiling of experimental Parkinson’s disease: direct analysis of peptides and proteins on brain tissue sections by MALDI mass spectrometry. J. Proteome Res. 3, 289–295

7. Chaurand, P., DaGue, B. B., Pearsall, R. S., Threadgill, D. W., and Caprioli, R. M. (2001) Profiling proteins from azoxymethane-induced colon tumors at the molecular level by matrix-assisted laser desorption/ionization mass spectrometry. Proteomics 1, 1320–1326

8. Yanagisawa, K., Shyr, Y., Xu, B. J., Massion, P. L., Larsen, P. H., White, B. C., Roberts, J. R., Edgerton, M., Gonzales, A., Nadaf, S., Moore, J. H., Caprioli, R. M., and Carbone, D. P. (2003) Proteomic patterns of tumour subsets in non-small-cell lung cancer. Lancet 362, 433–439

9. Valdivielso, J. M., Rivas-Cabanero, L., Morales, A. I., Arévalo, M., M. López-Novo, J., and Pérez-Barriocanal, F. (1999) Increased renal glomerular endothelin-1 release in gentamicin-induced nephropathy. Int. J. Exp. Pathol. 80, 265–270

10. Nagai, J., and Takano, M. (2004) Molecular aspects of renal handling of aminoglycosides and strategies for preventing the nephrotoxicity. Drug Metab. Pharmacokinet. 19, 159–170

11. Mazzon, E., Britti, D., Sarro, A. D., Caputti, A. P., and Cuzzocrea, S. (2001) Effect of N-acetylcysteine on gentamicin-mediated nephropathy in rats. Eur. J. Pharmacol. 424, 75–83

12. Schwartz, S. A., Reyzer, M. L., and Caprioli, R. M. (2003) Direct tissue analysis using matrix-assisted laser desorption/ionization mass spectrometry: practical aspects of sample preparation. J. Mass Spectrom. 38, 699–708

13. Aerni, H.-R., Cornett, D. S., and Caprioli, R. M. (2006) Automated acoustic matrix deposition for MALDI sample preparation. Anal. Chem. 78, 827–834

14. Golu, T., Slonim, D., Tamayo, P., Huard, C., Gaasenbeek, M., Keswani, J., Coller, H., Loh, M., Downing, J., Caligiuri, M., Bloomfield, C., and Lander, E. (1999) Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science 286, 531–537

15. Mobeley, J. A., Lam, Y. W., Lau, K. M., Pais, V. M., L’Esperance, J. O., Steadman, B., Fuster, L. M., Blute, R. D., Taplin, M. E., and Ho, S. M. (2004) Monitoring the serological proteome: the latest modality in prostate cancer detection. J. Urol. 172, 331–337

16. Kelleher, N. L., Lin, H. Y., Valasukov, G. A., Aaserud, D. J., Fridriksson, E. K., and McLafferty, F. W. (1999) Top down versus bottom up protein characterization by tandem high-resolution mass spectrometry. J. Proteome Res. 3, 806–812

17. Nemeth-Cawley, J. F., and Rouse, J. C. (2002) Identification and sequencing of intact proteins via collision-induced dissociation and quadrupole time-of-flight mass spectrometry. J. Mass Spectrom. 37, 270–282

18. Sundin, D. P., Sandoval, R., and Molitoris, B. A. (2001) Gentamicin inhibits renal protein and phospholipid metabolism in rats: Implications involving intracellular trafficking. J. Am. Soc. Nephrol. 12, 114–123

19. Schnellmann, R. G. (2001) In Casaret and Doul’s Toxicology: The Basic
Biomarker Discovery by Imaging Mass Spectrometry

Science of Poisons (Klaassen, C. D., ed) 6th Ed., pp. 491–514, McGraw-Hill, New York

20. Bernstein, L., and Ingenbleek, Y. (2002) Transthyretin: its response to malnutrition and stress injury—clinical usefulness and economic implications. *Clin. Chem. Lab. Med.* 40, 1344–1348

21. Ingenbleek, Y., and Young, V. (1994) Transthyretin (prealbumin) in health and disease: nutritional implications. *Annu. Rev. Nutr.* 14, 495–533

22. DeNayer, P. (2002) Historical overview of analytical methods for the measurement of transthyretin. *Clin. Chem. Lab. Med.* 40, 1271–1273

23. Lasztity, N., Biro, N., Nemeth, E., Pap, A., and Antal, M. (2002) Protein status in pancreatitis: transthyretin is a sensitive biomarker of malnutrition in acute and chronic pancreatitis. *Clin. Chem. Lab. Med.* 40, 1320–1324

24. Sreedhara, R., Avram, M., Blanco, M., Batish, R., and Mittman, N. (1996) Prealbumin is the best nutritional predictor of survival in hemodialysis and peritoneal dialysis. *Am. J. Kidney Dis.* 28, 937–942

25. Zhang, Z., Bast, R. C., Yu, Y., Li, J., Sokoll, L. J., Rai, A. J., Rosenzweig, J. M., Cameron, B., Wang, Y. Y., Meng, X.-Y., Berchuck, A., Haaf ten-Day, C. v., Hacker, N. F., de Brujin, H. W. A., van der Zee, A. G. J., Jacobs, I. J., Fung, E. T., and Chang, D. W. (2004) Three biomarkers identified from serum proteomic analysis for the detection of early stage ovarian cancer. *Cancer Res.* 64, 5882–5890

26. Sousa, M. M., Norden, A. G. W., Jacobsen, C., Willnow, T. E., Christensen, E. I., Thakker, R. V., Verroust, P. J., Moestrup, S. K., and Saraiva, M. J. (2000) Evidence for the role of megalin in renal uptake of transthyretin. *J. Biol. Chem.* 275, 38176–38181

27. Verroust, P. J., Birn, H., Nielsen, R., Kozyraki, R., and Christensen, E. I. (2002) The tandem endocytic receptors megalin and cubulin are important proteins in renal pathology. *Kidney Int.* 62, 745–756

28. Moestrup, S. K., Cui, S., Vorum, H., Bregengard, C., Bjorn, S. E., Norris, K., Gliemann, J., and Christensen, E. I. (1995) Evidence that epithelial glycoprotein 330/megalin mediates uptake of polybasic drugs. *J. Clin. Invest.* 96, 1404–1413

29. Nagai, J., Tanaka, H., Nakanishi, N., Murakami, T., and Tanako, M. (2001) Role of megalin in renal handling of aminoglycosides. *Am. J. Physiol.* 281, F337–F344

30. Cui, S., Verroust, J. P., Moestrup, S. K., and Christensen, E. I. (1996) Megalin/gp 330 mediates uptake of albumin in renal proximal tubules. *Am. J. Physiol.* 271, F900–F907

31. Steiner, G., Suter, L., Boess, F., Gasser, R., DeVera, M. C., Albertini, S., and Ruepp, S. (2004) Discriminating different classes of toxicants by transcript profiling. *Environ. Health Perspect.* 112, 1236–1248