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Review

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Mass Spectrometry Imaging in Drug Development

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Drug discovery and development is a high-risk, expensive endeavor and it commonly takes about 10−15 years to obtain marketing approval from project inception.[1] Research efforts during the early stages of the process aim to generate and support pharmaceutically relevant hypotheses, for example, that the inhibition or activation of a protein or pathway will have a positive therapeutic effect in a particular disease state. These studies lead to the selection of a target, which is then thoroughly explored to identify a drug-like small molecule or biological therapeutic that can be advanced into preclinical and clinical development and ultimately approved and marketed as a medicine.[2,3]

Drugs can only elicit their full benefit if they are present in the vicinity of the target to which they bind at high enough concentrations for an appropriate amount of time. To understand the complex biochemical interactions between drugs and their targets, it is necessary to identify the molecular entities involved and determine their abundance and spatial distribution in tissues. It is also important to understand why new compounds are (or are not) efficacious and the reasons for their toxicity, if any. Acquiring such information is therefore an important task in pharmaceutical development and requires the use of analytical tools that provide as much insight as possible into the identities and topographical distributions of all the chemical relevant species present within biological samples including the therapeutic agent, the endogenous ligand, the target protein, or biomarkers of the drug effect.[2,3] The major factors that influence a drug's distribution are well-known; they include its relative tissue and plasma protein binding affinities, pH effects, membrane affinities, transporter interactions, and membrane permeability. However, these factors cannot yet be used to reliably predict how a given agent will behave in the body.[4,5] In addition, drug or drug metabolite levels measured in plasma often do not correctly represent the levels present within tissues or organ subcompartments and therefore cannot be relied upon when seeking to explain the efficacy or toxicology of drugs within the body.[6]

Molecular imaging attempts to characterize and quantify biological processes and drug distributions. Experimental techniques including computed tomography, ultrasound, magnetic resonance imaging, and positron emission tomography (PET) have been successfully used in the noninvasive monitoring of anatomy (structural imaging) and physiology (functional imaging) and also to image specific molecules and their targets (molecular imaging).[3] Recent advances in fluorescence and bioluminescence imaging, as well as near-infrared probes, have made these methodologies particularly attractive for drug discovery.[2] Although many of them were initially developed for clinical applications, they are increasingly being used in drug discovery and development as well. Several of these imaging technologies are superior to established methods for studying compound localization and distribution because they are noninvasive and can be performed *in vivo*. Unfortunately, they either do not provide molecular information or rely on radiolabeled tracers, necessitating the administration of imaging agents or reporter probes. Developing appropriate labeled tracers is often complicated and expensive. In addition, it is usually impossible to discriminate between the labeled parent drug compound and drug metabolites that retain the label.[7] Therefore, an imaging technique that could directly and quantitatively visualize compound localization within tissue samples without relying on a label would be useful. It would also be very desirable for new imaging technologies to be capable of multiplex analysis, enabling the simultaneous detection of multiple endogenous compounds and pharmaceutical agents in a single experiment.

Mass spectrometry imaging (MSI) is an *in vitro* technology that can provide information complementary to that obtained via other analytical methods and imaging modalities used in drug discovery and development studies.[6,8,9] As such, it has rapidly found diverse applications in both industrial and

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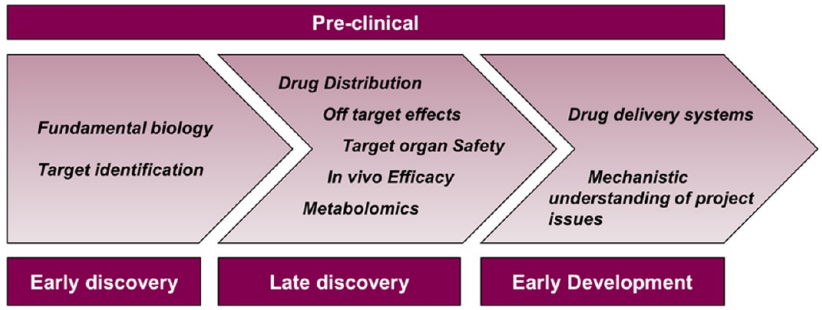

Figure 1. Overview of where MSI is able to impact preclinical drug discovery/development.

academic pharmaceutical research. The speed, sensitivity, and molecular specificity of modern mass spectrometers enable the direct simultaneous imaging of molecules such as drugs, their metabolites, and endogenous biomolecules in tissue sections and specific tissue microstructures at near-cellular spatial resolutions.[10−12] Unlike most clinically useful imaging methods, MSI can image and quantitate endogenous analytes without the use of target-specific molecular labeling reagents. This enables highly multiplexed analyses that can be used both for targeted assays and to drive the discovery of new biology. Furthermore, MSI is capable of detecting phenotypic changes (such as those that may occur after drug administration), which may provide new insights into the molecular biology of diseases and their treatments. The information provided by modern MSI methods could thus be tremendously useful in the selection or improvement of drug molecules during discovery and development.

This review focuses on existing MSI technologies and presents some of their potential applications at various stages of the drug discovery and development process (Figure 1). We begin by briefly describing the common pharmaceutically applicable MSI techniques along with their advantages, challenges, and limitations. We then discuss the application of MSI in various areas of drug discovery and development, namely, (i) drug metabolism and pharmacokinetics (DMPK) and liberation, absorption, distribution, metabolism, and excretion (LADME); (ii) safety toxicology; (iii) assessing penetration of the blood-brain barrier; and (iv) studies on biomarkers and the effects of drugs. Finally, we conclude by outlining likely developments in the field of MSI and its applications in pharmaceutical research.

OVERVIEW OF COMMON PHARMACEUTICALLY APPLICABLE MSI TECHNIQUES

Mass spectrometry imaging relies on an increasing variety of techniques that enable discrete and direct ionization from the surface of a sample, which is typically a tissue section. The ion signals generated in this way are detected by MS and processed, enabling the virtual reconstruction of the sample surface in a way that shows the distribution of individual molecular species within it.

The most widely used method for sample ionization is matrix-assisted laser desorption ionization (MALDI), followed by secondary ion mass spectrometry (SIMS) and desorption electrospray ionization (DESI). The chosen ionization technique is paired with one of an increasingly large range of ever more sensitive mass analyzers, to yield a molecular imaging system that can rapidly generate 2D visualizations of the distributions of diverse molecular species including endogenous small ions, lipids, and small size proteins as well as exogenous pharmaceutical compounds and their metabolites.[9,13−17] Many hundreds of ionized molecular species can be detected simultaneously while preserving their spatial distribution information at micrometer resolutions or even submicrometer resolutions when using SIMS. Moreover, MSI does not require any labeling of the target analytes. This facilitates the detection and imaging of both known and unknown targets. Because of these properties, MSI is broadly applicable during early drug discovery and development, when multiple related compounds are tested *in vivo*.

There is a large body of literature on the optimization, applicability, and limitations of the existing sample ionization techniques and mass analyzers and on the preparation of samples for MSI. These studies are not reviewed in detail here, although some of their results are mentioned. The interested reader is directed to more focused reviews for more detailed information.[6,9,12,13,18−21]

The first MSI studies used SIMS ionization and aimed to map the distribution of elemental ions in tissue sections.[22,23] SIMS works by focusing a primary ion beam onto the sample's surface to generate and desorb secondary ions. The introduction of time-of-flight (TOF) mass analyzers greatly increased the versatility of SIMS and enabled its use in biomolecular imaging; SIMS is now almost invariably used in conjunction with TOF analyzers. The most attractive quality of SIMS imaging, and the one that justifies the investment in the costly equipment and laborious sample preparation it requires, is that it can achieve very high spatial resolutions. This high spatial resolution makes SIMS imaging a powerful tool for pharmaceutical R&D, pharmaceutical formulation, and manufacturers,[24−27] but the technical challenges it presents and its limited sensitivity mean that it is most useful in specific and focused investigations rather than being a general purpose MSI approach.

In contrast, the more recently developed technique DESI requires no sample preparation beyond tissue sectioning. This simplicity, together with its good sensitivity with respect to a wide range of low molecular weight target ions makes it a straightforward and general ionization technique. In DESI, the sample is sprayed with an electrically charged solvent mist (i.e., an electrospray) at an angle that causes the ionization and desorption of various molecular species.[28] DESI-MSI can thus be used to generate two-dimensional maps showing the distribution and relative abundance of selected ions in the surface layer of a tissue sample.[29] This less-destructive ionization technique enables the reuse of the imaged tissue sections in traditional histological imaging because they are readily amenable to hematoxylin and eosin (H&E) and immunohistochemistry (IHC) staining. In addition, because

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ions are only desorbed from the outermost surface of the sample, there is no need to use tissue sections thicker than those typically used in histological analyses (typically 4−6 μm). Some alternative ionization techniques, such as MALDI, may require thicker tissue sections.

The most widely used ionization technique in modern MSI is MALDI, which can be coupled with diverse types of common mass analyzers including TOF, TOF-TOF, quadrupole time-of-flight (qTOF), ion trap, and Fourier transform ion cyclotron resonance (FTICR) instruments. Again, MALDI-MSI enables direct label-free mapping of the distributions of molecular species in tissue sections. Ions are generated by focusing a pulsed laser beam onto the sample, which is coated with an assistive medium known as the matrix.[30] MALDI-MSI is typically performed on 10−20 μm thick tissue sections. The relatively high thickness of the tissue sections increases the quantity of analytes available for cocrystallization with the matrix. Commonly used MALDI matrixes are organic acids such as α-cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA), and 2,5-dihydroxybenzoic acid (DHB). As in all MSI experiments, an ordered array of mass spectra is acquired from a set of raster-defined grid points on the sample surface or collected using continuous ionization and subsequent regression to align the ionization collection position with the relative abundance of each mass-to-charge ratio (m/z) assigned to each pair of x and y coordinates. Images showing the distributions of selected molecules provide information on each compound's relative abundance at every point on the sample's surface, revealing their spatial localization.[12,13] By varying the MALDI matrix it is possible to preferentially ionize target molecules and increase the sensitivity of their detection. Moreover, matrix tuning makes it possible to retain the ionization efficacy of a preferred matrix while shifting the m/z of unwanted matrix adduct ions that may mask a target ion. For example, the replacement of CHCA with a deuterated analog facilitated the analysis of certain low molecular weight targets, which could be particularly useful in drug distribution imaging.[31]

All MSI techniques offer different degrees of sensitivity, specificity, and spatial resolution. It is therefore important to carefully select the most appropriate MSI method for a given application in order to obtain the most useful possible data. Furthermore, it is often necessary to use multiple MSI strategies to detect all compounds of interest.[32] In drug discovery and development, the selection of an MSI system should be guided by the question that is to be answered and the stage one is at in the discovery and development pipeline. If the highest possible spatial resolution is required then SIMS imaging might be the preferred choice because it is capable of submicrometer resolutions and can determine the subcellular distribution of target analytes. However, this power comes at an increased financial cost and may be accompanied by a relatively low sensitivity. In contrast, if one is interested in a wider range of analytes, ranging from small molecules to peptides and small size proteins, MALDI may be used. Unfortunately, its flexibility and capacity for multiple target analysis in the low and intermediate mass ranges comes at the expense of spatial resolution and necessitates the use of a MALDI matrix, which introduces a possible source of intersample variation and may cause target diffusion during sample preparation.

DESI analysis, in contrast, is not complicated by any further sample preparation other than tissue sectioning. This means there is no risk of analyte diffusion due to matrix application. It also makes subsequent post-MSI analysis possible, so traditional histology is often performed on the same tissue section.[33,34]

For all MSI methods, it is crucial to use a sample preparation protocol that preserves the distribution and abundance of the target analytes. The protocol must also be sufficiently robust and reproducible to enable intersample comparison without introducing excessive variability. Special care must be taken when collecting tissue samples for MSI; in particular, it is important to ensure that all samples are prepared at approximately the same time post mortem. If a target analyte is degraded or modified during sampling or sample preparation, it may be converted into a new compound that will generate ions with a different mass to that expected. Such degradation can occur quite quickly, affecting the detected abundances of both large and small molecules.[35−37] The reproducibility of MSI results and the extent of tissue preservation can sometimes be increased by rapidly removing dissected organs and snap-freezing tissue samples in a liquid nitrogen or chilled-isopentane bath.[38] Thin tissue sections are then prepared using a cryostat microtome and placed on MSI-compatible plates for analysis. Sections to be analyzed by MALDI-MSI are usually placed on metal, metal-coated glass, or regular glass slides according to the requirements of the instrument, whereas nonconductive glass slides are used for DESI-MSI. Whole body MSI analysis can also be performed,[39] allowing noncovalently bound drug and metabolite distributions to be mapped in a similar way to quantitative whole-body autoradiography (QWBA).[39] However, it is usually dissected organs that are analyzed by MSI because this approach makes it possible to achieve higher spatial resolutions and use optimized tissue-specific sample preparation protocols while also eliminating the need to correct for interorgan ionization suppression.[40] In addition, it takes several minutes for the core to cool to the required temperature during whole animal freezing, which may cause the degradation of certain targets or modify their distribution. A wide range of optimized sample collection and preparation protocols have been developed for different MSI technologies with the aim of optimizing ionization while minimizing the degree of introduced variation. These protocols are not discussed here because they have been reviewed in detail by other authors.[19,41] However, it should be noted that the application of the matrix during sample preparation for MALDI-MSI can significantly affect the quality of the results obtained, whether it is deposited as a series of microspots or in a uniform layer or by sublimation. Despite such complications, it is clear that MSI has considerable potential to facilitate drug discovery and development and the process of bringing new medicines to market.

■ ADVANTAGES, CHALLENGES, AND LIMITATIONS OF MSI

A great range of analytical techniques are likely to be used during the long and often meandering journeys that compounds take from hit identification and lead optimization, through to candidate selection, and then onward to preclinical and clinical testing. Even highly specific plasma liquid chromatography (LC)-MS methods (the standard approach to drug exposure assessment) may not provide an accurate picture of a compound's fate or effects in tissues. For example, plasma measurements will not accurately represent a compound's intratumor concentration if the tumor is poorly vascularized, and studies on homogenized samples of the same tumor will enable the determination of the compound's average

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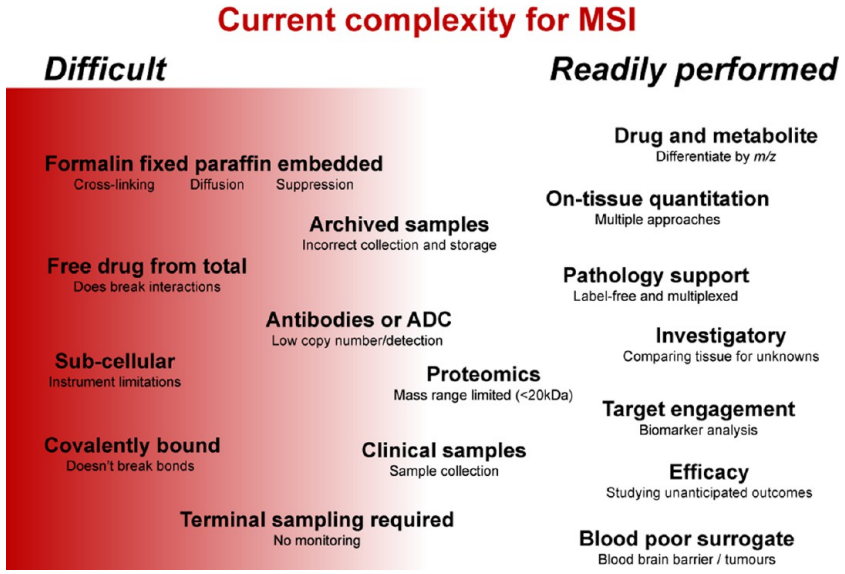

Figure 2. Summary of areas where MSI could play a role, arranged on a scale ranging from difficult to "readily performed".

intratumor abundance but say nothing about its extent of penetration/accumulation into the tumor or the tumor's microenvironment. Additionally, methods that rely on the direct labeling of an analyte only provide information on the abundance and distribution of the label rather than the drug *per se*. If the drug is metabolized, the label may be retained on the metabolite, which will then become indistinguishable from the original compound. This problem can be overcome by sample homogenization followed by LC separation and MS analysis, with scintillation detection to determine the ratio of the radioactive parent to its metabolites. However, once again all spatial information is lost. Such problems can be avoided entirely with MSI.

When considering the applications of MSI (or any new analytical imaging technique), it is important to recall that drugs have successfully been developed and marketed for decades using established traditional methods. Furthermore, there is currently no regulatory requirement to measure or monitor concentrations of drug candidates or metabolites in tissue samples (by any method) when preparing for clinical trials or drug registration. Consequently, investments in the emerging collection of MSI technologies must be justified on the basis of their potential contributions to the drug discovery and development process. These contributions may involve facilitating timely decision making on key issues or supporting problem solving by providing important insights that would not be accessible using more conventional or widely used technologies. Figure 1 outlines the key areas where MSI can have a meaningful impact on a drug development project by supporting decision making, reducing compound attrition, and facilitating the assessment of efficacy and safety. All of these applications rely on the power of MSI to measure the abundance and distribution of both targets and unknown endogenous compounds whose concentrations change in response to treatment in ways that may reflect target engagement or adverse toxicological events. Because MSI has the potential to be an important addition to the arsenal of analytical technologies, it is important to clearly state its current advantages, the challenges associated with its use (which may be overcome in future), and its limitations in the context of drug discovery and development. At present there still remains a limitation to the capabilities of current MSI technologies with respect to some of the types of samples or assays that pharmaceutical researchers would like to undertake. There are also a group of experiments that, while possible, are harder to perform than standard MSI studies. Figure 2 summarizes these groups from those readily performed using current MSI technologies to those that remain a challenge.

Advantages. The advantages of MSI over traditional methods for studying the distribution of compounds within tissue sections are that it can either provide more information, greater selectivity, or more accuracy. Alternatively, it can provide similar results with less investment of time or resources. Therefore, the increasing breadth of MSI technologies, with increased speed, accuracy, and spatial resolution for mapping endogenous and exogenous compounds are the driving forces behind MSI technologies being widely used in both academic and industrial drug research and development.

As previously described, there are key areas where MSI overcomes complications associated with traditional approaches to tissue bioanalysis, including the use of tissue homogenates, the analysis of plasma as a surrogate for tissues of interest, and labeling or probe-based assays. The analysis of homogenates in particular is antithetical to the approach adopted in MSI analysis. It generally involves extraction of the homogenate followed by chromatographic separation and MS. This necessarily means that the concentration of any given target is averaged over the entire sample, so the results obtained will never provide information on the (in)homogeneity of the analyte's distribution within a tissue, in tissue microenvironments, or across specific cell types. Homogenate-based analyses have the advantage of providing a quick and simple measurement of absolute concentrations. Although there are situations where this may be adequate, loss of all spatial distribution is, in many circumstances, a major impediment, especially for highly heterogeneous tissues (e.g., kidney and brain) where the spatial relationship between target compounds (drug, metabolite, or endogenous) is important. A key reason for using MSI techniques in tissue analysis is that they can provide high-resolution data on the concentrations of drugs at sites (targeted or not) where they may exert effects, which is highly beneficial for efficacy and safety assessments.

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Plasma and blood measurements of compounds or biomarkers are staple procedures in all pharmaceutical DMPK laboratories. The combination of high-resolution chromatographic separation and sensitive MS analysis enables multiple measurements of microsamples from small rodents[20] and detailed pharmacokinetic measurements on compounds during discovery and early development. Significantly, there is no requirement for extensive assay optimization or expensive label synthesis and application. Modern drug development is strongly driven by the free drug hypothesis, which asserts that the concentration of the unbound ("free") drug is tightly correlated with its effect and that at equilibrium the free drug concentration in the tissue is generally equal to that in plasma. Hence, estimated free plasma concentrations are critical parameters for pharmacokinetic/pharmacodynamic (PKPD) models and drug design. However, the hypothesis that plasma analysis can provide sufficiently robust surrogates for tissue abundance is only valid if the ratio of free drug concentrations in plasma and tissue is one to one. This is generally true for highly permeable drugs, but there are exceptions where true tissue concentrations markedly differ, for instance due to the action of drug transporters (see summary in Figure 3).

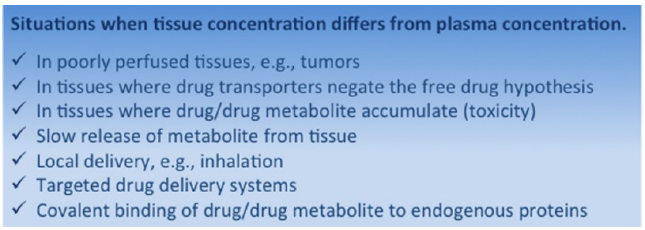

Figure 3. Summary box on situations when tissue concentrations might differ from plasma concentration, making the use of MSI and measurements of true tissue concentrations important.

Furthermore, in tissues with limited or compromised vasculature, such as tumors or ischemic tissue, drug distribution may be modulated not only by drug transporters but also by perfusion limitations, and oxygen, pH, and interstitial pressure gradients.[42] MSI is particularly valuable in such cases, as it can provide potent insights into the true tissue penetration of a compound.[43] Conversely, MSI analysis (but not blood monitoring) can readily detect accumulation of metabolites within a tissue, if they are not released into systemic circulation.[24] Indeed, MSI is highly valuable in all situations where free plasma concentrations do not correlate well with tissue concentrations, notably for drugs delivered locally (e.g., inhaled or topically) or via any system yielding a protracted or tissue-targeted release profile (e.g., tumor-targeted nanoparticles).

Complications associated with the blood brain barrier (BBB) must also be considered. The ability to penetrate the BBB is important for all pharmaceuticals: agents targeting the brain must be able to reach their sites of action, while those targeting other parts of the body should ideally be incapable of penetrating the BBB in order to minimize potential adverse effects. However, measurements of plasma levels provide no information on events in the cerebral environment. Traditionally, such information is acquired by whole brain homogenization and extraction, followed by concentration determination and adjustment of the measured concentrations using a correction factor to account for the quantity of drug present in residual blood. However, this is at best an approximation for compound penetration, and again all spatial information is lost, which is highly significant in a very inhomogeneous organ such as the brain. In contrast, by utilizing the high spatial resolution afforded by MSI, researchers can detect blood markers (heme) and infer precise degrees of drugs' BBB penetration from ratios of their concentrations to the marker concentrations.[44] The technique was applied in a similar, highly effective manner in a study where the impact of orthotropic brain tumors on the BBB was assessed through concentrations of markers in brain and tumor tissues.[45] Aspects of MSI related to BBB analysis are considered in more detail below, in the section on the applications of MSI in drug development.

An alternative targeted strategy is to radiolabel the molecule (usually with ^{3}H or ^{14}C) prior to administration and subsequently track its biodistribution, for instance by QWBA. However, it is important to note that MSI is by no means an alternative to QWBA. This regulatory staple is used to generate experimental data pertaining to drug and metabolite distribution for drug registration in a longitudinal rat WBA study, using a radiolabeled version of the drug in male and pregnant animals. This allows identification of both the organs and tissues that are exposed to the highest concentrations of compound-related materials (i.e., the sum of the drug and metabolite levels). Data from such investigations are used to assess potential exposures in human radiolabeled drug metabolism/excretion balance studies and potential levels of fetal exposure. They are also used to identify tissues with protracted retention of radioactivity, which are likely to accumulate material on repeat dosing, possibly because of covalent interactions between the tested agent and endogenous macromolecules. Because appropriate radiolabeling greatly facilitates tracking of all drug-related materials and identifying covalently bound adducts by MSI is challenging, MSI is not an alternative approach to QWBA from either a technical or a regulatory perspective. However, MSI is complementary as it adds differentiated information about drug and metabolite localization.

Those working in pharmaceutical R&D may be drawn to MSI based on its ability to map the abundance of targets and metabolites at ever increasing spatial resolution and speed. However, the most attractive aspect of MSI in pharmaceutical research and development is probably its utility in the untargeted interrogation of tissues to detect and identify biomarkers in studies on efficacy (target engagement) and safety (toxicity). Such studies require extensive follow-up experimentation and validation to translate unknown mass-to-charge ratios into a mechanistic understanding of molecular activities in focal tissues.

Limitations. While it would be easy to focus on the advantages of MSI in drug research, development, and clinical evaluation, it is important for researchers to be aware of its limitations in order to mitigate risks and avoid overinterpretation of the acquired data. Many of the complications of MSI are similar to those associated with traditional MS bioanalyses. Notably, in both MSI and traditional analyses it is impossible to tell whether failure to detect a compound in a tissue is due to its absence or insufficient sensitivity of the analytical technique. This problem can be reduced (but never eliminated) by applying multiple ionization methods, with wide ranges of sensitivity and spatial resolution, to determine whether the analyte is present but not detectable by the preferred method.[32,46]

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The sensitivity of MSI is also highly compound-dependent, and although matrixes, MS parameters, and ionization techniques can all be optimized, the amount of material entering the mass spectrometer (and hence sensitivity) is greatly reduced when using high spatial resolution and hence smaller sampling areas. Thus, it is important to apply MSI at the most appropriate spatial resolution to maximize the probability of successful detection of targets. It is also worth remembering that despite these limitations, MSI may provide greater ability to detect compounds localized in small sites than homogenization followed by extraction and LC−MSMS.

DMPK and LADME researchers should also be aware of other limitations associated with MSI data in order to mitigate their effects. For example, when ionizing targets in tissue samples it is impossible to distinguish between total, protein-bound, and unbound fractions, which are usually parameters in pharmacokinetic calculations. In addition, pathologists usually prepare (or receive) tissues fixed in formalin, ready for paraffin embedding and subsequent sectioning. This has been a mainstay for toxicological studies throughout the history of modern drug discovery. However, such methods are not compatible with MSI analysis of small targets, as the delocalization of small molecules (both endogenous and exogenous) will result in inaccurate target distribution measurements. Even if this problem is overcome, there is risk that the paraffin will suppress ionization. There are methods for large molecule proteomic analysis of fixed samples,[47,48] but the harsh washing and processing required to reverse disulfide cross-linking in proteins also complicates subsequent small molecule MSI analysis. Therefore, it is suggested that if MSI is to be used, studies should be designed to accommodate it from the outset. This will ensure that material specifically earmarked for MSI analysis (which could subsequently be homogenized and used in conventional LC−MS analyses) is collected under optimal conditions for MSI rather than relying on archived samples that may be unsuitable. Also, while plasma PK studies can provide data over the course of a study, it should be noted that if plasma levels indicate high levels of compound elimination by the study's end, then tissues are likely to have similarly low levels.

Antibody therapeutics and antibody drug conjugates represent exciting avenues of development for the pharmaceutical industry. Unfortunately, the molecular size of these agents and their low copy numbers in tissues mean that it is not currently possible to study their abundance or distribution in tissue samples by MSI. However, MSI could potentially support the development of such therapeutics by providing information on the efflux of endogenous compounds in response to the treatment. For smaller targets, the upper limits on the molecular masses detectable with current MSI mass analyzers are high enough to permit proteomic analysis, but the technique has primarily been used in academic contexts rather than in pharmaceutical research. This may be because enzymatic digestion and incubation are required for the detection and identification of larger proteins in tissue samples. This enables the detection of very abundant proteins but sacrifices a degree of spatial resolution. It is however possible to perform multiplexed MSI and histochemical studies by probing tissues with MSI-detectable labels (either photocleavable tags or heavy metals[49,50]). Although, this approach shifts the analysis toward label reliance and does not exploit the main advantage of MSI, namely, its capacity for untargeted full-spectrum analysis.

APPLICATIONS OF MSI IN DRUG DEVELOPMENT

The ongoing development of MSI technologies means that it is gradually becoming applicable to areas that were once considered unsuitable or off limits. It is likely that increases in instrument sensitivity, the development of new and improved methods of ionization, and sample preparation will continue to increase the applicability of MSI to various sample types at every stage of drug discovery and development. In addition to this great potential, there are many areas where MSI is currently being used to support drug research; this section reviews some of the most important.

LADME. A multimodal imaging technology that enables simultaneous and label-free quantitative analysis of drugs and metabolites across multiple tissue types is ideally suited for the study of drug LADME. Despite its limitations, MSI is broadly applicable in almost all areas of LADME research and the ionization methods discussed in the preceding sections (SIMS, DESI, and especially MALDI) are widely used to map the distributions of both endogenous and exogenous compounds in tissue samples at near cellular resolution.

As stated previously, QWBA is the commonly used method for studying the spatial distribution of drug related molecules and *in vivo*, even though it is incapable of distinguishing the administered compound from its labeled metabolites. However, *ex vivo* studies based on MSI (specifically, MALDI-MSI) are arguably more suitable for answering questions about the distribution of drugs and their metabolites. Interestingly, Drexler et al. have shown that QWBA and MALDI-MSI can be regarded as complementary methods for phototoxicity studies: QWBA provides the necessary quantitative imaging capabilities with an appropriate resolution while MALDI-MSI generates multiplexed data on individual molecular ions.[39] Radio-labeled and unlabeled proprietary drugs targeting the eyes were used in complementary QWBA and MALDI-MSI studies. QWBA autoradiogram data indicated that the radio-labeled compounds (which could be either the parent drug or its metabolites) were localized in the uveal tract and not the cornea, while MALDI-MSI experiments indicated that it was the drug rather than any potential metabolites that was generating the QWBA signal.

There is a need to improve and standardize quantitative MSI methodologies, and the era of simply generating impressive relative molecular abundance images by MSI is drawing to an end. This can be challenging in MSI studies because the ion signal intensity is not solely dependent on the concentration of the analyte; it is also sensitive to local suppression effects and the extractability of the relevant chemical species. We have previously reported a quantitative MALDI-MSI methodology in which a standard curve is created by spotting known concentrations of the target analyte onto control tissue sections and comparing this curve to the signal intensity observed in adjacent dosed sections. This technique was used to determine the absolute concentrations of tiotropium in different structures of the lung, revealing a concentration gradient formed within 15 min of inhalation: the drug concentration ranged from around 12 nmol/g tissue in the central airways to 7 nmol/g in the lung parenchyma and pleura.[10] Further, the potential of MSI in this area was demonstrated by a proof of principle study in which MALDI-MSI was used to map the distribution of ipratropium, an inhaled bronchodilator, in human bronchial biopsies that were extracted shortly after administration.[51] The ipratropium signal was detected in individual airway wall biopsies, and the

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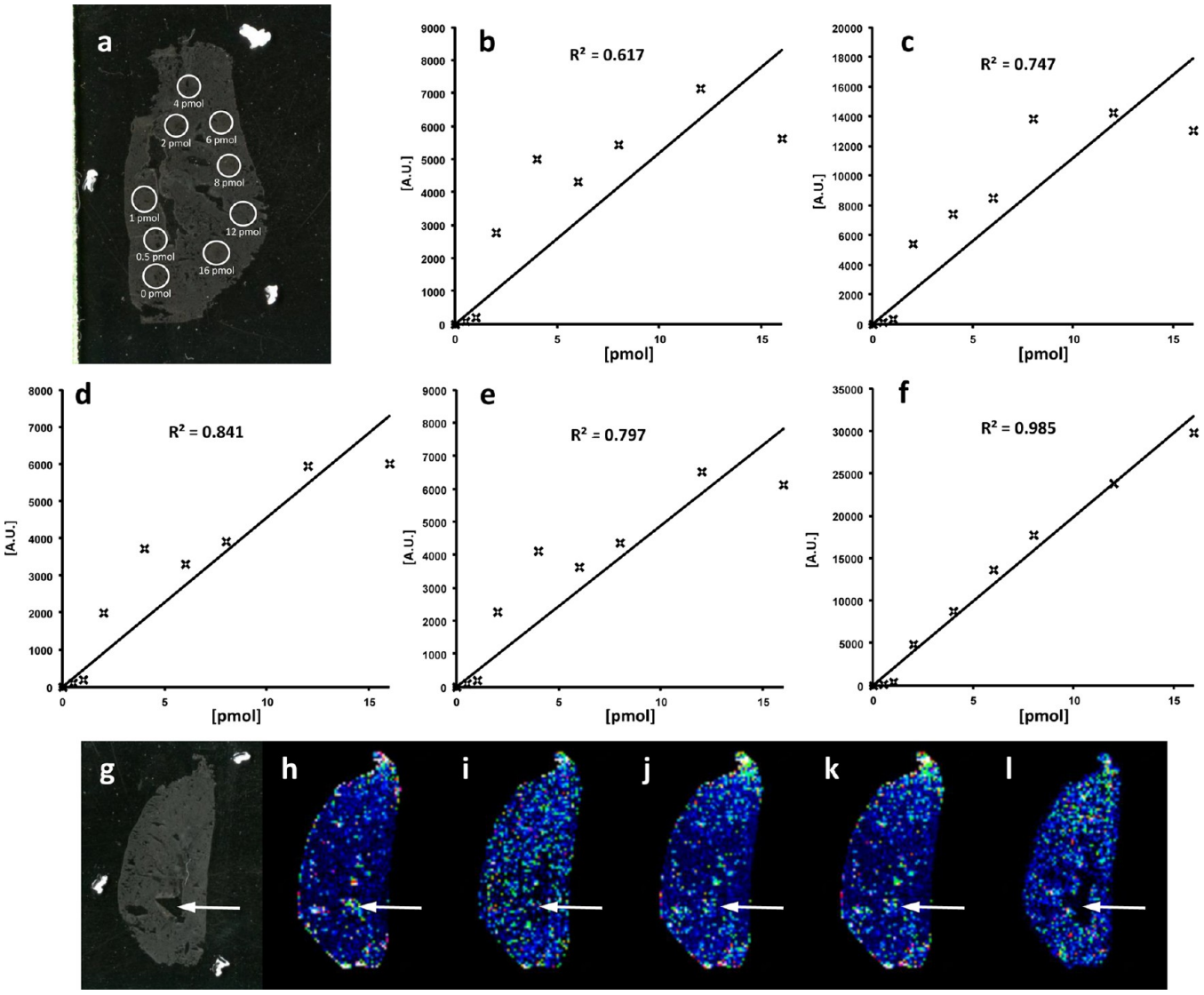

Figure 4. Effects of normalization strategies on the linearity of quantitative MSI data exemplified by calibration curves for imipramine standards. Calibration curve plots of imipramine standards using different normalization methods. (a) Digital photograph of a lung tissue section shown with different amounts of imipramine (blank, 0.5, 1, 2, 4, 6, 8, 12, and 16 pmol in 0.2 μL) applied on a rat lung tissue section. The average diameter of the spots was calculated to 1.51 mm. The correlation coefficients (R^2) for the concentration standards were calculated using the different normalization methods, (b) non-normalization, (c) median normalization, (d) total ion current (TIC) normalization, (e) root mean square (RMS) normalization, and (f) labeled normalization. The labeled normalization generated the best fit (R^2 = 0.985). MSI spatial distribution images of imipramine on central lung tissue sections from rats using different normalization methods. (g) Digital photographic images of lung tissue sections where the arrows are indicating areas inside the lung tissue section where no tissue is available. Drug distribution MSI images are shown using (h) non-normalization, (i) median normalization, (j) TIC normalization, (k) RMS normalization, or (l) labeled normalization methods. Only the labeled normalization method was able to compensate for the areas in the tissue section without tissue available. Reprinted from *Journal of Proteomics*, 75/16, Patrik Källback, Mohammadreza Shariatgorji, Anna Nilsson, Per E. Andrén, Novel mass spectrometry imaging software assisting labeled normalization and quantitation of drugs and neuropeptides directly in tissue sections, 4941−4951, ref 55. Copyright 2012, with permission from Elsevier.

highest signal intensities were associated with areas rich in smooth muscle. This study also resulted in the development of an strategy for quantitative MALDI-MSI in cases where the tissue sample is too small to accommodate the series of microliter external calibration standard droplets required to construct a calibration curve. Because the standards could not be accommodated on the tissue sample of interest, the human tissue samples of interest were imaged alongside a section of rat lung, onto which the exogenous standard was spotted; it was assumed that the relative distribution of cells and the average volume and surface area of most cell types would be consistent between the two mammalian species.[52] Quantification at high spatial resolution and accuracy has real potential for improving understanding of drug efficacy and the duration of effect of inhaled compounds.

The accuracy of quantitative MALDI-MSI can be increased further by introducing a normalization factor known as the tissue extinction coefficient (TEC) to account for ion suppression effects.[40,53] This approach enabled the quantitative imaging of exogenous propranolol and olanzapine in mouse kidney, lung, and brain tissue sections.

A similar concept to TEC has been introduced to quantify drug concentrations in tissue samples using DESI-MSI,[54] where clozapine was quantified in coronal rat brain tissue sections by

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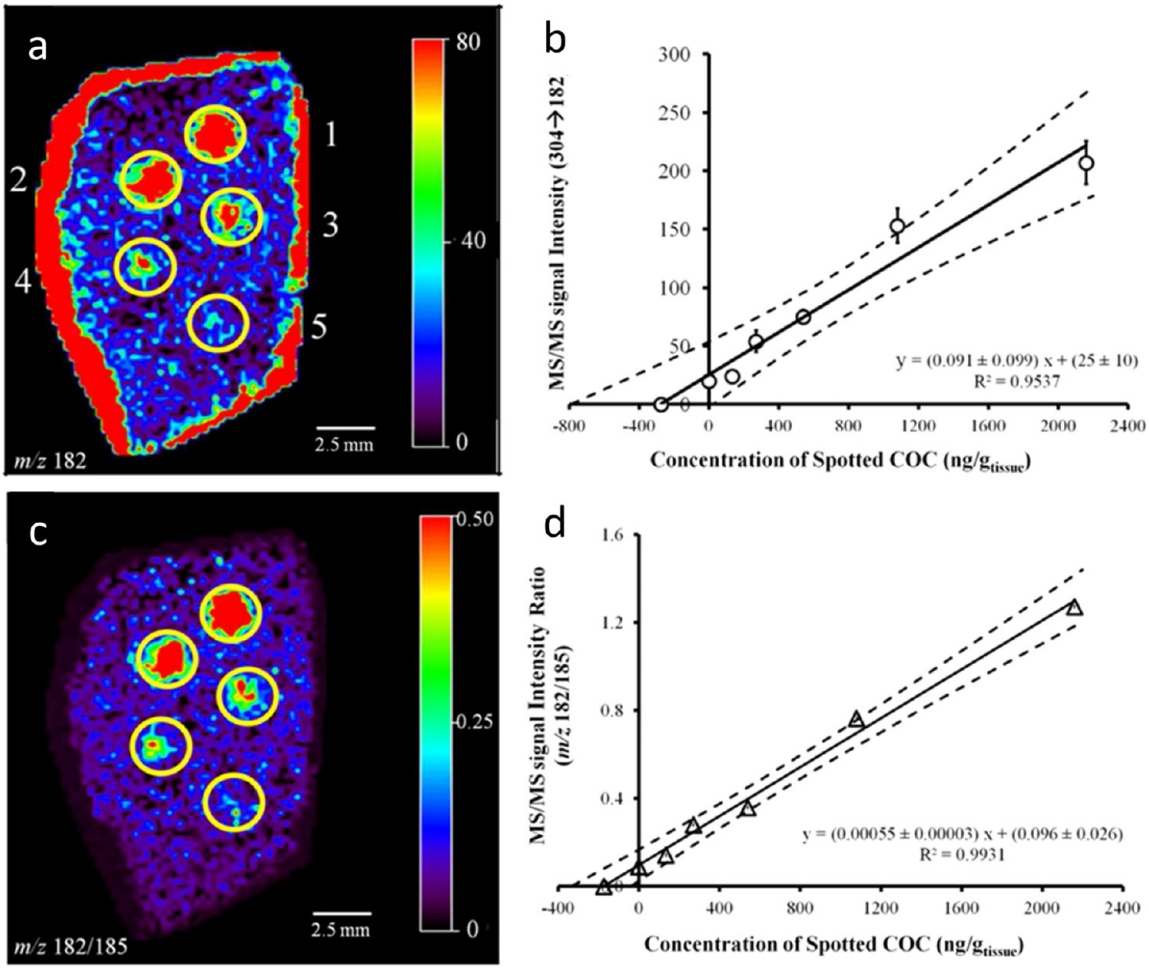

Figure 5. Results of wide-isolation MS/MSI using a Q-TOF instrument for the quantitative analysis of cocaine (COC) in rat nucleus accumbens. (a) Ion intensity of m/z 182 and (c) the ratio of m/z 182/185 were used to generate MS/MS images. Calibration spots increase in concentration from 200 (spot 5) to 2200 ng/g_{tissue} (spot 1). Corresponding standard addition plots for quantitation with (b) m/z 182 ion intensity and (d) the ratio of m/z 182/185. Normalization to the COC-d_3 improved the linearity (as indicated by the R^2 value) and the error of the calibration plot (as indicated by the relative standard deviation of the slope and intercept), leading to improved quantitative estimation of COC in the tissue. Confidence bands were calculated from the 95% confidence interval, and error bars represent the standard deviations of the mean. Reprinted from ref 63. Copyright 2013 American Chemical Society.

spotting external standards onto the tissue sections to create a calibration curve.

The quality of the MSI quantitation protocol were further improved by applying a labeled internal standard homogeneously across the tissue sample in order to detect and compensate for variations in signal strength arising from local variation in analyte extraction efficiency, cocrystallization, and ionization[55] (Figure 4). The internal standard could be a labeled version of the analyte,[55−59] a structural analogue,[60] or an endogenous molecule.[61,62] Pirman et al. reported a variant of this approach in which deuterated cocaine was used as the internal standard, and both the calibration spots and the internal standard were applied to the underside of the tissue sample rather than being placed on top of it. A wide-isolation MS/MS technique was used to detect both the analyte and the internal standard in the same MS^n scan (Figure 5). This significantly increased signal reproducibility and calibration curve linearity, and the quantitative results obtained compared very well to data from LC−MS/MS analyses of tissue extracts.[63]

Further improvements in the accuracy of quantitation were reported by Porta et al., who investigated the number of pixels needed for accurate quantitation by MALDI-MSI. Their findings suggested that accurate quantitation could not be achieved at the single pixel level and that 4−5 pixels were needed to achieve a precision better than 10−15% in MALDI single reaction monitoring (SRM) MSI quantitation.[64] This level of precision satisfies the requirements of most guidelines for quantitation within bioanalysis and toxicology.

The growing understanding of quantitative MSI and its increasing performance have prompted suggestions that it could soon become a standard tool used by DMPK groups.[65] It is worth noting that as MSI analysis becomes more powerful and more widely exploited by bioanalytical groups, the demand for instrument time is likely to increase, necessitating the development of more effective strategies for integrating MSI into the high throughput workspace of pharmaceutical research. Cassette dosing is one such strategy and has the potential to be very powerful in preclinical drug plasma pharmacokinetic analysis.[66] It has also become increasingly attractive in the early stages of the drug discovery process as a way of increasing screening throughput while decreasing the number of animals that must be used. Label-free imaging techniques with the

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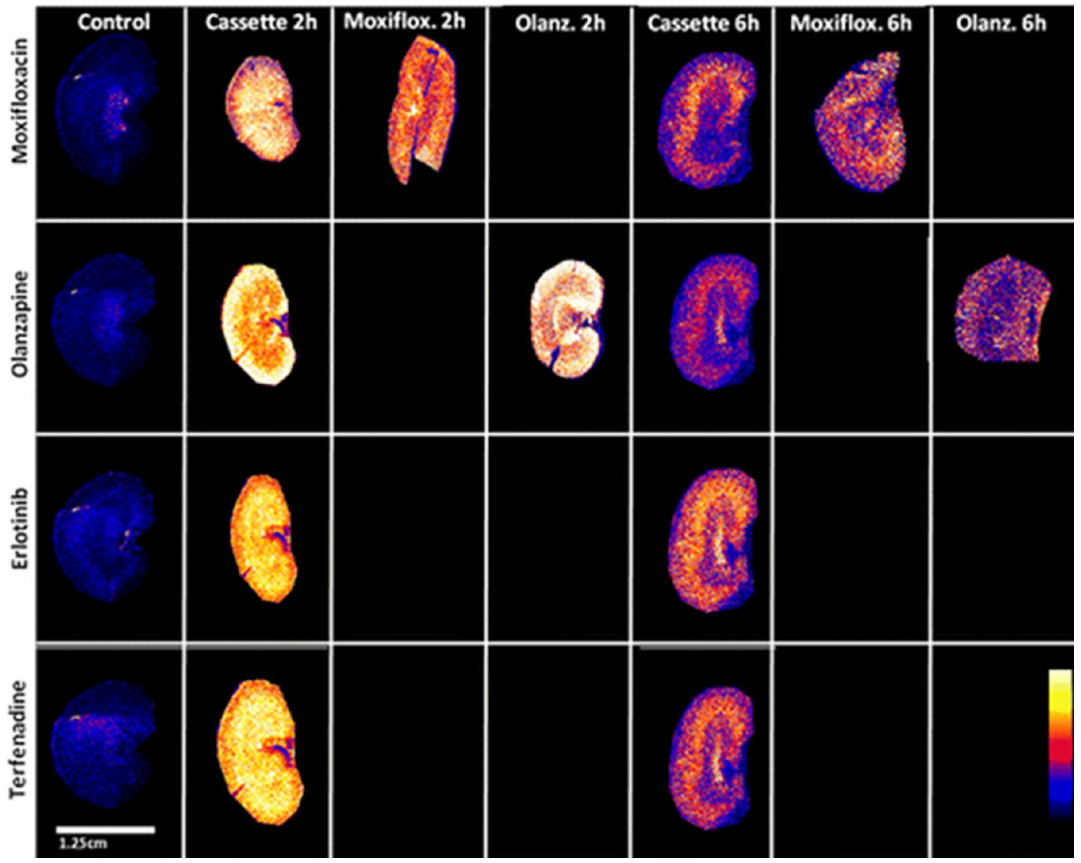

Figure 6. MALDI-MSI images showing distributions of the abundance of cassette- and discrete-dosed compounds in rat kidney sections (14 μm) at 2 and 6 h post oral dose. Moxifloxacin and olanzapine distributions are comparable at the two time points validating the cassette approach. Positive ion MALDI-MSI (using a MALDI Synapt Q-TOF instrument, Waters, Manchester, U.K.) was employed to analyze the kidney sections. Data were normalized by total ion count, at a spatial resolution of 100 μm and with a consistent signal thresholding across samples. Reprinted from ref 32. Copyright 2014 American Chemical Society.

capacity for multiplex data acquisition should arguably be preferred for such studies.

Swales et al. successfully used an array of MS based imaging technologies including MALDI-MS, liquid extraction surface analysis (LESA)-MS/MS, and DESI-MS to image the spatial distribution of multiple drugs as well as endogenous compounds in multiple tissues after oral and intravenous cassette dosing[32] (Figure 6). MALDI-MSI was successfully used to map the distribution of four compounds in tissue sections from orally cassette dosed animals with spatial resolutions of 15 and 100 μm while DESI-MS and LESA-MS/MS were used to profile the relative distributions of intravenously cassette-dosed drugs.

The unreliability of plasma measurements as indicators of tissue drug concentrations has already been touched upon but will now be considered again. Anticancer drugs must efficiently penetrate into and distribute themselves within tumors in order to have a significant therapeutic effect. MALDI-MSI has been used extensively to visualize the penetration and distribution of drugs and metabolites in tumors and healthy tissues.[67−72] However, there are two major problems that complicate MSI and specifically MALDI-MSI of small molecule drugs including anticancer agents. The first is the fact that most pharmaceuticals are small molecules whose molecular ions fall into m/z ranges that may be dominated by matrix fragments and cluster signals. The second challenge is that the drugs' concentrations in the relevant tissues may be very low when administered at therapeutic doses; in some cases they may be below the limit of detection of the chosen MSI method.[73] New matrixes[74,75] and matrix combinations[76] have been developed to address the first problem, along with modified variants of established matrix agents such as deuterated CHCA.[31] At present, the best way of overcoming the second problem is to selectively derivatize problematic small molecules to increase their ease of ionization and thereby effectively enhance the sensitivity of the chosen MSI technique.[77]

An interesting new matrix developed for pharmaceutical applications of MSI was reported by Morosi et al., who used TiO_2 as a medium to facilitate the laser desorption and ionization of the anticancer drug paclitaxel in normal and neoplastic mouse liver and kidney tissue sections. Imaging with a spatial resolution of 75 μm was achieved using this approach.[75]

Effective MALDI-MSI of drugs is strongly dependent on both the type of matrix used and the uniformity of the matrix layer. One study involves the use of dry finely ground CHCA as a matrix. This approach was adopted in a study on the quantitation and spatial mapping of two PET ligands, raclopride and SCH 23390, in rat brain and kidney tissue sections.[78] Both compounds were predominantly localized in the cerebral cortex and their detected concentrations decreased as the time after dosing increased. On-tissue quantitation was achieved by comparing the signal intensities of the dosed tissues to those observed for standard solutions spotted on

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control tissue sections. Absolute quantitation based on this approach revealed that the average raclopride concentration was approximately 60 nM after treatment with a single dose at 7.5 mg/kg (1 min post dosing) and about 15 nM after treatment with a single dose at 2 mg/kg (1 min post dosing). In addition, changes in the detected concentrations of PET ligands as a function of time after administration was investigated by imaging brains and kidney sections collected 1, 5, and 30 min post dosing. The intensity of the raclopride signal in the tissue samples declined heavily over this period.

Although SIMS imaging offers the highest spatial resolution of all MSI techniques, and has the potential to yield information on subcellular distributions, it has mainly been limited to the imaging of small ions and highly abundant lipids. It has successfully been used in combination with scanning electron microscopy (SEM) to address targeted question about issues such as drug penetration through the external barrier of the skin.[79] SEM was used to identify the individual skin layers of infected mouse ears, and TOF-SIMS imaging was used to overlay data on the distribution of roflumilast, tofacitinib, and ruxolitinib onto the SEM images. This dual approach indicated that the drugs were primarily distributed inside or on the top of the stratum corneum. The major drawback of using SIMS ionization for such applications is its low sensitivity: it can only detect compounds present at millimolar concentrations or above, which is well above the normal pharmacologically relevant concentration range.[79] However, much higher concentrations are common for drugs that are applied and delivered topically.

The potential of MALDI-MSI for assessing the penetrative capacity and localization of pharmaceuticals has also been evaluated using skin samples.[80] Indium tin oxide glass slides were modified by coronal discharge to increase the adhesion of skin tissue sections, enabling their analysis by MSI. Porcine skin samples were then treated with the model compound Nile Red and imaged by MALDI-MS at a spatial resolution of 30 μm to study the agent's skin penetration. Nile red signals were detected in the epidermis and in the vicinity of the hair follicles. The MSI data from these experiments were simultaneously used to investigate the distribution of endogenous cholesterol sulfate (a regulator of protective barrier formation) in the skin.

MALDI-MSI has also been used to characterize the liberation/release of theophylline and propranolol hydrochloride from lipid-based/slow release implants.[81] A model was constructed by embedding the cylindrical implant in gelatin, which was then frozen, sectioned, and coated with matrix solution. MALDI-MSI data provided important information on changes in the inner structure of the controlled drug delivery system before and at multiple time points after exposure to the release medium.

While most drug research is focused on small molecule targets, it is becoming increasingly important to consider proteomic changes in response to treatments or during disease progression. It could also be desirable to image therapeutic molecules or targeting systems. The mass selectivity of MSI techniques and specifically MALDI-MSI make them potentially useful tools for peptide imaging.

There is great interest in tracing the conversion and metabolism of peptides, such as the potent vasoconstrictor angiotensin II (ANG II), because insights into their biotransformation and mechanisms of action could lead to the development of new treatment strategies for important diseases.[82,83] MALDI-MSI has been used to map the distribution and relative abundances of Ang II and its degradation products in mouse kidney tissue sections that were incubated with different concentrations of Ang II for different lengths of time.[84] It was found that the peptide's various biotransformation products had distinct localization patterns and that the enzymatic processing of Ang II is dose- and time-dependent.

One way of increasing the sensitivity of MSI studies and facilitating the identification of various signals is to use an MS/MS fragmentation strategy. Bonnel et al. have demonstrated that the complementary use of different modes of MALDI-MSI such as MS, MS/MS, SRM, and multiple reaction monitoring (MRM) can be extremely valuable in LADME and DMPK studies.[85] In one notable example, olanzapine (OLZ), a neuroleptic drug for treating psychological disorders such as bipolar disorder or schizophrenia, was used as the model compound in a DMPK study focusing on mouse kidneys. The drug's spatial distribution was visualized 2 and 6 h post administration. OLZ and its metabolite, *N*-desmethyl-OLZ, were detected 2 h post administration; OLZ was primarily localized in the cortex and the medulla but the metabolite was mostly found in the renal pelvis. Studies on tissues harvested 6 h after administration indicated that the drug had largely been metabolized by this point because its signal intensity was significantly reduced while the detected concentration of the metabolite had increased substantially. In the same study, MALDI-MSI was used for the LADME analysis of BDM31343, a compound that potentiates the second-line antibiotic ethionamide, which is used to treat tuberculosis. Whole body imaging of a treated mouse using MS/MS and SRM was used to visualize the drug's distribution, showing that it was primarily localized in the brain, lung, liver, and intestine.

MSI is developing a role complementing established LADME assays due to the ability to retain the localization of parent and metabolite simultaneously. Such analysis is offering new insights that enable better understanding of compound safety and efficacy that could be missed using traditional analysis methods.

Safety Toxicology. The primary objective of toxicology studies in the drug discovery process is to evaluate the safety of potential drug candidates. The ultimate goal is then to translate the animal responses into an understanding of the risk that the agent presents in human subjects. Rapid and accurate elucidation of toxicological events enables early stage identification of problematic drug candidates that will require redevelopment or cancellation. Unfortunately, it is common for a drug candidate's efficacy in target organs to be investigated in detail during the early stages of drug development whereas associated toxicological events are analyzed less rigorously.[86]

Common techniques to assess toxicity are histopathology analysis and LC−MS and MS/MS analyses of tissue extracts. MALDI-MSI, with the ability to provide a greater level of spatial resolution than achievable by LC−MS after manual dissection of the structures of interest, enables direct comparisons of compounds' distributions and morphological patterns. The fact that the analysis can be performed in an untargeted way also enables the detection of secondary toxicity events and discrimination between the direct effects of the drug and consequences of its undesired accumulation or that of its metabolites. These factors make MSI a potentially very valuable technology for safety assessments and the investigation of toxicological events.

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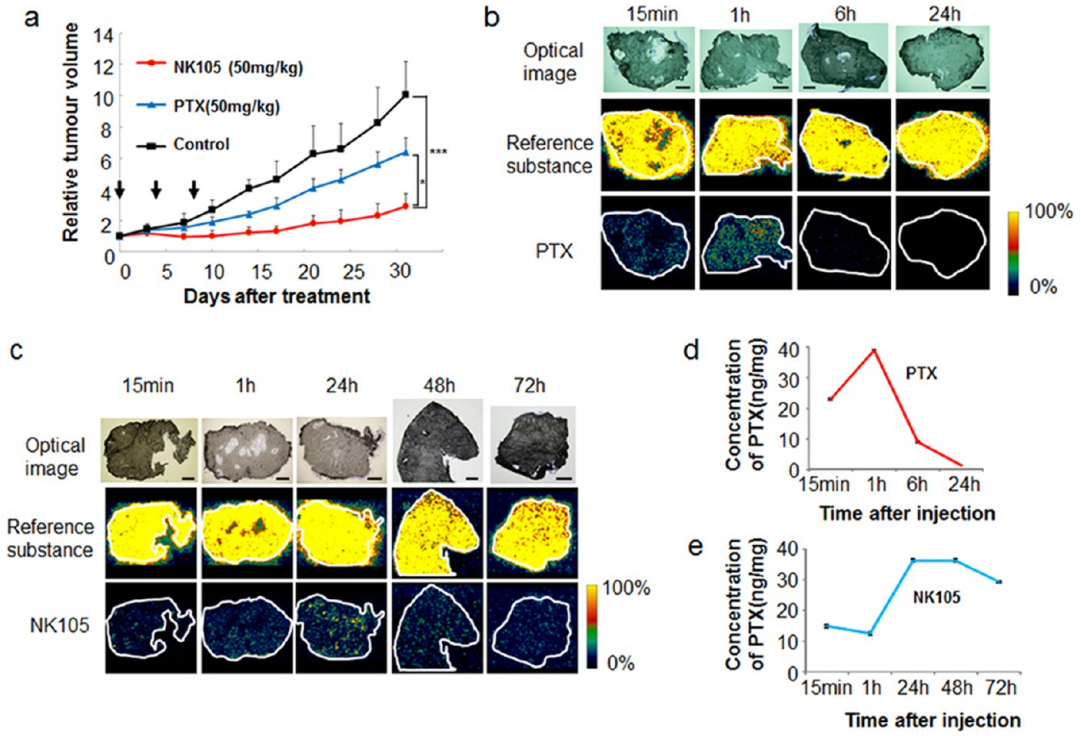

Figure 7. (a) Antitumor activity was examined in an animal model with BXPC3 xenografts. NK105, PTX, or saline (as a control) was administered at a PTX equivalent dose of 50 mg/kg on days 0, 4, and 8. *P < 0.05 (PTX vs NK105), ***P < 0.001 (saline vs NK105). Bar = SD (b, c) Imaging of PTX within the tumor was performed after PTX (b) or NK105 (c) administration at a dose of 100 mg/kg. The upper, middle, and lower columns display the optical images, reference substance (an arbitrary signal of m/z 824.6), and PTX (specific signal of m/z 892.3 $[M + K]^+$), respectively. Bar, 1 mm. (d, e) LC−MS analysis of the PTX concentration in the tumors treated by PTX (d) or NK105 (e) on tissue extracts from sections serial to those shown in parts b and c. Reprinted by permission from Macmillan Publishers Ltd.: *Scientific Reports*, ref 90 Copyright 2013.

Several studies in toxicology research utilizing MALDI-MSI have been reported including investigations into drug effects on ocular toxicity,[39] the characterization of crystal deposits in the kidney and spleen,[11,87,88] and studies on neuronal toxicity.[89,90] Identifying the contents of crystalline deposits or similar kidney lesions can be very laborious (and sometimes almost impossible) using conventional MS-coupled extraction techniques. However, this task has been performed with relative ease using TOF-SIMS[91] and MALDI-MSI in various contexts, providing important information that can be fed back into drug development projects and which could potentially support a redesign of the drug candidate to prevent the formation of e.g., toxic metabolites. More selective approaches may be required to address specific toxicological effects such as phototoxicity, which should be studied for any compound that absorbs light in the 290−700 nm range and generates a positive response in a standard in vitro phototoxicity assay.[39] Drug candidates that satisfy both criteria should be studied *in vivo* to determine the extent of their distribution (and that of their metabolites) into the substructures of the eye, because their accumulation in these tissues may influence subsequent decisions on the compound's advancement. Drexler et al. showed that MALDI-MSI and QWBA can provide complementary information on the localization and identity of small molecule drugs and their metabolites in the eyes, with QWBA providing quantitative data on the abundance of labeled species and MALDI-MSI supplying their identities.[39]

Other valuable information can be obtained by comparing different drug delivery systems in terms of their toxicity. Yasunaga et al. explored the delivery of paclitaxel into tumors with and without the nanoparticle delivery agent NK105. MSI visualization indicated that the use of NK105 enabled extended drug delivery, reducing paclitaxel's neurotoxicity in mice[90] (Figure 7). The ability to monitor drugs, their release, and their delivery systems *in situ* as a function of the target tissue's architecture will provide bioscientists and formulation groups with important insights to guide the development of improved delivery vehicles.

TOF-SIMS has also been used in safety and toxicological applications. For example, it has been used to assess the safety of sunscreen formulas containing TiO_2 or ZnO when applied to the skin, and the cytotoxicity of ZnO nanoparticles in a cell model based on human keratinocytes.[25,92] It has also been used for the localization and quantitation of a preservative, benzalkonium, on rabbit eye sections.[24]

In some cases, standard histological staining protocols are incapable of supporting meaningful diagnosis or tissue classification. MSI can be used to perform tissue classification at the molecular level and could thus be very useful to pathologists as an alternative or complement to histology for the diagnosis and characterization of toxicological findings.

Blood-Brain Barrier Permeability and Brain Distribution. The exchange of nutrients, endogenous compounds, and xenobiotics between the central nervous system (CNS) and the blood is regulated by the BBB and the blood-cerebrospinal fluid barrier (BCSFB). Although passive diffusion is the main transport pathway, transporter proteins play very important roles in BBB penetration.[93−96] The ATP- binding cassette (ABC) transporters P-glycoprotein (Pgp, MDR1, ABCB1) and breast cancer resistance protein (BCRP, ABCG2) are the most important BBB efflux proteins, with BCRP being expressed more strongly than MDR1 in the human BBB.[97] As many drugs

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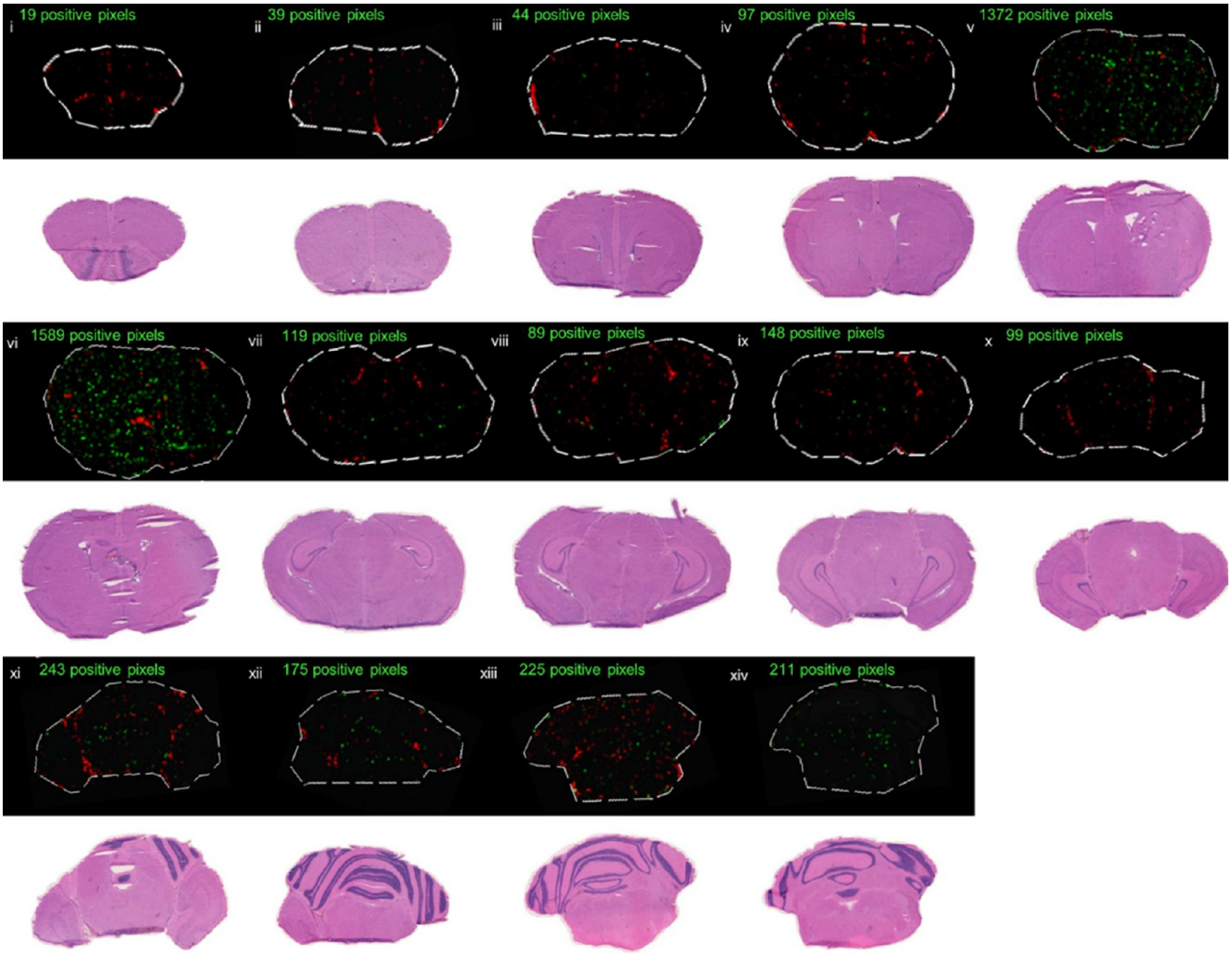

Figure 8. MALDI-MSI images of serial coronal mouse brain sections (10 μm thick) followed by the hematoxylin and eosin (H&E) stained sister sections (magenta). Sections were collected at 500 μm intervals and imaged at 100 μm spatial resolution by MALDI-MSI. Overlay images of signals from heme (red, m/z 616.1 $\pm$ 0.1) and the anticancer agent BKM120 (green, m/z 411.2 $\pm$ 0.1) show no colocalization for a significant proportion of the drug indicating that it penetrates the BBB. Regions where the drug diffusion is relatively high are also visible (v, vi, xii–xiv). Reprinted by permission from Macmillan Publishers Ltd.: *Scientific Reports*, ref 44 Copyright 2013.

are MDR1 or BCRP substrates, these proteins play central roles in determining the brain distributions of drug compounds and strongly influence drug–drug interactions. Phase I and phase II drug metabolizing enzymes such as CYP1B1 and CYP2U1 and glutathione-S-transferases (GST), respectively, have also been detected in the BBB, but they seem to be less important than the transporters.[98]

BBB penetration is particularly important in the treatment of pathological conditions such as neurological disorders (e.g., Parkinson's and Alzheimer's disease), brain tumors, or HIV-induced dementia. Drugs designed to treat these conditions must pass through the BBB in order to exert their pharmacological effects, and treatment failures in these conditions are often related to the inability of the applied drugs to cross the BBB. On the other hand, BBB penetration by peripherally acting compounds can cause side effects and CNS toxicity. It is therefore essential to determine a drug candidate's BBB permeability during the early stages of drug development.

Established methods for estimating BBB permeability include *in vitro* methods such as the parallel artificial membrane permeability and Madin–Darby canine kidney cell line permeability assays and *in vivo* techniques such as the brain perfusion assay, determinations of CSF concentrations, and *in vivo* microdialysis. There are also *in silico* and rule-based approaches, which are based on the consideration of compound physicochemical parameters that influence BBB permeability such as the polar surface area and molecular weight.[99] Imaging methods such as PET have also been employed.[100] However, there is still a need to increase the throughput of these techniques and improve their reliability.

MSI techniques could also be useful in assessing BBB permeability. As mentioned above, their major advantage over radiolabeled imaging methods such as autoradiography and PET is that they can be used to simultaneously detect the parent drug and its metabolites. In addition, they can provide detailed information about the drug's distribution in specific regions or structures of the brain, along with data on changes in neurochemical and toxicological factors associated with drug administration. However, issues related to the ionization efficiency of the studied compounds together with sensitivity limitations of MSI and the high spatial resolution required for characterization of BBB permeability must also be considered.

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There have been relatively few studies on the use of MSI techniques to assess BBB permeability, most of which have focused on MALDI-MSI or DESI-MSI. Liu et al. used MALDI-MSI to investigate the BBB permeability of three anticancer drugs.[44] They began their study by validating the usefulness of heme as a biomarker of vasculature in the brain and then demonstrated the ability of their MSI approach to detect regional differences in BBB integrity and to simultaneously image drugs and their metabolites in the brain (Figure 8). The simultaneous detection of both the parent drug and its major metabolite was also achieved for astemizole using MALDI-MSI. A considerable amount of the drug appeared to be distributed in the brain regions where its target receptors are localized, while its metabolite was detected primarily around the three ventricle sites, implying that it remains in the CSF rather than crossing the BBB.[101] It should be noted that the FTICR mass analyzer used in this work was particularly effective at distinguishing between the metabolite and the parent drug because of its high mass accuracy.[44] Another study used MALDI-MSI to investigate the BBB permeability of anticancer agents, revealing that the differences in their distribution profiles within brain tumors and healthy brain regions correlated strongly with their interactions with MDRI and BCRP and therefore with their efflux kinetics.[45] MALDI-MSI has also been used in conjunction with LC−MS to examine the pharmacokinetic and toxicological behavior of the non-nucleoside reverse transcriptase inhibitor fosdevirine in patients with HIV in order to understand its CNS toxicity. The authors investigated the BBB and BCSFB penetration of fosdevirine and its metabolites as well as its metabolism in the brain and brain endothelium in several species (rabbit, minipig, monkey). One of this study's most interesting findings was that there were species-dependent differences in the brain localization profiles of fosdevirine (monkey, gray matter) and its cysteine metabolite (rabbit and minipig, white matter).[89] The utility of MALDI-MSI as a tool for the development of anti-Alzheimer's drugs was evaluated by pairing it with a high-throughput amyloid thioflavin competitive binding optical assay to screen a set of potential amyloid-binding compounds.[102] DESI-MSI has also been used to study drug distribution in order to better understand the factors that govern brain disposition. More specifically, the whole-body distributions of four compounds were investigated by DESI-MSI. The results obtained suggested that lipophilicity was important for brain distribution because only the most lipophilic compounds from the set of four were detected in the CNS.[103] However, the small number of compounds considered limits the generalizability of these findings. Although DESI-MSI has an advantage over MALDI in that it does not require a matrix and thus avoids problems arising from matrix-related ion suppression, its maximum achievable spatial resolution is appreciably lower than that for MALDI-MSI. In addition, the limited scope for absolute quantitation using DESI-MSI has yet to be addressed.

The studies discussed above demonstrate that MSI, and particularly MALDI-MSI, have great potential in the study of drug transport across the BBB.

Biomarkers and Effects of Drugs. MSI and especially MALDI-MSI has been put forward as a versatile tool for identifying biomarkers associated with diverse diseases[104−108] and/or detecting biomolecular changes due to drug treatment.[69,109−111] Its inherent connection to histology makes it very suitable as a diagnostic tool because it generates profile signatures that can complement histopathological data. This usage has been facilitated by the development of software that enables coregistration of high spatial resolution MS images with histologically stained sections. The uniqueness in the analysis is that one can perform an untargeted analysis *in situ* that simultaneously provides information on the distributions and abundances of drugs, metabolites and endogenous compounds. MALDI-MSI has been used in this way in proteomic and metabolomic studies examining changes in the concentration of proteins,[109,110,112,113] neuropeptides,[114] lipids,[115−118] and amino acids,[119] as well as fluctuations in energy metabolism[111,120−122] associated with disease states or drug responses. The information obtained from such studies can be beneficial during drug development because it offers insights into metabolic changes linked to drug administration, providing a more complete picture of the drug response and unwanted deleterious side effects or toxicity.

One approach is to use MSI to identify markers linked to a therapeutic regime. For instance, MALDI-MSI has been used to identify markers of taxane sensitivity in breast cancer patients.[109] The taxane paclitaxel was administered with radiation and the patient cohort were divided into responders and nonresponders following treatment. MSI of tumor samples from the two cohorts revealed three significantly overexpressed proteins in the responder group. These results were confirmed by IHC. Similarly, an MSI analysis of early proteomic changes successfully predicted the treatment responses of mammary tumors in transgenic mice.[110] Early biomarkers could also be of great importance in screening new compounds for toxicity or protective efficacy. An MSI study on proteomic changes following administration of the nephrotoxicant gentamicin revealed the accumulation of the transthyretin fragment (Ser28-Gln146) in the cortex regions of the kidney, suggesting that this species may be a useful marker of nephrotoxicity.[113]

Several studies have examined the effects of drugs on energy metabolites. Atkinson et al. designed a study using AQ4N (banoxatrone), a bioreductive prodrug that is converted into a topomerase II inhibitor AQ4 in hypoxic cells. MALDI-MSI was used to determine the distibution of AQ4N and AQ4 in tumor xenographs along with the abundance and distribution of ATP. The results indicated that there was little overlap between the distributions of AQ4N and AQ4 and that no conversion of AQ4N occurred in the nonhypoxic regions where ATP levels were high. By inhibiting topoisomerase II only in hypoxic regions, AQ4N sensitizes the tumor to existing chemo- and radiotherapy treatments.[69]

In a murine model of kainate-induced seizure, Sugiura et al. imaged the distribution patterns of the energy related metabolites ATP, ADP, and AMP in the hippocampus.[111] Kainate induces severe seizures that can be observed 30 min post administration. The MSI analysis showed that ATP levels were greatly reduced whereas those of ADP and AMP changed only moderately and not at all, respectively. There was also a secondary effect during seizure that was probably caused by ATP depletion: glycolysis accelerated and TCA cycle activity may have also increased. A note of caution about these results is necessary; however, it is essential to consider the sample quality and post mortem degradation when studying the abundance of rapidly degraded biomolecules such as ATP. In the cited study, great care was taken to keep post mortem times exactly the same in all experiments, but even so it was noted that the adenylate levels observed in the brain samples were somewhat lower than those reported in studies using *in situ* freezing methods.[111,123] Similar differences between denatured and

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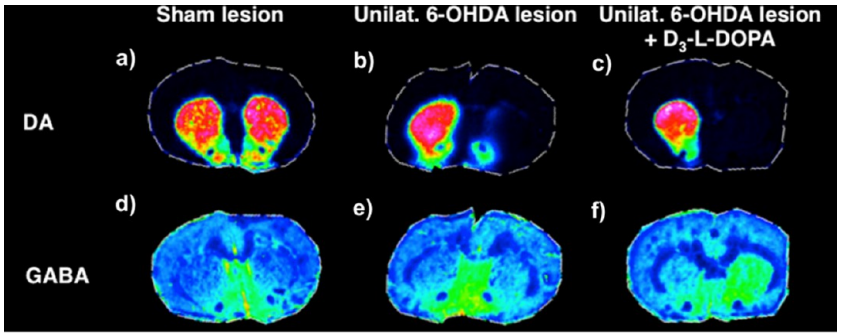

Figure 9. MALDI-MS images of dopamine and GABA in coronal rat Parkinson's Disease model tissue sections (a–f). Imaging experiments were conducted on brain tissue sections from unilateral sham-lesioned-, unilateral 6-OHDA-lesioned, and unilateral 6-OHDA-lesioned animals that were treated with subchronic L-DOPA for 4 weeks, with the final dose being given as deuterated (D_3)-L-DOPA. The images show the distributions of DA (a–c) and GABA (d–f) in sham-lesioned (a,d), 6-OHDA-lesioned (b,e), and 6-OHDA-lesioned L-DOPA-treated animals (c,f). Sham-lesioned rat brain tissue sections (a) had a normal DA distribution in their striatal regions, but the intensity of the DA signal was significantly lower in the lesioned side of the brain, with or without D3-L-DOPA treatment (b,c). The experiment also showed that L-DOPA/D3-L-DOPA treatment increased striatal GABA levels in the lesioned side of the brain (f) relative to those in sham- and 6-OHDA-lesioned brain sections (d,e). MS images were acquired using a MALDI-FTICR mass spectrometer. Data are shown using a rainbow scale, normalized against the total ion count. Scale bar, 2 mm; spatial resolution = 150 μm. Reprinted from *Neuron*, 84/4, Mohammadreza Shariatgorji, Anna Nilsson, Richard J. A. Goodwin, Patrik Källback, Nicoletta Schintu, Xiaoqun Zhang, Alan R. Crossman, Erwan Bezard, Per Svenningsson, and Per E. Andren, Direct Targeted Quantitative Molecular Imaging of Neurotransmitters in Brain Tissue Sections, 697–707, ref 77 Copyright 2014, with permission from Elsevier.

fresh-frozen tissues have previously been reported in studies on ATP levels[124] and the concentrations of other small endogenous metabolites.[36]

MSI has also been used to address the effects of a drug on triglyceride (TG) accumulation in arterial tissue, which is associated with the development of cardiovascular disease.[118] Cilostazol, a selective inhibitor of phosphodiesterase 3 with antiplatelet and vasodilative effects, were administered in a rat model of cartid artery ligation orally for 4 weeks. MSI studies revealed a reduction in TG accumulation in the animals that were given cilostazol compared to control animals.

A newly developed method for the simultaneous MSI detection and quantitation of of multiple neurochemicals has made it possible to visualize the effects of different drugs on the levels of selected neurotransmitters.[77] The methodology targets primary amine neurotransmitters such as GABA, dopamine, serotonin, and glutamate and facilitates their detection by *in situ* derivatization to enhance their ionization efficiency. Results from the analysis of a unilaterally lesioned rodent Parkinson's disease model revealed an upregulation of GABA in the lesioned hemisphere following L-DOPA treatment (Figure 9). The same study explored the effect of a centrally acting choline esterase inhibitor, tacrine, on acetylcholine (ACh) levels in mouse brain. The detection of ACh presents a different problem to that encountered when dealing with primary amines: its signal is masked by a major matrix peak when using CHCA as the ionization matrix. The ACh signal was unmasked by replacing CHCA with a deuterated analogue,[31,77] revealing that a 10 mg/kg intraperitoneal dose of tacrine caused ACh to be upregulated by a factor of 7 while the concentration of its precursor alpha-GPC declined (Figure 10).

MALDI-MSI cannot yet match the performance of established methods for intracellular imaging. This is a problem because many drug act different in different intracellular compartments, so it is necessary to measure their concentration at the subcellular level in order to accurately predict their effects. Measurements of intracellular concentrations are not easy, but some progress in this direction has been made by using the SIMS and nanoSIMS ionization techniques.[125] For example, SIMS was used to investigate the effect of the chemotherapeutic drug cisplatin on the cellular level at a spatial resolution of 500 nm in a pig kidney cell line. Cisplatin is known to induce cell death but its mechanism of action is not yet clear.[126−128] Further details of its mechanism are urgently needed because it is associated with severe nephrotoxicity. The SIMS analysis revealed that cells treated with cisplatin exhibited disrupted calcium homeostasis and effects on sodium and potassium were also observed for some of the treated cells.[129]

Although MSI and in particular MALDI-MSI have proven successful in detecting potential biomarkers for a disease state or a drug effect, much work remains to be done before these markers can be used in the clinic or drug development. However, MSI has the potential to greatly complement existing tools such as WBA, conventional LC–MS and MS/MS proteomic analyses, and histopathology.

FUTURE PERSPECTIVES

MSI has gained acceptance as a valuable tool for drug and metabolite studies. The ability to directly and rapidly localize drugs, metabolites, and other endogenous molecules simultaneously *in situ* without needing any prelabeling presents an advantage over conventional techniques such as WBA.

Even though MSI sometimes suffers from sensitivity problems, its technical advancement over the past few years has enabled analyses at ever-greater spectral and spatial resolutions and dramatic increases in sensitivity.[77,130−133] There is however still a need for improvement of these parameters as well as sampling speed and improved data analysis/handling programs. New emerging techniques for MSI ionization and sampling might also complement information obtained by methods such as MALDI-MSI. By better defining the questions we want answered by MSI, it will be possible to make a more informed decision concerning which technique should be used in a given case, for example, it will be possible to determine whether to prioritize high spatial resolution or sensitivity. Moreover, as researchers come to accept that a failure to detect a compound may just reflect a lack of sensitivity, there will hopefully be a movement away from high resolution "pretty pictures" to analyses with lower spatial resolutions but also lower limits of detection by tissue

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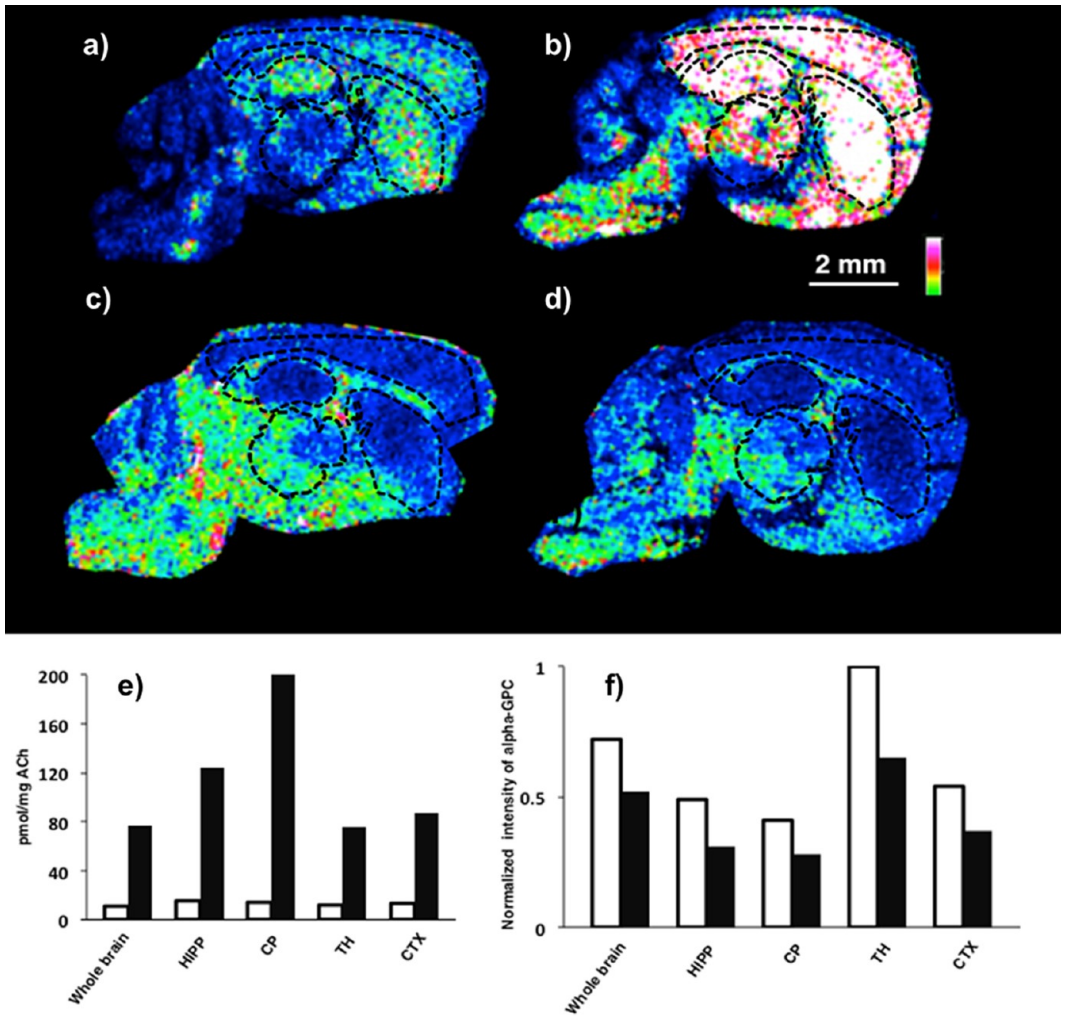

Figure 10. MALDI-MS images of ACh and Alpha-GPC concentrations in sagittal mouse brain sections from a control animal and after administration of 10 mg/kg of the cholinesterase inhibitor tacrine (a–d). The absolute concentration of ACh in the control brain section (a) was about 7 times lower than that observed following the tacrine injection (b). Conversely, the concentration of the ACh precursor alpha-GPC was higher in the control sample (c) than in the tacrine-treated brain (d). The overall levels of alpha-GPC were reduced following tacrine treatment. The absolute concentrations of ACh in the control (A) and tacrine-treated (B) mouse brains were quantified using a calibration standard curve. (e and f) Bar graphs showing the absolute concentration of ACh (e) and relative concentration of alpha-GPC (f) with (black bars) and without (white bars) administration of tacrine in whole brains and selected structures. MS images were acquired using a MALDI-TOF/TOF mass spectrometer. Data are shown using a rainbow scale over a single range, normalized against the total ion count. Scale bar, 2 mm; spatial resolution = 100 μm. Caudate-putamen, CP; hippocampus, HIPP; cerebral cortex, CTX; thalamus, TH. Reprinted from *Neuron*, 84/4, Mohammadreza Shariatgorji, Anna Nilsson, Richard J. A. Goodwin, Patrik Källback, Nicoletta Schintu, Xiaoqun Zhang, Alan R. Crossman, Erwan Bezard, Per Svenningsson, and Per E. Andren, Direct Targeted Quantitative Molecular Imaging of Neurotransmitters in Brain Tissue Sections, 697–707, ref 77 Copyright 2014, with permission from Elsevier.

profiling.[134,135] On the other hand, answers to some questions really do require accurate and detailed information at the cellular level, so improvements in absolute spatial resolution will continue to be a major factor in many areas of pharmaceutical MSI, with near- and subcellular analysis required for the study of multiple diseases and therapeutic areas on a single instrument or within a single experiment.[131]

There is also great scope for improvement in sample preparation techniques. The development of new matrixes that are more suitable for a wider range of molecules in the low molecular weight area and on-sample chemistry that enhances sensitivity for a specific molecule or group of molecules are two areas that should be explored further. The idea is to increase the ionization efficiency of target compounds and hence aid their detection and quantitation. Derivatization protocols have been applied to detect drugs,[136] neurotransmitters,[77,137] and endogenous neutral steroids.[138] Finally, another area that could be further developed is photocleavable mass-tag technologies such as those used to probe tissues.[50,139] A small molecule mass-tag approach is less complex than an untargeted, full spectrum analysis and may thus be useful in enabling the future analysis of larger molecular weight biologics.

Aside from technical and practical aspects, there is great potential for more widespread use of MSI in drug discovery and in other areas of pharmaceutical research and development such as formulation/production, galenics, and clinical studies. MSI is already established within many DMPK departments, where experts in bioanalysis have embraced the ability to performing label-free compound and metabolite biodistribution studies in conjunction with (or as an alternative to) traditional

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autoradiographic methods. MSI seems to be particularly attractive in fields where the ability to performing untargeted exploratory experiments is important, such as pharmatoxicology.[11] The technique also has the potential to provide a greater mechanistic understanding compounds' effects, which could facilitate candidate design and selection during the discovery phase, hopefully reducing attrition later on. In addition, it could be used to validate plasma concentration data, which often serve as a quantitative reference point for translation, or it could be a valuable tool for characterizing the heterogeneity of biomarker responses in target and nontarget tissues.

MSI is often referred to as a multiplex analysis that maps the masses of thousands of chemical species across tissue samples, although usually it is only the target mass and at best a few endogenous compounds that are characterized. In the future, it can be anticipated that there will be a better connection between the distributions of xenobiotics, endogenous metabolites, and larger molecules producing a more complete picture of "true molecular histology".

In conclusion, MSI techniques have evolved significantly over the last decade, such that the improvements in spatial resolution, sensitivity and overall robustness mean that MSI is becoming an attractive option for addressing key drug discovery and development issues. The range of ionization techniques available enables the distribution a wide range of drugs, metabolites and endogenous biomarkers to be described at near cellular resolution, addressing fundamental questions of drug efficacy and safety. Future enhancements in quantification protocols, particularly for the higher spatial resolution techniques, have the potential to revolutionize our understanding of drug disposition at the cellular and sub-cellular levels.

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REFERENCES

(1) Hughes, J. P.; Rees, S.; Kalindjian, S. B.; Philpott, K. L. *Br. J. Pharmacol.* **2011**, *162*, 1239−1249.
(2) Rudin, M.; Weissleder, R. *Nat. Rev. Drug Discovery* **2003**, *2*, 123−131.
(3) Willmann, J. K.; van Bruggen, N.; Dinkelborg, L. M.; Gambhir, S. S. *Nat. Rev. Drug Discovery* **2008**, *7*, 591−607.
(4) Ballard, P.; Brassil, P.; Bui, K. H.; Dolgos, H.; Petersson, C.; Tunek, A.; Webborn, P. J. H. *Drug Metab. Rev.* **2012**, *44*, 224−252.
(5) Rowland, M.; Peck, C.; Tucker, G. *Annu. Rev. Pharmacol. Toxicol.* **2011**, *51*, 45−73.
(6) Prideaux, B.; Stoeckli, M. *J. Proteomics* **2012**, *75*, 4999−5013.
(7) James, M. L.; Gambhir, S. S. *Physiol. Rev.* **2012**, *92*, 897−965.
(8) Prideaux, B.; Staab, D.; Stoeckli, M. *Methods Mol. Biol.* **2010**, *656*, 405−413.
(9) Shariatgorji, M.; Svenningsson, P.; Andren, P. E. *Neuropsychopharmacology* **2014**, *39*, 34−49.

DOI: 10.1021/ac504734s
Anal. Chem. 2015, 87, 1437−1455

(10) Nilsson, A.; Fehniger, T. E.; Gustavsson, L.; Andersson, M.; Kenne, K.; Marko-Varga, G.; Andren, P. E. *PLoS One* **2010**, *5*, e11411.
(11) Nilsson, A.; Forngren, B.; Bjurstrom, S.; Goodwin, R. J.; Basmaci, E.; Gustafsson, I.; Annas, A.; Hellgren, D.; Svanhagen, A.; Andren, P. E.; Lindberg, J. *PLoS One* **2012**, *7*, e47353.
(12) Norris, J. L.; Caprioli, R. M. *Chem. Rev.* **2013**, *113*, 2309−2342.
(13) Stoeckli, M.; Chaurand, P.; Hallahan, D. E.; Caprioli, R. M. *Nat. Med.* **2001**, *7*, 493−496.
(14) Seeley, E. H.; Schwamborn, K.; Caprioli, R. M. *J. Biol. Chem.* **2011**, *286*, 25459−25466.
(15) Kim, J. H.; Kim, J. H.; Ahn, B. J.; Park, J. H.; Shon, H. K.; Yu, Y. S.; Moon, D. W.; Lee, T. G.; Kim, K. W. *Biophys. J.* **2008**, *94*, 4095−4102.
(16) Bennet, R. V.; Gamage, C. M.; Fernandez, F. M. *J. Vis. Exp.* **2013**, e50575.
(17) Gode, D.; Volmer, D. A. *Analyst* **2013**, *138*, 1289−1315.
(18) Bich, C.; Touboul, D.; Brunelle, A. *Mass Spectrom. Rev.* **2013**, 442−451.
(19) Goodwin, R. J. *J. Proteomics* **2012**, *75*, 4893−4911.
(20) Solon, E.; Schweitzer, A.; Stoeckli, M.; Prideaux, B. *AAPS J.* **2010**, *12*, 11−26.
(21) Weaver, E. M.; Hummon, A. B. *Adv. Drug Delivery Rev.* **2013**, *65*, 1039−1055.
(22) Castaing, R.; Slodzian, G. *J. Microsc* **1962**, *1*, 395−410.
(23) Liebl, H. *J. Appl. Phys.* **1967**, *38*, 5277−5283.
(24) Desbenoit, N.; Schmitz-Afonso, I.; Baudouin, C.; Laprevote, O.; Touboul, D.; Brignole-Baudouin, F.; Brunelle, A. *Anal. Bioanal. Chem.* **2013**, *405*, 4039−4049.
(25) Lee, P. L.; Chen, B. C.; Gollavelli, G.; Shen, S. Y.; Yin, Y. S.; Lei, S. L.; Jhang, C. L.; Lee, W. R.; Ling, Y. C. *J. Hazard. Mater.* **2014**, *277*, 3−12.
(26) Pajander, J.; Haugshøj, K. B.; Bjørneboe, K.; Wahlberg, P.; Rantanen, J. *J. Pharm. Biomed. Anal.* **2013**, *80*, 116−125.
(27) Sole-Domenech, S.; Sjovall, P.; Vukojevic, V.; Fernando, R.; Codita, A.; Salve, S.; Bogdanovic, N.; Mohammed, A. H.; Hammarstrom, P.; Nilsson, K. P.; LaFerla, F. M.; Jacob, S.; Berggren, P. O.; Gimenez-Llort, L.; Schalling, M.; Terenius, L.; Johansson, B. *Acta Neuropathol.* **2013**, *125*, 145−157.
(28) Takats, Z.; Wiseman, J. M.; Gologan, B.; Cooks, R. G. *Science* **2004**, *306*, 471−473.
(29) Ifa, D. R.; Wiseman, J. M.; Song, Q. Y.; Cooks, R. G. *Int. J. Mass Spectrom.* **2007**, *259*, 8−15.
(30) Caprioli, R. M.; Farmer, T. B.; Gile, J. *Anal. Chem.* **1997**, *69*, 4751−4760.
(31) Shariatgorji, M.; Nilsson, A.; Goodwin, R. J. A.; Svenningsson, P.; Schintu, N.; Banka, Z.; Kladni, L.; Hasko, T.; Szabo, A.; Andren, P. E. *Anal. Chem.* **2012**, *84*, 7152−7157.
(32) Swales, J. G.; Tucker, J. W.; Strittmatter, N.; Nilsson, A.; Cobice, D.; Clench, M. R.; Mackay, C. L.; Andren, P. E.; Takats, Z.; Webborn, P. J.; Goodwin, R. J. *Anal. Chem.* **2014**, *86*, 8473−8480.
(33) Abbassi-Ghadi, N.; Veselkov, K.; Kumar, S.; Huang, J.; Jones, E.; Strittmatter, N.; Kudo, H.; Goldin, R.; Takats, Z.; Hanna, G. B. *Chem. Commun. (Cambridge, U. K.)* **2014**, *50*, 3661−3664.
(34) Veselkov, K. A.; Mirnezami, R.; Strittmatter, N.; Goldin, R. D.; Kinross, J.; Speller, A. V.; Abramov, T.; Jones, E. A.; Darzi, A.; Holmes, E.; Nicholson, J. K.; Takats, Z. *Proc. Natl. Acad. Sci. U.S.A.* **2014**, *111*, 1216−1221.
(35) Skold, K.; Svensson, M.; Norrman, M.; Sjogren, B.; Svenningsson, P.; Andren, P. E. *Proteomics* **2007**, *7*, 4445−4456.
(36) Goodwin, R. J.; Iverson, S. L.; Andren, P. E. *Rapid Commun. Mass Spectrom.* **2012**, *26*, 494−498.
(37) Goodwin, R. J. A.; Pennington, S. R.; Pitt, A. R. *Proteomics* **2008**, *8*, 3785−3800.
(38) Jehl, B.; Bauer, R.; Dorge, A.; Rick, R. *J. Microsc.* **1981**, *123*, 307−309.
(39) Drexler, D. M.; Tannehill-Gregg, S. H.; Wang, L.; Brock, B. J. *J. Pharmacol. Toxicol. Methods* **2011**, *63*, 205−208.
(40) Hamm, G.; Bonnel, D.; Legouffe, R.; Pamelard, F.; Delbos, J.-M.; Bouzom, F.; Stauber, J. *J. Proteomics* **2012**, *75*, 4952−4961.
(41) Amstalden van Hove, E. R.; Smith, D. F.; Heeren, R. M. A. *J. Chromatogr., A* **2010**, *1217*, 3946−3954.
(42) Jain, R. K. *Sci. Am.* **1994**, *271*, 58−65.
(43) Bianga, J.; Bouslimani, A.; Bec, N.; Quenet, F.; Mounicou, S.; Szpunar, J.; Bouyssiere, B.; Lobinski, R.; Larroque, C. *Metallomics* **2014**, *6*, 1382−1386.
(44) Liu, X.; Ide, J. L.; Norton, I.; Marchionni, M. A.; Ebling, M. C.; Wang, L. Y.; Davis, E.; Sauvageot, C. M.; Kesari, S.; Kellersberger, K. A.; Easterling, M. L.; Santagata, S.; Stuart, D. D.; Alberta, J.; Agar, J. N.; Stiles, C. D.; Agar, N. Y. *Sci. Rep.* **2013**, *3*, 2859.
(45) Salphati, L.; Shahidi-Latham, S.; Quiason, C.; Barck, K.; Nishimura, M.; Alicke, B.; Pang, J.; Carano, R. A.; Olivero, A. G.; Phillips, H. S. *Drug Metab. Dispos.* **2014**, *42*, 1110−1116.
(46) Passarelli, M. K.; Winograd, N. *Surf. Interface Anal.* **2011**, *43*, 269−271.
(47) Carter, C. L.; McLeod, C. W.; Bunch, J. *J. Am. Soc. Mass Spectrom.* **2011**, *22*, 1991−1998.
(48) Casadonte, R.; Caprioli, R. M. *Nat. Protoc.* **2011**, *6*, 14.
(49) Angelo, M.; Bendall, S. C.; Finck, R.; Hale, M. B.; Hitzman, C.; Borowsky, A. D.; Levenson, R. M.; Lowe, J. B.; Liu, S. D.; Zhao, S.; Natkunam, Y.; Nolan, G. P. *Nat. Med.* **2014**, *20*, 436−442.
(50) Stauber, J.; Ayed, M. E.; Wisztorski, M.; Salzet, M.; Fournier, I. *Methods Mol. Biol.* **2010**, *656*, 339−361.
(51) Fehniger, T. E.; Vegvari, A.; Rezeli, M.; Prikk, K.; Ross, P.; Dahlback, M.; Edula, G.; Sepper, R.; Marko-Varga, G. *Anal. Chem.* **2011**, *83*, 8329−8336.
(52) Crapo, J. D.; Young, S. L.; Fram, E. K.; Pinkerton, K. E.; Barry, B. E.; Crapo, R. O. *Am. Rev. Respir. Dis.* **1983**, *128*, S42−46.
(53) Hochart, G.; Hamm, G.; Stauber, J. *Bioanalysis* **2014**, *6*, 2775−2788.
(54) Vismeh, R.; Waldon, D. J.; Teffera, Y.; Zhao, Z. *Anal. Chem.* **2012**, *84*, 5439−5445.
(55) Kallback, P.; Shariatgorji, M.; Nilsson, A.; Andren, P. E. *J. Proteomics* **2012**, *75*, 4941−4951.
(56) Pirman, D. A.; Kiss, A.; Heeren, R. M.; Yost, R. A. *Anal. Chem.* **2013**, *85*, 1090−1096.
(57) Clemis, E. J.; Smith, D. S.; Camenzind, A. G.; Danell, R. M.; Parker, C. E.; Borchers, C. H. *Anal. Chem.* **2012**, *84*, 3514−3522.
(58) Porta, T.; Grivet, C.; Kraemer, T.; Varesio, E.; Hopfgartner, G. *Anal. Chem.* **2011**, *83*, 4266−4272.
(59) Reich, R. F.; Cudzilo, K.; Levisky, J. A.; Yost, R. A. *J. Am. Soc. Mass Spectrom.* **2010**, *21*, 564−571.
(60) Prideaux, B.; Dartois, V.; Staab, D.; Weiner, D. M.; Goh, A.; Via, L. E.; Barry, C. E., 3rd; Stoeckli, M. *Anal. Chem.* **2011**, *83*, 2112−2118.
(61) Rohner, T. C.; Staab, D.; Stoeckli, M. *Mech. Ageing Dev.* **2005**, *126*, 177−185.
(62) Wiseman, J. M.; Ifa, D. R.; Zhu, Y. X.; Kissinger, C. B.; Manicke, N. E.; Kissinger, P. T.; Cooks, R. G. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 18120−18125.
(63) Pirman, D. A.; Reich, R. F.; Kiss, A.; Heeren, R. M. A.; Yost, R. A. *Anal. Chem.* **2013**, *85*, 1081−1089.
(64) Porta, T.; Lesur, A.; Varesio, E.; Hopfgartner, G. *Anal. Bioanal. Chem.* **2014**, DOI: 10.1007/s00216-014-8315-5.
(65) Lietz, C. B.; Gemperline, E.; Li, L. *Adv. Drug Delivery Rev.* **2013**, *65*, 1074−1085.
(66) Smith, N. F.; Raynaud, F. I.; Workman, P. *Mol. Cancer Ther.* **2007**, *6*, 428−440.
(67) Troendle, F. J.; Reddick, C. D.; Yost, R. A. *J. Am. Soc. Mass Spectrom.* **1999**, *10*, 1315−1321.
(68) Cornett, D. S.; Frappier, S. L.; Caprioli, R. M. *Anal. Chem.* **2008**, *80*, 5648−5653.
(69) Atkinson, S. J.; Loadman, P. M.; Sutton, C.; Patterson, L. H.; Clench, M. R. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 1271−1276.
(70) Marko-Varga, G.; Fehniger, T. E.; Rezeli, M.; Dome, B.; Laurell, T.; Vegvari, A. *J. Proteomics* **2011**, *74*, 982−992.
(71) Sugihara, Y.; Vegvari, A.; Welinder, C.; Jonsson, G.; Ingvar, C.; Lundgren, L.; Olsson, H.; Breslin, T.; Wieslander, E.; Laurell, T.; Rezeli, M.; Jansson, B.; Nishimura, T.; Fehniger, T. E.; Baldetorp, B.; Marko-Varga, G. *Proteomics* **2014**, *14*, 1963−1970.

DOI: 10.1021/ac504734s
Anal. Chem. 2015, 87, 1437−1455

(72) Vegvari, A.; Fehniger, T. E.; Rezeli, M.; Laurell, T.; Dome, B.; Jansson, B.; Welinder, C.; Marko-Varga, G. *J. Proteome Res.* **2013**, *12*, 5626−5633.
(73) Morosi, L.; Zucchetti, M.; D'Incalci, M.; Davoli, E. *Curr. Opin. Pharmacol.* **2013**, *13*, 807−812.
(74) Wang, X.; Han, J.; Chou, A.; Yang, J.; Pan, J.; Borchers, C. H. *Anal. Chem.* **2013**, *85*, 7566−7573.
(75) Morosi, L.; Spinelli, P.; Zucchetti, M.; Pretto, F.; Carra, A.; D'Incalci, M.; Giavazzi, R.; Davoli, E. *PLos One* **2013**, *8*, e72532.
(76) Shanta, S. R.; Kim, T. Y.; Hong, J. H.; Lee, J. H.; Shin, C. Y.; Kim, K. H.; Kim, Y. H.; Kim, S. K.; Kim, K. P. *Analyst* **2012**, *137*, 5757−5762.
(77) Shariatgorji, M.; Nilsson, A.; Goodwin, R. J.; Kallback, P.; Schintu, N.; Zhang, X.; Crossman, A. R.; Bezard, E.; Svenningsson, P.; Andren, P. E. *Neuron* **2014**, *84*, 697−707.
(78) Goodwin, R. J.; Mackay, C. L.; Nilsson, A.; Harrison, D. J.; Farde, L.; Andren, P. E.; Iverson, S. L. *Anal. Chem.* **2011**, *83*, 9694−9701.
(79) Sjovall, P.; Greve, T. M.; Clausen, S. K.; Möller, K.; Eirefelt, S.; Johansson, B.; Nielsen, K. T. *Anal. Chem.* **2014**, *86*, 3443−3452.
(80) Enthaler, B.; Pruns, J. K.; Wessel, S.; Rapp, C.; Fischer, M.; Wittern, K. P. *Anal Bioanal Chem.* **2012**, *402*, 1159−1167.
(81) Kreye, F.; Hamm, G.; Karrout, Y.; Legouffe, R.; Bonnel, D.; Siepmann, F.; Siepmann, J. *J. Controlled Release* **2012**, *161*, 98−108.
(82) Der Sarkissian, S.; Huentelman, M. J.; Stewart, J.; Katovich, M. J.; Raizada, M. K. *Prog. Biophys. Mol. Biol.* **2006**, *91*, 163−198.
(83) Raizada, M. K.; Ferreira, A. J. *J. Cardiovasc. Pharmacol.* **2007**, *50*, 112−119.
(84) Grobe, N.; Elased, K. M.; Cool, D. R.; Morris, M. *Am. J. Physiol. Endocrinol. Metab.* **2012**, *302*, E1016−1024.
(85) Bonnel, D.; Legouffe, R.; Willand, N.; Baulard, A.; Hamm, G.; Deprez, B.; Stauber, J. *Bioanalysis* **2011**, *3*, 1399−1406.
(86) Pellegatti, M.; Pagliarusco, S. *Expert Opin. Drug Metab. Toxicol.* **2011**, *7*, 137−146.
(87) Drexler, D. M.; Garrett, T. J.; Cantone, J. L.; Diters, R. W.; Mitroka, J. G.; Prieto Conaway, M. C.; Adams, S. P.; Yost, R. A.; Sanders, M. *J. Pharmacol. Toxicol. Methods* **2007**, *55*, 279−288.
(88) Kim, C. W.; Yun, J. W.; Bae, I. H.; Lee, J. S.; Kang, H. J.; Joo, K. M.; Jeong, H. J.; Chung, J. H.; Park, Y. H.; Lim, K. M. *Chem. Res. Toxicol.* **2010**, *23*, 220−227.
(89) Castellino, S.; Groseclose, M. R.; Sigafoos, J.; Wagner, D.; de Serres, M.; Polli, J. W.; Romach, E.; Myer, J.; Hamilton, B. *Chem. Res. Toxicol.* **2013**, *26*, 241−251.
(90) Yasunaga, M.; Furuta, M.; Ogata, K.; Koga, Y.; Yamamoto, Y.; Takigahira, M.; Matsumura, Y. *Sci. Rep* **2013**, *3*, 3050.
(91) Levin, S.; Friedman, R. M.; Cortez, E.; Hribar, J.; Nicholas, M.; Schlessinger, S.; Fouant, M.; Khan, N. *Toxicol. Pathol.* **1999**, *27*, 38−43.
(92) Monteiro-Riviere, N. A.; Wiench, K.; Landsiedel, R.; Schulte, S.; Inman, A. O.; Riviere, J. E. *Toxicol. Sci.* **2011**, *123*, 264−280.
(93) Abbott, N. J.; Patabendige, A. A.; Dolman, D. E.; Yusof, S. R.; Begley, D. J. *Neurobiol. Dis.* **2010**, *37*, 13−25.
(94) Eyal, S.; Hsiao, P.; Unadkat, J. D. *Pharmacol. Ther.* **2009**, *123*, 80−104.
(95) International Transporter, C.; Giacomini, K. M.; Huang, S. M.; Tweedie, D. J.; Benet, L. Z.; Brouwer, K. L.; Chu, X.; Dahlin, A.; Evers, R.; Fischer, V.; Hillgren, K. M.; Hoffmaster, K. A.; Ishikawa, T.; Keppler, D.; Kim, R. B.; Lee, C. A.; Niemi, M.; Polli, J. W.; Sugiyama, Y.; Swaan, P. W.; Ware, J. A.; Wright, S. H.; Yee, S. W.; Zamek-Gliszczynski, M. J.; Zhang, L. *Nat. Rev. Drug Discovery* **2010**, *9*, 215−236.
(96) Pardridge, W. M. *J. Cereb. Blood Flow Metab.* **2012**, *32*, 1959−1972.
(97) Ohtsuki, S.; Ikeda, C.; Uchida, Y.; Sakamoto, Y.; Miller, F.; Glacial, F.; Decleves, X.; Scherrmann, J. M.; Couraud, P. O.; Kubo, Y.; Tachikawa, M.; Terasaki, T. *Mol. Pharmaceutics* **2013**, *10*, 289−296.
(98) Dauchy, S.; Dutheil, F.; Weaver, R. J.; Chassoux, F.; Daumas-Duport, C.; Couraud, P. O.; Scherrmann, J. M.; De Waziers, I.; Decleves, X. *J. Neurochem.* **2008**, *107*, 1518−1528.
(99) Di, L.; Kerns, E. H.; Carter, G. T. *Expert Opin. Drug Discovery* **2008**, *3*, 677−687.
(100) Syvanen, S.; Hammarlund-Udenaes, M. *Curr. Top. Med. Chem.* **2010**, *10*, 1799−1809.
(101) Li, F.; Hsieh, Y.; Kang, L.; Sondey, C.; Lachowicz, J.; Korfmacher, W. A. *Bioanalysis* **2009**, *1*, 299−307.
(102) McClure, R. A.; Chumbley, C. W.; Reyzer, M. L.; Wilson, K.; Caprioli, R. M.; Gore, J. C.; Pham, W. *Neuroimage Clin.* **2013**, *2*, 620−629.
(103) Liu, J.; Gingras, J.; Ganley, K. P.; Vismeh, R.; Teffera, Y.; Zhao, Z. *Rapid Commun. Mass Spectrom.* **2014**, *28*, 185−190.
(104) Balluff, B.; Rauser, S.; Meding, S.; Elsner, M.; Schone, C.; Feuchtinger, A.; Schuhmacher, C.; Novotny, A.; Jutting, U.; Maccarrone, G.; Sarioglu, H.; Ueffing, M.; Braselmann, H.; Zitzelsberger, H.; Schmid, R. M.; Hofler, H.; Ebert, M. P.; Walch, A. *Am. J. Pathol.* **2011**, *179*, 2720−2729.
(105) Elsner, M.; Rauser, S.; Maier, S.; Schone, C.; Balluff, B.; Meding, S.; Jung, G.; Nipp, M.; Sarioglu, H.; Maccarrone, G.; Aichler, M.; Ruchtinger, A.; Langer, R.; Jutting, U.; Feith, M.; Kuster, B.; Ueffing, M.; Zitzelsberger, H.; Hofler, H.; Walch, A. *J. Proteomics* **2012**, *75*, 4693−4704.
(106) Hardesty, W. M.; Kelley, M. C.; Mi, D.; Low, R. L.; Caprioli, R. M. *J. Proteomics* **2011**, *74*, 1002−1014.
(107) Skold, K.; Svensson, M.; Nilsson, A.; Zhang, X. Q.; Nydahl, K.; Caprioli, R. M.; Svenningsson, P.; Andren, P. E. *J. Proteome Res.* **2006**, *5*, 262−269.
(108) Ye, H.; Mandal, R.; Catherman, A.; Thomas, P. M.; Kelleher, N. L.; Ikonomidou, C.; Li, L. *PLoS One* **2014**, *9*, e92831.
(109) Bauer, J. A.; Chakravarthy, A. B.; Rosenbluth, J. M.; Mi, D.; Seeley, E. H.; De Matos Granja-Ingram, N.; Olivares, M. G.; Kelley, M. C.; Mayer, I. A.; Meszoely, I. M.; Means-Powell, J. A.; Johnson, K. N.; Tsai, C. J.; Ayers, G. D.; Sanders, M. E.; Schneider, R. J.; Formenti, S. C.; Caprioli, R. M.; Pietenpol, J. A. *Clin. Cancer Res.* **2010**, *16*, 681−690.
(110) Reyzer, M. L.; Caldwell, R. L.; Dugger, T. C.; Forbes, J. T.; Ritter, C. A.; Guix, M.; Arteaga, C. L.; Caprioli, R. M. *Cancer Res.* **2004**, *64*, 9093−9100.
(111) Sugiura, Y.; Taguchi, R.; Setou, M. *PLoS One* **2011**, *6*, e17952.
(112) Cole, L. M.; Djidja, M. C.; Bluff, J.; Claude, E.; Carolan, V. A.; Paley, M.; Tozer, G. M.; Clench, M. R. *Methods* **2011**, *54*, 442−453.
(113) Meistermann, H.; Norris, J. L.; Aerni, H. R.; Cornett, D. S.; Friedlein, A.; Erskine, A. R.; Augustin, A.; De Vera Mudry, M. C.; Ruepp, S.; Suter, L.; Langen, H.; Caprioli, R. M.; Ducret, A. *Mol. Cell Proteomics* **2006**, *5*, 1876−1886.
(114) Ljungdahl, A.; Hanrieder, J.; Falth, M.; Bergquist, J.; Andersson, M. *PLoS One* **2011**, *6*, e25653.
(115) Kurabe, N.; Hayasaka, T.; Ogawa, M.; Masaki, N.; Ide, Y.; Waki, M.; Nakamura, T.; Kurachi, K.; Kahyo, T.; Shinmura, K.; Midorikawa, Y.; Sugiyama, Y.; Setou, M.; Sugimura, H. *Cancer Sci.* **2013**, *104*, 1295−1302.
(116) Shimma, S.; Sugiura, Y.; Hayasaka, T.; Zaima, N.; Matsumoto, M.; Setou, M. *Anal. Chem.* **2008**, *80*, 878−885.
(117) Sugiura, Y.; Setou, M. *J. Neuroimmune Pharmacol.* **2010**, *5*, 31−43.
(118) Tanaka, H.; Zaima, N.; Ito, H.; Hattori, K.; Yamamoto, N.; Konno, H.; Setou, M.; Unno, N. *J. Vasc. Surg.* **2013**, *58*, 1366−1374.
(119) Toue, S.; Sugiura, Y.; Kubo, A.; Ohmura, M.; Karakawa, S.; Mizukoshi, T.; Yoneda, J.; Miyano, H.; Noguchi, Y.; Kobayashi, T.; Kabe, Y.; Suematsu, M. *Proteomics* **2014**, *14*, 810−819.
(120) Bao, Y.; Mukai, K.; Hishiki, T.; Kubo, A.; Ohmura, M.; Sugiura, Y.; Matsuura, T.; Nagahata, Y.; Hayakawa, N.; Yamamoto, T.; Fukuda, R.; Saya, H.; Suematsu, M.; Minamishima, Y. A. *Mol. Cancer Res.* **2013**, *11*, 973−985.
(121) Miura, D.; Fujimura, Y.; Wariishi, H. *J. Proteomics* **2012**, *75*, 5052−5060.
(122) Miura, D.; Fujimura, Y.; Yamato, M.; Hyodo, F.; Utsumi, H.; Tachibana, H.; Wariishi, H. *Anal. Chem.* **2010**, *82*, 9789−9796.
(123) Hattori, K.; Kajimura, M.; Hishiki, T.; Nakanishi, T.; Kubo, A.; Nagahata, Y.; Ohmura, M.; Yachie-Kinoshita, A.; Matsuura, T.;

DOI: 10.1021/ac504734s
Anal. Chem. 2015, 87, 1437−1455

Morikawa, T.; Nakamura, T.; Setou, M.; Suematsu, M. *Antioxid Redox Signal.* **2010**, *13*, 1157−1167.
(124) Sugiura, Y.; Honda, K.; Kajimura, M.; Suematsu, M. *Proteomics* **2014**, *14*, 829−838.
(125) Dollery, C. T. *Clin. Pharmacol. Ther.* **2013**, *93*, 263−266.
(126) Gonzalez, V. M.; Fuertes, M. A.; Alonso, C.; Perez, J. M. *Mol. Pharmacol.* **2001**, *59*, 657−663.
(127) Liang, X. J.; Shen, D. W.; Chen, K. G.; Wincovitch, S. M.; Garfield, S. H.; Gottesman, M. M. *J. Cell Physiol* **2005**, *202*, 635−641.
(128) Lieberthal, W.; Triaca, V.; Levine, J. *Am. J. Physiol.* **1996**, *270*, F700−708.
(129) Chandra, S. *Methods Mol. Biol.* **2010**, *656*, 113−30.
(130) Kawashima, M.; Iwamoto, N.; Kawaguchi-Sakita, N.; Sugimoto, M.; Ueno, T.; Mikami, Y.; Terasawa, K.; Sato, T. A.; Tanaka, K.; Shimizu, K.; Toi, M. *Cancer Sci.* **2013**, *104*, 1372−1379.
(131) Schober, Y.; Guenther, S.; Spengler, B.; Rompp, A. *Anal. Chem.* **2012**, *84*, 6293−6297.
(132) Shariatgorji, M.; Kallback, P.; Gustavsson, L.; Schintu, N.; Svenningsson, P.; Goodwin, R. J. A.; Andren, P. E. *Anal. Chem.* **2012**, *84*, 4603−4607.
(133) Zavalin, A.; Todd, E. M.; Rawhouser, P. D.; Yang, J.; Norris, J. L.; Caprioli, R. M. *J. Mass Spectrom.* **2012**, *47*, i.
(134) Blatherwick, E. Q.; Van Berkel, G. J.; Pickup, K.; Johansson, M. K.; Beaudoin, M. E.; Cole, R. O.; Day, J. M.; Iverson, S.; Wilson, I. D.; Scrivens, J. H.; Weston, D. J. *Xenobiotica* **2011**, *41*, 720−734.
(135) Eikel, D.; Vavrek, M.; Smith, S.; Bason, C.; Yeh, S.; Korfmacher, W. A.; Henion, J. D. *Rapid Commun. Mass Spectrom.* **2011**, *25*, 3587−3596.
(136) Manier, M. L.; Reyzer, M. L.; Goh, A.; Dartois, V.; Via, L. E.; Barry, C. E., 3rd; Caprioli, R. M. *J. Am. Soc. Mass Spectrom.* **2011**, *22*, 1409−1419.
(137) Manier, M. L.; Spraggins, J. M.; Reyzer, M. L.; Norris, J. L.; Caprioli, R. M. *J. Mass Spectrom.* **2014**, *49*, 665−673.
(138) Cobice, D. F.; Mackay, C. L.; Goodwin, R. J.; McBride, A.; Langridge-Smith, P. R.; Webster, S. P.; Walker, B. R.; Andrew, R. *Anal. Chem.* **2013**, *85*, 11576−11584.
(139) Wisztorski, M.; Lemaire, R.; Stauber, J.; Ait Menguellet, S.; Jardin-Mathe, O.; Day, R.; Salzet, M.; Fournier, I. *Med. Sci. (Paris)* **2007**, *23* (Spec. No. 1), 31−36.

DOI: 10.1021/ac504734s
Anal. Chem. 2015, 87, 1437−1455