CHAPTER SIX

Mass Spectrometry Imaging in Oncology Drug Discovery

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Abstract

Over the last decade mass spectrometry imaging (MSI) has been integrated in to many areas of drug discovery and development. It can have significant impact in oncology drug discovery as it allows efficacy and safety of compounds to be assessed against the backdrop of the complex tumour microenvironment. We will discuss the roles of MSI in investigating compound and metabolite biodistribution and defining pharmacokinetic -pharmacodynamic relationships, analysis that is applicable to all drug

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discovery projects. We will then look more specifically at how MSI can be used to understand tumour metabolism and other applications specific to oncology research. This will all be described alongside the challenges of applying MSI to industry research with increased use of metrology for MSI.

1. INTRODUCTION

Modern drug discovery and development is a lengthy, high-risk, and competitive business. It may take a decade and costs billions of dollars to get new medicines to market. High attrition rates, together with rising R&D and clinical trial costs, have presented significant challenges to the pharmaceutical industry over the last two decades. While the financial rewards for new blockbuster drugs are substantial, it is worth remember that a key driving force behind the scientists developing new therapeutics is the desire to get new medicines to patients to save or improve lives. This is particularly true for oncology drug discovery research teams, where there continues to be significant unmet medical need for treating this most devastating disease.

The primary reasons for drug attrition have consistently been lack of efficacy and toxicological or clinical safety risk (Kubinyi, 2003; Schuster, Laggner, & Langer, 2005). Oncology as a therapeutic area has suffered similarly high attrition compared to other diseases, but there are distinct trends that have shaped the modern drug discovery environment. The demand for effective new treatments is being met by oncology researchers developing ever more complex therapeutic regimes including use of new modalities, drug combinations, and delivery approaches. Advanced drug delivery technologies, such as nanomedicines, are being designed to increase therapeutic index by improving the delivery of drug to tumors relative to normal tissue, albeit with limited clinical success up to now (Hare et al., 2016). Historically, treatment of cancer relied predominantly on DNA-damaging radiotherapy and chemotherapy. In recent years, more specific target-based approaches have come to the fore. There has also been the tailoring of therapies to those specific patient populations most likely to benefit and increased use of oral administration. These developments, together with therapeutic and technological advances, have led to a greater emphasis on applying DMPK insight and know-how to develop oncology drugs with an acceptable pharmacokinetic profile while maximizing the therapeutic index and minimizing the drug–drug interaction potential.

The complexity of modern drug discovery and development, utilizing novel therapeutics, delivery systems, and innovative schedule design, provides certain challenges for the traditional bioanalytical methods employed across the value chain. While not replacing any established method for measuring and monitoring of drug exposure, metabolism, and disposition, mass spectrometry imaging (MSI) is increasingly being applied to complement these established bioanalysis approaches and is providing additional insights in both a timely and cost-effective way. MSI has been employed by both academic and industrial researchers for over a decade now, with increasing utility and breadth of applications reported in the literature. However, by the nature of commercial pharmaceutical research, the number of publications does not truly reflect the uptake and effectiveness of MSI in drug development. We will now outline the current and emerging applications of MSI within the field of oncology drug discovery, drug delivery, and clinical applications. Where no current examples from oncology have been reported, we will reference wider drug discovery examples. There are a number of comprehensive reviews on the range of MSI technologies, detailing their relative merits and utility to a wide range of applications, often with a focus on drug discovery (Cobice et al., 2015; Goodwin & Webborn, 2015; Nilsson et al., 2015). We will not therefore consider in detail the respective data acquisition speeds, mass analyzer sensitivities, and spatial and spectral resolutions of the increasing number of ionization systems that form the wide array of MSI platforms open to researchers. We will, however, focus on how MSI can impact oncology drug discovery. In recent times, the pharmaceutical industry has increased the diversity of approaches to therapeutic intervention, from traditional "small molecule" new chemical entities to "large molecule" biologic drug candidates, including peptides and monoclonal antibodies due to advances in biotechnology and apparent lower attrition rates relative to small molecules (Jiunn, 2009). Within this chapter, we will pay particular attention to the larger field of small molecule drug discovery, but we will also consider the analysis of new and emerging large molecule therapeutics.

2. HOW MSI CAN INFORM OUR UNDERSTANDING OF PHARMACOKINETIC–PHARMACODYNAMIC RELATIONSHIPS

The consideration and application of pharmacokinetic principles in drug discovery are now ubiquitous due to the recognition of the role of free

drug concentrations as a surrogate for measuring pharmacological effects. A basic tenet of pharmacology is that the magnitude of a pharmacological response is a function of the drug concentration at the site of action. Thus the objective of therapy may be achieved by maintaining sufficient concentration of drug at the site of action for the necessary duration, yet not so high as to elicit a deleterious effect. The concentration of drug has been typically measured in blood or plasma as the direct measurement of the available compound at the site of action, including tumors, can be challenging. However, the relevance of this surrogate site, and the drivers for the observed changes over time, will depend upon a number of factors and assumptions. Our understanding of such factors has been via the science of pharmacokinetics (PK), which is defined as the study of change of drug concentration over time and describes a systematic approach to relating dose to amount of drug in the body. Pharmacodynamics (PD) is the study of how drug concentration relates to effect. By modeling the mechanism of drug action, quantitative pharmacology or pharmacokinetics–pharmacodynamics (PKPD) describes the relationship between dose, concentration, and intensity and duration of response. The relatively recent use of new analytical tools including MSI in drug discovery and development now can provide insight into not just the identity and spatial distributions of therapeutic modalities in both normal tissues and tumors but also endogenous substrates, ligands, target proteins, and downstream biomarkers of target engagement and drug effect. Factors influencing the distribution of candidate drugs to the site(s) of pharmacological effect include intrinsic membrane permeability, the relative affinity for components in tissue vs blood, and whether or not the candidate drugs are substrates for drug transporters. Candidate drug or metabolite (which may be active or inactive against the same or different pharmacologically relevant targets) levels measured in plasma may not represent those present at the site of action and therefore cannot explain any observed efficacy or toxicological sequelae. It is important to stress that even in the oncology therapeutic area, for solid tumors, it remains the goal preclinically to build a PKPD relationship from the plasma compartment. This facilitates translatability to the clinical situation where sample availability and analysis from the plasma will be more achievable than from tumor biopsies. Understanding tumor PK and PD supports the building of this pharmacokinetic–pharmacodynamic–efficacy (PK/PD/E) relationship. Moreover, tumor PK can bridge the gap between plasma PK and tumor PD kinetics. A key question in oncology drug discovery that needs to be asked includes whether in vivo pharmacodynamics (and efficacy) can be robustly modeled using plasma PK? Of course, this assumes that unbound

concentrations of drug candidates are determined relative to PD endpoints via a time course of total plasma level measurements. This requires applying a correction for plasma protein binding. Preferably all measurements are determined from experiments at a range of doses via the relevant administration route.

3. BIODISTRIBUTION

To date, the core role for MSI in pharma R&D has been to provide projects with data on the biodistribution of compounds in situations when plasma measurements are thought to poorly represent tissue concentration. For oncology, this is not limited to abundance and distribution within a tumor but across all tissues. The MSI data generated also should not be considered in isolation but related to the range of complementary in vivo, in vitro, and in silico assays. However, when successfully performed, MSI can generate insights in a timely and cost-effective way. Determining the biodistribution of a compound can require several iterative MSI experiments to be performed. The first that a researcher could consider performing, to obtain an overall view on distribution of target analyte, would be whole body tissue section MSI (Shahidi-Latham, Dutta, Prieto Conaway, & Rudewicz, 2012). While this can generate images that appear initially impressive and be interpreted in a similar way as the radiolabeled mainstay in DMPK of quantitative whole body autoradiography (QWBA), the collected data come with many complications that need to be interpreted (Drexler, Tannehill-Gregg, Wang, & Brock, 2011). These have been discussed in previous reviews (Amstalden van Hove, Smith, & Heeren, 2010; Goodwin, 2012), but in brief the main issues are localized ionization suppression requiring every tissue to need a correction factor to allow interorgan comparison (Hamm et al., 2012; Källback, Shariatgorji, Nilsson, & Andrén, 2012) and requirement to compromise on spatial resolution. Therefore, an alternative approach for preliminary analysis is the collection of the target tissues and analysis at higher spatial resolution. Regardless of size of the sample, subsequent MS/MS analysis is usually performed to provide confirmation and validation of target, albeit this means that no additional metabolite or biomarker distributions can be simultaneously detected when acquiring MS/MS data. Therefore, analysis often involves iterative qualitative and quantitative analysis at a range of spatial resolutions, as required to address specific project needs. Quantitative MSI can also be performed in either full spectrum or targeted MS/MS mode and allows inferences beyond relative distribution to be made and is discussed in more detail later.

As regularly asserted, the power of MSI is that, unlike other probe or labeling assays, thousands of endogenous molecular species are simultaneously detected at the same time as any exogenous compound or metabolite. This allows MSI experiments to relate the distribution of the drug to the distribution of endogenous metabolites. The endogenous molecules also provide molecular maps of the target tissue or the tumor architecture (Mascini et al., 2016) and can be biomarkers for compound efficacy or safety and these applications will be explored later.

4. TUMOR METABOLISM: MSI ANALYSIS FOR MORE THAN JUST DRUG DISTRIBUTION

Before identifying new therapeutic targets, a key component is to understand the target tumor metabolism. Increased knowledge of cancer metabolism shows that the metabolic processes are extremely heterogeneous, derived from factors including genetic diversity, multiple and redundant metabolic pathways, and the tumor microenvironment. Metabolomic analysis has been extensively studied since the discovery that cancer cells rely on glycolysis followed by lactic acid fermentation for energy production, even in the presence of oxygen (Vander Heiden, Cantley, & Thompson, 2009; Warburg, 1956). While the Warburg effect remains debated, the consensus is that cancer cells can salvage organic carbon for the synthesis of large quantities of the biomolecules required for cell proliferation. Therefore, understanding tumor metabolism, crucial in identifying new druggable targets, requires technologies that are able to measure the level and flux of metabolism within the tumor microenvironment (Zhou & Lu, 2016). Examples of metabolic changes, often linked to specific genetic alterations, include the following: (1) reductive glutamine metabolism involving cytoplasmic IDH1 or mitochondrial IDH2 (Maddocks et al., 2013; Metallo et al., 2012; Wise et al., 2011); (2) oncogenic KRAS that promotes nonoxidative pentose phosphate pathway metabolism in pancreatic tumors (Ying et al., 2012); (3) oncogenic BRAF V600E that upregulates a ketogenic enzyme 3-hydroxy-3-methylglutaryl-CoA lyase, leading in turn to the increased induction of the ketone body acetoacetate that selectively enhances BRAF V600E to MEK1 binding to promote MEK–ERK signaling (Kang et al., 2015); (4) inappropriate activation of PI3K/Akt also promotes glycolysis (Deprez, Vertommen, Alessi, Hue, & Rider, 1997; Gottlob et al., 2001; Kohn, Summers, Birnbaum, & Roth, 1996; Rathmell et al., 2003) and stimulates de novo lipogenesis through the direct phosphorylation and activation of ACL (Berwick, Hers,

Heesom, Moule, & Tavare, 2002); and (5) mTORC1-mediated activation of SREBF (Porstmann et al., 2008). Metabolic changes are also observed for tumor cells with loss or mutation of well-established tumor suppressors, such as for p53, where p53-null cells fail to complete the response to serine deprivation, resulting in oxidative stress-induced inhibition of cell viability and proliferation (Maddocks et al., 2013). To further exacerbate the situation, cancers also elicit extremely high metabolic flexibility, making some metabolite synthesis-targeted therapeutic strategies fail. For example, fatty acid synthase (FASN) inhibitors, targeting a key enzyme involved in neoplastic lipogenesis, can block de novo fatty acid synthesis. However, this leads to reliance on the circulating lipid pool for the synthesis of new membranes, resulting in slower tumor growth but not complete remission (Flavin, Peluso, Nguyen, & Loda, 2010). Therefore, the MSI analysis of metabolomic profiles in tumor samples, at both baseline and following therapeutic interventions, will be of significance in both understanding and characterizing the tumor microenvironment in the preclinical and clinical setting.

Steroid concentrations within tissues, modulated by intracellular enzymes, can influence hormone-dependent cancers and have proven elusive endogenous targets for bioanalysis. However, mapping and quantifying the distribution of steroids within tissue sections has been possible by simple adaptation to standard MSI protocols. Effective detection of previously poorly detected steroid targets has been accomplished using on-tissue derivatization with Girard T reagent. This modified the targets to generate increased ionization, so quantification of substrate and product (11-dehydrocorticosterone and corticosterone) of the glucocorticoid-amplifying enzyme 11β-HSD1 could be made (Cobice et al., 2013). The use of on-tissue derivatization is proving to be an effective strategy for poorly detected targets (Shariatgorji et al., 2014). While not simple or quick, it does mean whole classes of endogenous and exogenous targets can be analyzed by MSI. There are a number of sample processing and optimization strategies that can be used to increase the success of MSI in drug development, and the importance of sample preparation will now be discussed.

5. SAMPLE PREPARATION

Having considered the endogenous molecular targets, we will now consider aspects of sample collection and processing. Sample preparation is crucial in performing robust and accurate MSI experiments. Any failings early in the MSI analysis can render subsequent interpretation void. Specifics of the sample preparation for the different MSI technologies have been extensively

reviewed (Goodwin, 2012) so will not be described here. However, before starting a study, adequate consideration must be made into how each tissue, biopsy, or tumor sample will be analyzed to allow effective interassay comparisons. Each preclinical or patient sample may be analyzed by a range of imaging and molecular assays; therefore, tissue collection is the first point where protocol optimization and standardization is required for a multiassay tissue study. As discussed, MSI can be performed on whole rodent body sections, dissected tissues, or biopsies, each with advantages and challenges in the subsequent analyses. Dissected tissue analysis is probably the most common approach for studying PK groups, and using such tissues means that control and dosed samples can be readily analyzed within a single experiment if tissue sections are placed on the same slide. Typically, six rat organ sections can be placed on the same MS compatible slide. To allow a full study means running interslide standards, utilizing effective quantification strategies, and mixing groups so no bias is introduced due to the order of analysis. It is important to note that time taken to section can affect the detected abundance of endogenous (Goodwin, Dungworth, Cobb, & Pitt, 2008; Goodwin, Pennington, & Pitt, 2008) or exogenous compounds (Goodwin, Iverson, & Andren, 2012). This can be mitigated by reducing sample preparation time through the embedding of multiple samples into a support media, allowing all samples to be sectioned using a cryomicrotome at the same time rather than sequentially. This means that all samples spend the same period in cryostat and all sections on a given slide will undergo identical treatment for the remainder of the experiment. Common embedding media are carboxymethyl cellulose and gelatin as they do not cause detectable sample contamination yet provide sufficient support for sectioning thin (<5 μm) tissue sections required for optimal histology (immunohistochemistry—IHC and hematoxylin and eosin—H&E) of matched MSI samples. New MSI embedding support media (Strohalm et al., 2011) and even blends of embedding media (Nelson, Daniels, Fournie, & Hemmer, 2013) are used for optimal tissue support. For analysis of small tumors, such as those from efficacy studies, embedding is the only practicable option. This is also the case for the analysis of tissue needle biopsies from clinical studies. Larger biopsies and resection material can be treated in a similar way to dissected rodent organs and sectioned either embedded in media or with minimal mounting media.

Postsample sectioning, tissue washing (briefly bathing tissue sections placed on slides into water and ethanol solutions) can be performed to remove low molecular weight molecules and salts that limit the ionization and detection of larger peptides and proteins. In our experience, this has

limited usefulness if attempting analysis of either lower molecular weight endogenous biomarkers or therapeutics as they are also removed or delocalized. Researchers have however demonstrated that by modifying the pH of the wash solution to a level where the target compound is poorly soluble, tissues can be washed and efficiency of detection of the target can be improved by removal of ionization suppressing material (Shariatgorji et al., 2012). In a similar way to derivatization, this is not a strategy that is widely used in the MSI analysis due to the extra optimization and validation time required. However, it is particularly effective within a pharma environment when a single target is often pursued and teams are able to make more in-depth investments in analysis time for each target.

6. QUANTITATION

Drug quantitation measured directly from tissue sections is now routinely performed by MSI, though there remain many complications and caveats that need to be considered (Nilsson et al., 2010). The standard approach is to spot an array of known concentration droplets onto the surface of adjacent control tissue sections and analyze in parallel to the target tissue. This allows a simple calibration curve to be generated and subsequent calculation of concentration of target analyte in the sample. However, this method fails to take into account any localized suppression or enhancement of analyte ionization caused by the architecture of the sample. This can be overcome by correcting for ionization efficiency using a stable-labeled analogue of the drug. The compound is sprayed over the tissue at an appropriate concentration to give moderated detection by subsequent MSI analysis (Källback et al., 2012). Note that too strong a detected response may cause suppression of the target analyte. The application of the labeled standard should be homogeneous and in minimal solvent to prevent delocalization of analytes within the tissue section. This is typically performed using similar systems for applying the even matrix coating required for MALDI MSI. Correction for ionization suppression can then be made pixel by pixel and has been shown to be an improvement over other normalization correction factors such as total ion count. However, a stable-labeled analogue of the compound is often not available for researchers working in drug discovery when multiple compounds in a chemical series are being evaluated. In such situations, two alternative approaches can be attempted. Control tissue can be coated with the drug, as just described, and regions of ionization suppression and enhancement identified and excluded from subsequent quantification. Alternatively a compound

with similar structure, physiochemical properties, and ionization efficiency can be used as a substitute for the labeled analogue. Neither method is ideal; however, the analysis is often performed to differentiate and identify major differences between candidates that will be more fully characterized and assessed as they are progressed. So, for example, precoating a tissue sample with the candidate drug may show that a calibration curve needs to be applied to multiple tissue regions or over entire samples for more accurate quantification calculations (Chumbley et al., 2016).

Safety and efficacy biomarkers, discussed later, can also be quantified directly from tissue sections. Such analysis is also routinely performed, but the main complication is the fact that control tissue will also contain the endogenous molecular target, either at higher or at lower abundance depending on the effect of compound or disease state. This means that the calibration spots need to contain a labeled target. Labeled endogenous metabolites are readily available for purchase with various degrees and positioning of the labeling. It should be checked that the selected labeled standard is sufficiently different in mass to the unlabeled version to avoid overlap of the isotope peaks of the endogenous molecule. A further complication for many efficacy biomarkers for oncology projects is endogenous metabolites, which may be readily turned over and unstable during standard bioanalysis (homogenization and LC-MS quantification). Care must therefore be taken to evaluate the stability of the calibration stock solutions as well as the stability of the endogenous metabolites within the tissue section. While this can be problematic, the MSI analysis can actually prove to be superior to homogenization in this regard. The sample preparation for MSI, performed on frozen samples, does not cause mixing of the endogenous metabolites, enzymes, and reactive molecules in an extraction solution such as used for homogenization. So, MSI can provide means to relate biomarker distribution to drug distribution and tissue microenvironment. Arguably, MSI provides a simple, more accurate means to quantify the absolute abundance of unstable endogenous metabolites (Fujimura & Miura, 2014). In summary, quantitation by MSI has many challenges to be overcome, but it does provide multiple opportunities to add value to imaging analysis of tissues and tumors. Though it is imperative, given the number of possible complications, all MSI quantitative data should be carefully reviewed and where possible related to complementary data.

7. TOXICITY AND SAFETY ASSESSMENT

While oncology researchers may typically countenance lower therapeutic safety/toxicity margins, due to the serious and life-threatening nature

of the disease, relative to other more chronic diseases, the primary objective of oncology animal toxicology studies remains evaluating the safety profile of drug candidates. Preclinical analysis and effective understanding and translation to man of any toxicological finding in drug discovery can lead to the termination of a compound. Ideally, information will feed into the subsequent optimization of the chemical series to avoid any off-target pharmacology (adverse pharmacologic effects at proteins other than the primary therapeutic target) or the termination of the project against a target with the elucidation of an unacceptable on-target effect. While it is customary for a compound's efficacy in tumors to be investigated in detail during drug discovery, possible adverse sequelae in the range of target organs from toxicological studies can be extremely challenging to assess (Pellegatti & Pagliarusco, 2011). In our experience, MSI can and will increasingly provide significant insight into the elucidation of safety and toxicity signals. Again, this technique is most powerful when performing in combination with other technologies such as histopathological analysis and LC-MS of tissue homogenates and blood/plasma bioanalysis. Multiple examples of investigatory MSI analysis in drug discovery have been reported to date, but few have specifically related to oncology drug development and/or drug safety. The following examples may have been derived from oncology candidate drugs, demonstrating either on- or off-target toxicity. Their inclusion is to exemplify how MSI can be used for investigatory toxicity studies. Early reported toxicity studies reported the characterization of crystal deposits within rat kidney or spleen following administration of compounds. MSI has able to identify the crystal deposits as metabolites of the compound compounds, in situ and with sufficient spatial resolution to map the deposition of the crystals (Drexler et al., 2007; Kim et al., 2010; Nilsson et al., 2012).

The effectiveness of MSI to measure, monitor, and investigate a compound's ability to penetrate the blood–brain barrier (BBB) and subsequent accumulation within the central nervous system (CNS) will be considered in detail later. Sample homogenization can be sufficient for certain tissues, when an averaged abundance is an acceptable comprise for increased sensitivity (using sample cleanup and separation by liquid chromatography). However, it is a poor substitute when looking to study subtle or slight concentration changes within the brain architecture. MSI toxicity studies have therefore been readily applied to the study of the CNS, such as reported by researchers at GlaxoSmithKline who assessed the disposition and metabolism of fosdevirine, a nonnucleoside reverse transcriptase inhibitor, on the CNS (Castellino et al., 2012). Using MSI detection and quantitation of multiple neurotransmitters, and monitoring effects of xenobiotics can be a crucial

component of drug safety programs. Recent improvement in the detection of neurotransmitters has been reported, and the utility is exemplified by following the example including drug treatments using MALDI (Shariatgorji et al., 2014) and DESI analysis (Shariatgorji et al., 2016). Methods used for on-tissue derivatization, for example, targeting primary amines in neurotransmitters such as GABA, dopamine, serotonin, and glutamate, are increasingly being employed. Chemical modification increases the ionization efficiency and hence detection and mapping in target tissue samples. Such endogenous metabolites may be measured to assess off-target effects that cause toxicity or safety concerns. The same endogenous markers may also be used as efficacy biomarkers. Oncology MSI drug safety studies have also reported that a compound's toxicity can be mitigated through the use of nanoparticle delivery systems. The MSI analysis has shown that delivery of paclitaxel into tumors in a nanoparticle formulation manipulated exposure. This was also associated with less neurotoxicity in mice (Yasunaga et al., 2013). Moreover, in our laboratory, the ability to monitor in situ drug concentrations and rate of active pharmaceutical ingredient (API) release, using novel delivery systems, in relation to the target tissue architecture offers powerful insights to bioscientists and medicine formulation groups.

8. BIOMARKERS FOR EFFICACY

Rapid feedback to medicinal chemistry-led design teams providing estimates of potency, selectivity, predictions of human PK, and therapeutic dose is pivotal to the efficient optimization of lead compounds into candidate drugs. The design-make-test-analyze (DMTA) cycle is the multidisciplinary engine of lead optimization and drug discovery screening cascades. The most efficient DMTA cycles are based on screening cascades appropriate for projects typically starting with a panel of assays in "Wave I" determining physicochemical properties and basic in vitro data such as potency against the target, key selectivity screens, metabolic stability, and perhaps cytochrome P450 inhibition assessment. Subsequent studies further profile lead compounds, and these include establishing in vivo PK, PD, and efficacy relationships only with quality compounds. There are many available PK optimization approaches to input into compound design and to predict key human PK properties with differing power, cost, and complexity, ranging from purely in silico methods via a multitude of in vitro assays and in vivo models, to physiologically based pharmacokinetic techniques, which

characterize drug distribution to specific organs in terms of physiologically relevant variables (Grime, Barton, & McGinnity, 2013). MSI is a powerful tool for analyses of known targets and is valuable in PK tissue analysis. However, the ability to also monitor endogenous as well as exogenous compounds makes it suitable for mapping and quantifying the abundance of biomarkers of compound efficacy in PD studies. Over a decade ago, researchers described solid tumor distribution of drug and active metabolite and related distribution simultaneously to intratumor distribution of ATP, indicating that the cytotoxic metabolite was confined to hypoxic tumor regions (Atkinson, Loadman, Sutton, Patterson, & Clench, 2007). Analysis of endogenous molecular changes can also be expanded to include identification of new biomarkers, and their characterization and distribution within the sample, to evaluate disease or toxicity in preclinical and clinical samples (Balluff et al., 2011; Elsner et al., 2012; Ye et al., 2014). Such MSI analysis can also monitor disease progression or regression in response to therapeutic intervention. Endogenous masses can be matched with metabolite databases to identify unknown markers within the samples. This typically requires high mass resolving mass analyzers or a hypothesis-driven experiment where MS/MS data from the tissue can be compared to the theorized molecular target. It is worth noting that many reported biomarkers are proteomic (Bauer et al., 2010; Cole et al., 2011; Meistermann et al., 2006; Reyzer et al., 2004) or for endogenous metabolites that related to energy metabolism (Bao et al., 2013; Miura, Fujimura, & Wariishi, 2012; Miura et al., 2010).

9. DRUG DELIVERY

To mitigate toxicity and to allow for optimal exposure profiles, modern therapeutics can be delivered by a number of methods. While oral administration is preferred, intravenous chemotherapy is a common route and other more advanced methods also exist, such as slow release depot implants. MSI has been used to assess the release of theophylline and propranolol hydrochloride from such implants (Kreye et al., 2012). The researchers used a cylindrical implant embedded in gelatin, frozen and sectioned as standard for tissues. The MSI analysis showed the change in the structure of the implant over a range of time points and release into external medium. A number of oncology researchers have attempted to incorporate tumor targeting by enhanced permeability retention effect (EPR) at target site. The aim is increased efficacy through higher localized exposure at the target site (e.g., tumor) and lower systemic exposure, thus increasing the therapeutic

index and reducing the dose-limiting toxicities. In oncology-targeted approaches, the premise is that by using larger molecules, delivery vesicles, or particles, therapeutics accumulate more in tumors than in the surrounding normal tissues (Gabizon & Papahadjopoulos, 1988; Golan et al., 2015; Maeda, Fang, Inutsuka, & Kitamoto, 2003). The encapsulation of the drug in a delivery particle can also protect it from early degradation or metabolism. The relevance of the EPR phenomenon in increasing therapeutic index is still hotly contested, in part because the clinical translation of a specific EPR effect has yet to be unequivocally demonstrated and to date most anticancer nanoparticle medicines on the market take form of a small molecule drug within a liposome with diameters of 30–150 μm.

Some feel that the full impact of carrier-mediated drugs remains to be fully utilized, as exemplified in recent meta-analysis of clinical and preclinical studies comparing the anticancer efficacy of liposomal vs conventional non-liposomal doxorubicin (Petersen, Alzghari, Chee, Sankari, & La-Beck, 2016) and summarized in Table 1. The researchers concluded that the optimal dosing regimen for carrier-mediated agents has not been thoroughly investigated, and it is possible that this will differ from that of the conventional formulation. Moreover, the contribution of the EPR effect and the tumor microenvironment to clinical efficacy remains to be fully elucidated. These are areas where MSI could make a significant impact. As MSI is able to detect

Table 1 Anticancer Nanoparticles Approved for Clinical Use

Trade Name	Carrier	Drug Cargo	Size	Approved Indications
Doxil/ Caelyx	PEGylated liposome	Doxorubicin	90 nm	AIDS-related Kaposi sarcoma, multiple myeloma, ovarian cancer
Myocet	Liposome	Doxorubicin	150 nm	Metastatic breast cancer
DaunoXome	Liposome	Daunorubicin	45 nm	AIDS-related Kaposi sarcoma
Marqibo	Liposome	Vincristine	100 nm	Acute lymphoblastic leukemia
Onivyde	PEGylated liposome	Irinotecan	110 nm	Pancreatic adenocarcinoma
DepoCyt	Liposome	Cytarabine	3–30 μm	Lymphomatous meningitis

Reproduced with permission from Petersen, G. H., Alzghari, S. K., Chee, W., Sankari, S. S., & La-Beck, N. M. (2016). Meta-analysis of clinical and preclinical studies comparing the anticancer efficacy of liposomal versus conventional non-liposomal doxorubicin. *Journal of Controlled Release, 232*, 255–264.

multiple molecular targets in every mass spectrum, researchers are able to determine the abundance of the API as well as constituents of the nanoparticle. This can offer insights into the total abundance of drug, released API, and possible carrier nanoparticle accumulation within tumors. If a labeled or tagged method was to be used, information would only be obtained on one component. For example, a radiolabeled drug could show the distribution with the tumor but could not differentiate if API was still within the nanoparticle or released. This is further complicated if there is localized metabolism and radiolabel remains on the metabolite. The inverse scenario is also true. A fluorescent nanoparticle could be formulated, and the distribution measured within tumor sections. However, no information on whether API was still encapsulated, released or if the nanoparticle had degraded and the label itself was accumulated at target site. MSI has the potential to assess such situations in a pixel-by-pixel and spectra-by-spectra fashion (Ashton et al., 2016).

Looking forward, many pharmaceutical companies have aspirations of combining small molecule treatments with a range of new modalities including macromolecules and gene therapy therapeutics. Recent reviews of advances on nanoparticles have highlighted that the use of delivery systems can circumvent the induction of immune response through the use of degradable and biocompatible polymeric particles. Again, delivery of such systems provides biocompatibility and prolonged systemic exposure, prevents payload degradation, and possibly benefits tumor targeting via the EPR effect. However, such delivery systems still have limitations to be overcome, including suitable stability and optimized releases (Mokhtarzadeh et al., 2016). Future oncology treatments may employ even more advanced drug delivery systems such as carbon-based nanomaterials. These include a range of structures such as carbon nanotubes, graphene, and carbon nanodots that have been proposed as drug delivery vehicles (Cai et al., 2005; Miyako et al., 2012). Directly assessing the biodistribution of such carbon carriers by MSI is complicated due to the larger molecular weights of the materials. However, researchers recently demonstrated the suborgan distribution of carbon nanomaterials using laser desorption/ionization MSI to map the intrinsic carbon cluster fingerprint signal of the nanomaterials (Chen et al., 2015).

10. TUMOR MICROENVIRONMENT

As an oncology compound progresses from in vitro to in vivo studies, researchers must use a range of preclinical models that are derived from

human or rodent cell lines. The four predominant models are (1) cell line xenograft models, (2) patient-derived xenograft models, (3) syngeneic mouse models, and (4) genetically engineered mouse model. Each model system has advantages and challenges as summarized in Table 2 reproduced from the comprehensive review by Gould, Junttila, and de Sauvage (2015).

Table 2 Preclinical In Vivo Efficacy Models Used for Oncology Drug Discovery: Strengths and Caveats

	Principal Components	Benefits	Caveats
Cell line xenograft models	Established human tumor cell lines transplanted into immune-deficient host	(i) Numerous established and well-annotated cell lines (ii) Representation from various human tumor types (iii) Features of the tumor microenvironment, including vascular and stromal cells incorporated within the tumor (iv) Tumors are easily and precisely measured	(i) Immune deficient (ii) Subcutaneous location may not foster important tissue-specific stromal infiltrate (iii) Cross-species disconnect, stromal components are mouse, whereas tumor cells are human (iv) Limited or no genetic heterogeneity is present within the tumor
Patient-derived xenograft models	Human tumor explants grown in immune-deficient host	(i) Genetic diversity and heterogeneity within tumors (ii) Representation from various human tumor types (iii) Features of the tumor microenvironment, including vascular and stromal cells incorporated within the tumor (iv) Tumors are easily and precisely measured	(i) Immune deficient (ii) Subcutaneous location may not foster important tissue-specific stromal infiltrate (iii) Surgical implantation required (iv) Cross-species disconnect, stromal components are mouse, whereas tumor cells are human (v) Genetic and phenotypic drift with passage

Table 2 Preclinical In Vivo Efficacy Models Used for Oncology Drug Discovery: Strengths and Caveats—cont'd

	Principal Components	Benefits	Caveats
Syngeneic models	Established mouse tumor cell lines transplanted into immune-competent host	(i) Presence of an intact immune system (ii) Features of the tumor microenvironment, including vascular and stromal cells incorporated within the tumor (iii) All cell types within the tumor are of mouse origin (iv) Tumors easily and precisely measured	(i) Limited number of established cell lines, poorly annotated (ii) Strong immunogenicity of some lines promotes spontaneous regression (iii) Rapid growth rate of many lines limits use in longer-term studies
Genetically engineered mouse models	Genetic modification that permits spontaneous or induced tumor development	(i) Tumors develop in the tissue of origin (ii) Presence of an intact immune system (iii) All cell types within the tumor are of mouse origin (iv) Incorporates features of the tumor microenvironment, including vascular and stromal cells and immune components	(i) Limited genetic mosaicism and heterogeneity of tumors (ii) Technical hurdles for monitoring tumor response when on internal organs (iii) Low throughput and high investment

Reproduced with permission from Gould, S. E., Junttila, M. R., & de Sauvage, F. J. (2015). Translational value of mouse models in oncology drug development. *Nature Medicine, 21*(5), 431–439.

A common challenge for all preclinical models is the heterogeneity within the tumor. This complex microenvironment is composed of multiple cell types, with oxygenation ranging from normoxia to hypoxia, and with huge interplay between viable and necrotic regions. This provides a challenge for pharmacokinetic and biomarker studies using tissue homogenization and analysis. However, this challenge is increasingly being met by MSI with the suite of technologies allowing researchers to map the architecture of the microenvironment in preclinical models and patient samples using

endogenous metabolites and lipids (Calligaris et al., 2014; Eberlin et al., 2012, 2014). However, pharmaceutical research assessment of the tumor microenvironment can go beyond simple comparison of endogenous and exogenous compounds in ex vivo tumor sections. Recent work has demonstrated an approach whereby in vivo imaging can be combined with ex vivo MSI analysis, using MRI techniques. Researchers described how following administration of a contrast reagent, tumors were excised and analyzed by DESI MS imaging. Researchers were able to map the distribution of the contrast reagent gadoteridol as well as endogenous lipids (Tata et al., 2015).

As the tumor microenvironment is thought to play a role in solid organ cancer development, it is also worth noting that microenvironment characterization is not restricted to preclinical models but has also been applied to patient samples. Such work has exemplified by groups investigating lipidomic differences between cancerous and healthy colorectal tissue (Mirnezami et al., 2014). Such data can feed back to drug discovery environment to generate better preclinical models as well as offer insights into the molecular events in tumor environment that can help researchers understand the lack of efficacy following transfer into the clinic.

11. ASSESSING HYPOXIA

Recent refinement of the lipid microenvironment mapping allows characterization of the regions of hypoxia. This has been achieved by relating changes in lipid profiles to IHC for hypoxia (Chughtai, Jiang, Greenwood, Glunde, & Heeren, 2013), thereby allowing co-registered MSI, IHC, and H&E data for multivariate image analysis. This allows statistical interrogation of the lipid abundances, which when validated can be used in subsequent MSI experiments for the same cell line to define tumor regions. The process can be refined by addition of specific IHC markers of tissue hypoxia, injected prior to tissue extraction, which can be directly detected by MSI. Such has been reported for pimonidazole, an exogenous hypoxia marker detected in tumor sections by MALDI MSI (Mascini et al., 2016).

12. BBB PENETRATION

As previously alluded to, there are scenarios in which there is a discrepancy between the measured circulating plasma levels of a compound and the abundance in a target tissue, and in such situations, MSI analysis

can prove particularly beneficial. Such may be the case for tumors or when tissue-targeting delivery strategies are used. However, during drug discovery a common such situation occurs when trying to understand the exposure to the brain. There will be situations where researchers want to make sure that compounds do not penetrate the CNS, risking off-target deleterious toxicity, as well as situations when brain penetration is highly desirable for a compound in order to have required neuropharmacological efficacy or to reach the site of brain tumors. The BBB tight junctions present between the brain microvessel endothelial cells, in combination with efflux transporters such as P-glycoprotein (P-gp), multidrug resistance-associated proteins, or organic anion-transporting polypeptides, prevent endogenous and exogenous compounds from affecting neurological activity. Therefore, it is important to determine BBB permeability of drug candidates during drug discovery both in animal models and in human. A range of alternative analytical strategies can be used to predict the impact of the BBB on new therapeutics (Löscher & Potschka, 2005), but clearly using the spatially resolving MSI can be a rapid and cost-effective tactic. Analysis can be further complicated if researchers are trying to make assessment of the exposure of compound not just to the brain but to brain tumors, with associated damage and leakage to BBB (Agarwal, Manchanda, Vogelbaum, Ohlfest, & Elmquist, 2013). Therefore, imaging methods that move analysis away from brain homogenization and calculation for peripheral blood contamination are an improvement. The simplest approach to detect if the compound has crossed the BBB is to use a low spatial resolution technology like liquid extraction surface analysis (LESA). This allows researchers to profile or probe tissue sections rather than higher resolution imaging. With spatial resolution of approximately 1 mm, such methods are suitable for analysis of rat brain sections but may not be suitable for smaller mouse brains or for analysis of brain tumors. However, the large sampling area means that such an approach can be very sensitive. By measuring for Heme β (m/z 616) as a marker for blood, any compound detected can be shown to either correlate to blood distribution or show BBB penetration (Swales et al., 2015). The validation of heme as a biomarker of vasculature in the brain has been performed at higher spatial resolution using MALDI MSI analysis and relating the MSI detected distribution of the blood to the fluorescence image distribution of fluorescein as reproduced in Fig. 1 (Liu, Ide, et al., 2013). The authors went on to show detection and distribution of RAF265, a small molecule inhibitor of the RAF serine/threonine protein kinases by MALDI MSI and the impact of aberrant tumor vasculature on compound distribution.

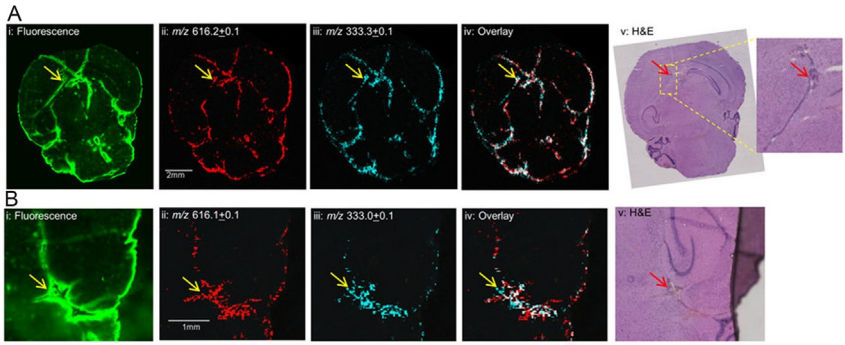

Fig. 1 Comparison of heme and fluorescein images from MALDI TOF MSI at 50 μm resolution with fluorescence image in the same mouse brain section (10 μm thickness) with preinjected fluorescein. (i) Fluorescence image of blood vessels from fluorescein (Ex 490 nm, Em 520 nm); (ii) heme image (*red*, *m/z* 616.2 ± 0.1) from MALDI MSI; (iii) fluorescein image (*blue*, *m/z* 333.3 ± 0.1) from MALDI MSI; (iv) overlay of heme (*red*) and fluorescein (*blue*) from MALDI MSI; (v) H&E staining of a sister section from (A) with the expanded view showing the lateral ventricle. The *yellow arrow* indicates the lateral ventricle delineated by fluorescein with the absence of heme. The *red arrow* shows blood in the H&E staining image. (B) Selected view of heme and fluorescein images from MALDI MSI under 25 μm resolution and fluorescence image in the same mouse brain section. (i) Fluorescence image of blood vessels from fluorescein (Ex 490 nm, Em 520 nm); (ii) heme image (*red*, *m/z* 616.1 ± 0.1) from MALDI MSI; (iii) fluorescein image (*blue*, *m/z* 333.0 ± 0.1) from MALDI MSI; (iv) overlay of heme (*red*) and fluorescein (*blue*) from MALDI MSI; (v) H&E staining of a sister section. The *arrow* shows the region of blood. *Reproduced with permission from Liu, X., Ide, J. L., Norton, I., Marchionni, M. A., Ebling, M. C., Wang, L. Y., . . . Agar, N. Y. R. (2013). Molecular imaging of drug transit through the blood-brain barrier with MALDI mass spectrometry imaging.* Scientific Reports, 3, *2859.*

MSI can also be used to study effects of efflux transporters for poorly brain penetrative compounds. The recent elegant study by Genentech highlighted the utility of MSI in a study investigating the role of P-gp on the brain penetration and brain PD activity of the MEK inhibitor cobimetinib (Choo et al., 2014). The researchers stated that the objective of the study was to determine—in vitro and in vivo—whether cobimetinib is a substrate of P-gp and/or breast cancer resistance protein (Bcrp1). The aim was also to assess the implications of efflux on cobimetinib PK, brain penetration, and target modulation. The data showed that for the preclinical study, the ability of cobimetinib to elicit pathway modulation in the brain is prevented by P-gp efflux. The study exemplified how MSI data can be effectively integrated into a wider PK/PD study. A combination of the approaches outlined in the previous two studies was recently undertaken

for assessing alectinib distribution in murine brains. The quantitative MSI analysis, validated by LC-MS data, again showed correlation of compound to heme, but the researchers also analyzed the effect on PK profiles of the multidrug resistance protein-1 (Mdr1) using Mdr1a/b knockout mice (Aikawa et al., 2016).

13. BEYOND SMALL MOLECULES

Traditionally, therapeutics have been small molecules that fall within the Lipinski's rule of five (i.e., a molecule with a molecular mass less than 500 Da, no more than 5 hydrogen bond donors, no more than 10 hydrogen bond acceptors, and an octanol–water partition coefficient log *P* not greater than 5). Such molecules are often detected by the various MSI technologies as we have previously discussed. However, there are emerging new therapeutic modalities that are much more complex and have moved way beyond traditional low molecular weight compounds. Nevertheless, MSI can still have a role to play in their development. Macromolecule therapeutics range in size and include oligonucleotides, proteins, antibodies, and antibody–drug conjugates (ADCs). Most of these molecules are currently beyond the size of analytes detectable by MSI technologies. While MALDI time of flight mass spectrometers are able to detect antibodies >100 kDa, they are not currently able to detect them in tissue sections. The simplest role MSI currently has to play in large molecule therapeutics is to measure and monitor biomarkers for efficacy or safety in the same way as employed for traditional small molecule work. Attempts have been made to develop strategies for larger molecules. Proteins, for example, can be detected and mapped indirectly by performing on tissue tryptic digestion and subsequent detection of the resulting lower mass peptides. This unfortunately generates multiple peptide fragments for each protein, so the most abundant proteins will swamp the detectable mass range and risk the detection of any lower abundance target. Another complication is the delocalization of targets, as samples need to be stored in humid atmosphere for enzymatic reaction to take place. However, with carefully controlled protocols, researchers have reported high-quality data at 50 μm spatial resolution (Schober, Guenther, Spengler, & Römpp, 2012). To increase the sensitivity for lower abundance targets a labeling strategy is required. While this moves us away from the label-free analysis approach that is integral to the success of MSI, multiplexed label detection is possible, where traditional IHC or other probe methods are restricted to single-target detection. A recent development has

been termed spectroimmunohistochemistry, which combines the use of specific antibodies against targets and mass spectrometric imaging detection (Longuespée et al., 2013). While further optimization would be required to assess the robustness of this technique and to allow quantitative measurements to be obtained, such a methodology has the potential to be applied to both clinical and pharmacological applications. The early data looks promising and is a novel solution to an obstacle for determining novel large molecule therapeutics. The same group developed a technique called Tag-Mass for mapping MSI-specific targets. The approach allows quantification and identification of biological macromolecules such as proteins and peptides by using photocleavable tag peptides of known sequences, attached to antibody probes on specific antibodies for a given biomarker (Stauber, Ayed, Wisztorski, Salzet, & Fournier, 2010). Once photocleaved, the peptide mass tags are detected by mass spectrometry. The researchers reported the distribution of 180-kDa carboxypeptidase D membrane protein at 50 μm resolution (Lemaire et al., 2007). The cleavage is induced by the MALDI laser, so this technique could have wide uptake as the most commonly employment mass spectrometer for MSI researchers has MALDI ionization. This method was reported to be highly efficient and reproducible, but the design of the linked antibodies has been considered expensive. However, for the specificity reported, such an approach could prove effective in large molecule drug development.

Finally, we should consider the first reported—and what may well be the most effective—tag-based MSI method for tissue-wide distribution of large molecule targets. The approach uses laser ablation-inductively coupled plasma mass spectrometry (ICP-MS). Researchers reported imaging of human gastric mucosa for a target cancer biomarker at 10 μm resolution (Fig. 2) (Seuma et al., 2008). This was achieved by coupling antibodies to metal tags. The ICP-MS has sensitivity for metals at subparts per billion when analyzing tissue samples. Therefore, this approach offers high spatial resolution with a large dynamic range for target quantitation. The use of this approach with a range of lanthanides offers real multiplexing and multitarget detection.

All the tag-based MSI methods are equally applicable to analysis of either large molecule therapeutics or endogenous large molecules that may be target receptors or biomarkers for efficacy or safety endpoints. There is one key point that should be considered before undertaking complex generation of the MS-labeled probe. Can adequate information be achieved by combining traditional IHC for target distribution with tissue homogenization for quantitation? This can allow higher spatial resolution IHC to be combined with

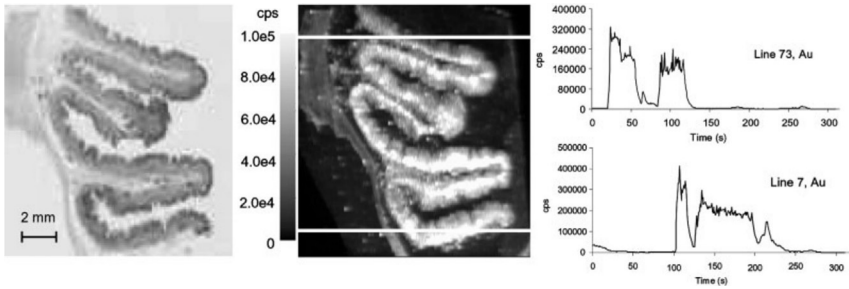

Fig. 2 Photomicrograph (*left*) of normal human gastric mucosa stained for MUC-1 antigen using the immunogold technique, and corresponding Au distribution by LA-ICP-MS (81 line rasters) (*center*). *Right*: Au single-line rasters. *Reproduced with permission from Seuma, J., Bunch, J., Cox, A., McLeod, C., Bell, J., & Murray, C. (2008). Combination of immunohistochemistry and laser ablation ICP mass spectrometry for imaging of cancer biomarkers.* Proteomics, 8*(18), 3775–3784.*

traditional proteomics or Western blots. However, MSI-labeled analysis offers the ability to multiplex significantly the number of targets mapped and quantified, simultaneously, while still allowing other nonlabeled molecular species to be detected in the same sample simultaneously. To date, such methods have not been widely used in pharmaceutical research, but it can be anticipated that with the increasing research into new larger molecule therapeutics, particularly within an oncology setting where tissue homogenization is a poor substitute for the complexity of a tumor microenvironment, the use of small molecule MSI analysis with large molecule labeling MSI will be increasingly performed.

Therapeutic ADCs are large molecule monoclonal antibodies coupled to potently cytotoxic drugs by chemical linkers with labile bonds. ADCs offer the possibility of tumor-targeted treatments. Targeting with the antibody allows lower dosing levels, reducing the risk of off-target effects of the cytotoxic payload. However, the bioanalysis, biodistribution, and monitoring of cytotoxic moiety required to mitigate toxicity remain challenging. The large molecule antibody and low copy number cytotoxic moiety push MS analysis to the limits. However, analysis of small molecule ADC catabolites in rat liver and tumor tissue by LESA coupled to microcapillary liquid chromatography/tandem mass spectrometry (LESA-μLC/MS/MS) has been reported. This alternative to tissue imaging using mass spectrometry is tissue profiling. Profiling MS can be described as direct and discrete position analysis used to compare different tissue regions. Imaging is like profiling but repeated at reparative distances to allow generation of molecular images. Therefore,

the profiling of tissues, rather than high resolution imaging, offers researchers at least some direct tissue measurements of hard to detect targets (Lanshoeft et al., 2016). The analysis described generated both quantitative and qualitative information for the spatial distribution of ADCs and their related catabolites in tissue sections. The manuscript also highlighted how the LESA-μLC/MS/MS and QWBA were complementary. For complex bioanalysis, it is through a combination of traditional bioanalysis of plasma and tissue homogenates, related to traditional histopathological imaging, label-based assays, and a variety of tissue profiling and imaging by mass spectrometry that will allow researchers to discover and develop new safe and effective therapies. Finally, it is worth noting that LESA sampling but without LC separation has been used for direct tissue imaging but at low resolution (1 mm) for low molecular weight targets that are hard to detect using the more common higher resolution MALDI and DESI MSI methods (Swales et al., 2015, 2014).

14. CLINICAL TRANSLATION

What we have considered so far is how MSI can play an effective role in the discovery and development of new drugs. This work is predominantly performed using preclinical assays and models (in vitro, in vivo, and ex vivo samples) but to transform a compound from the lab bench to a medicine requires extensive clinical trials. This is where any compound attrition can result in loss of hundreds of millions of research dollars. Therefore, translation of preclinical to clinical data is vital and MSI has an important role to play in contributing to oncology translational science. One crucial area is in the analysis of both diseased tissue biopsies and tissue following surgical segmental resection. Application of MSI analysis to the latter is proving useful in understanding disease progression and metabolic phenotyping (Guenther et al., 2015) as well as providing molecular information for intraoperative MS analysis by techniques using rapid evaporative ionization mass spectrometry (REIMS and iKnife) (Balog et al., 2013). It is worth remember that any clinical analysis by MSI is best related to gold standard match IHC data for validation, as exemplified by the analysis for the discrimination of lymph node metastases using DESI MSI imaging (Abbassi-Ghadi et al., 2014). Clinical pathology using MSI endpoints is being explored, and this includes traditional tissue samples that have been formalin fixed and paraffin embedded. However, this work is beyond the remit of this discussion so will not be considered further now, but is well reviewed by Aichler and Walch (2015).

A natural extension from the tissue section MSI analysis of material collected during surgery is the collection and analysis of tissue biopsies. Such an approach needs higher resolution analysis for the smaller needle biopsies. However, with spatial resolutions now routinely sub-50 μm, no major technical obstacles remain. Work in this area is led by groups like that of Natalie Agar using MSI as for surgical decision making (Calligaris et al., 2013). Applying such methods to understanding drug penetration and localization within solid tumors in clinical trials will impact future drug development. Such ongoing work has been reported by researchers from National Cancer Center, Japan, where tumor biopsies analyzed for patients with solid tumors. In their analysis, biopsies were taken following treatment with olaparib (a PARP-1 inhibitor). They have reported that the distribution of drug was detected in the tumor region by MALDI MSI and the signal detected in areas of necrosis was higher than that observed in living cell areas (Shimoi et al., 2014). To date, this exciting example of drug distribution analysis by patient needle biopsy MSI analysis has yet to be fully published. A recent example of the effectiveness of needle biopsy analysis by MSI describes element bioimaging of liver needle biopsy specimens from patients with Wilson's disease by LA-ICP-MS (the ionization and mass analyzer method discussed earlier for detection of antibodies with MS detectable metal tags) (Hachmöller et al., 2016). In this study, there was no metal-tagged probe; rather patients had a rare genetic dysfunction of the copper metabolism, which causes the accumulation of the metal in different organs (including the liver and the CNS). This means that the LA-ICP-MS was able to detect the copper in liver needle biopsies at 10 μm spatial resolution as highlighted in Fig. 3. Due to the sensitive of the ionization and mass analyzer, this needle biopsy methodology would be highly effective at the analysis of metal-containing drugs such as cisplatin.

15. EMERGING APPLICATIONS: SPHEROIDS

With the ability to perform MSI at greater spatial resolutions and with increased sensitivities, the technologies are now being applied to the analysis of microphysiological systems. Microphysiological systems are 3D cell cultures increasingly used in early drug discovery for efficacy and safety screening. Early utility was demonstrated by the Hummon group who first demonstrated protein and peptide distributions in 3D culture systems (Li & Hummon, 2011) before the group reported the utility of the application to drug discovery (Liu & Hummon, 2015). Their proof-of-concept

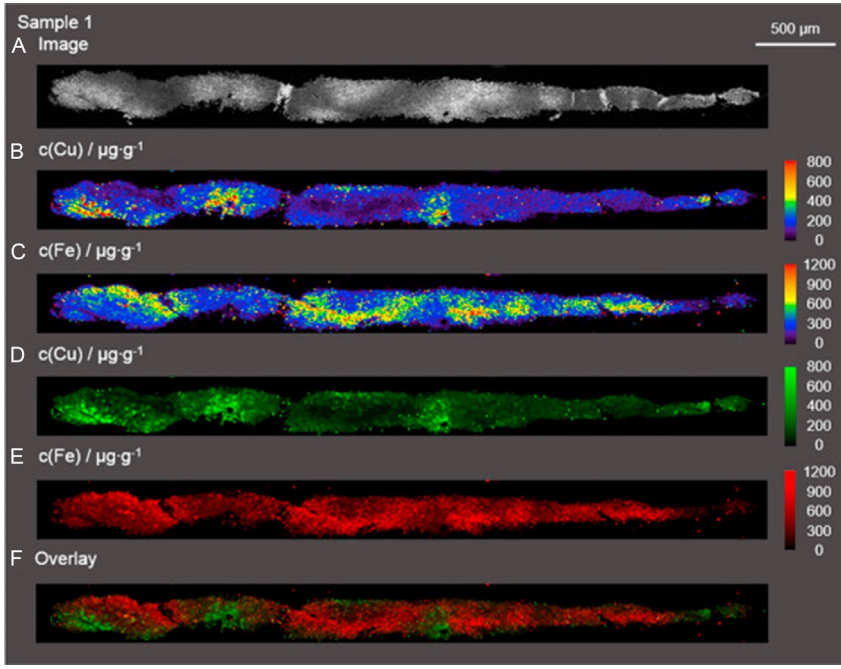

Fig. 3 Autofluorescence microscopic image of the Wilson's disease liver sample 1 (A) investigated by LA-ICP-MS, quantitative distribution maps of copper (B, D) and iron (C, E), and overlay of the copper and iron distribution (F). *Reproduced from Hachmöller, O., Aichler, M., Schwamborn, K., Lutz, L., Werner, M., Sperling, M., . . . Karst, U. (2016). Element bioimaging of liver needle biopsy specimens from patients with Wilson's disease by laser ablation-inductively coupled plasma-mass spectrometry.* Journal of Trace Elements in Medicine and Biology, 35*, 97–102.*

experiment, using in HCT 116 colon carcinoma multicellular spheroids, was to assess the distribution of the anticancer drug, irinotecan (Liu, Weaver, & Hummon, 2013). They were able to demonstrate time-dependent penetration of drug, as well as simultaneously the abundance and distribution of three metabolites as seen in Fig. 4. For smaller dimension samples, analysts are able to combine and relate the MSI analysis with other spectroscopic technologies such as fluorescence or Raman microscopy, allowing 3D imaging studies to be performed (Ahlf, Masyuko, Hummon, & Bohn, 2014).

There has been a recent explosion in use and analysis of microphysiological system and MSI analysis endpoints, a flavor of which includes the use of MSI technologies like laser ablation ICP-MS imaging of multicellular tumor spheroids as a tool in the preclinical development of metal-based anticancer drugs (Theiner et al., 2016), or sample processing

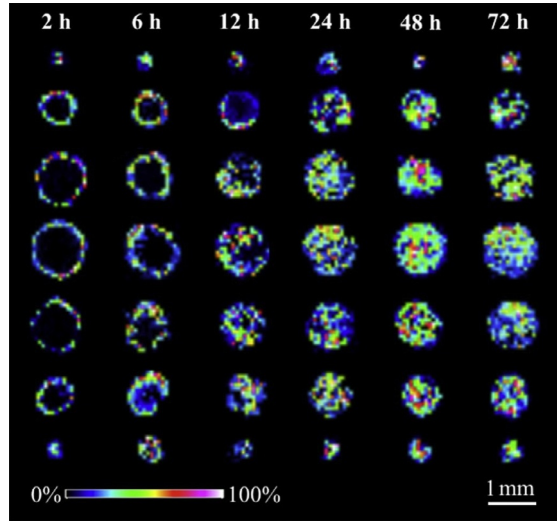

Fig. 4 Time-dependent penetration of irinotecan (*m/z* 587) in HCT 116 spheroids analyzed by MALDI-IMS. Spheroids were treated with 20.6 μM irinotecan for 2, 6, 12, 24, 48, and 72 h (from *left* to *right*). For every treatment duration, color gradient intensity maps were generated from seven consecutive 12 μm slices from a single spheroid in 120 μm vertical intervals. *Reproduced from Liu, X., Weaver, E. M., & Hummon, A. B. (2013). Evaluation of therapeutics in three-dimensional cell culture systems by MALDI imaging mass spectrometry.* Analytical Chemistry, 85*(13), 6295–6302.*

improvements using ultrathin matrix coating for tumor microenvironment model to study epithelial-to-mesenchymal transitions (Wang et al., 2015). To aid such analysis, there has also been development of new sample generation methods, such as the generation and functional assessment of 3D multicellular spheroids in droplet-based microfluidics platform (Sabhachandani et al., 2016) and the development of mixed hydrogel bead-based tumor spheroid formation for anticancer drug testing (Wang & Wang, 2014). Combined, such reported methods for spheroid generation and MSI analysis demonstrate that MSI and MPS can be efficiently scaled in production and analysis for effective screening of new therapeutics for efficacy and safety studies.

16. INCREASED SPATIAL RESOLUTION

One crucial aspect not yet considered in detail is that most MSI technologies do not allow intracellular analysis. Intracellular measurements are possible using secondary ion mass spectrometry (SIMS) and nanoSIMS

but are not easily performed. Such methods reveal the relative abundance of drug or endogenous metabolites at a subcellular level (Dollery, 2013; Passarelli et al., 2015). To date, the various SIMS instruments have not widely been used in the bioanalysis of tissues or cells by pharma though this is likely to increase as awareness of the technologies is further disseminated and next-generation instruments arrive. However, they have found use in medicine formulation and production where higher abundance of target compensates for the smaller site of sample ionization (Bich, Touboul, & Brunelle, 2013; Pajander, Haugshøj, Bjørneboe, Wahlberg, & Rantanen, 2013; Qu et al., 2015).

17. METROLOGY FOR MS IMAGING

Oncology bioscience and drug discovery are a highly interdisciplinary field requiring measurements from many techniques. As described through this chapter, MSI is emerging as a powerful suite of methods for the fundamental study of cancer biology and efficacy of therapeutics. While we have discussed the advantages and opportunities these relatively new and highly complex methods afford, we have not considered the metrology and regulatory factors of applying new technologies in R&D. One limitation is the confidence to apply data generated by new techniques in a regulatory environment or to clinical samples. In the majority of methods discussed in this chapter, there is a strong interdependence between sample preparation, instrument-operating conditions, and resulting data and suitability of data mining approaches. The correctness, reproducibility, and repeatability of the data produced are of great consequence for effective use, interpretation, and decision making. There is an increasing need for metrology and standards to support the continued use and development of MSI in these research areas. Standards ensure that the measurements are fit for purpose between different systems, users, laboratories, and instrument models. The goal of many researchers is to elevate MSI to an analytical technique validated for use in a clinical setting for measuring drug and biomarkers and as a diagnostic. This aim undoubtedly requires further work. For example, in MALDI MSI, the wide variety of matrices, sample preparation methods, instrumentation, analytical parameters, and mass analyzers (Caprioli, Farmer, & Gile, 1997; Gusev, Vasseur, Proctor, Sharkey, & Hercules, 1995) means that there have been few attempts to standardize methodologies, create reference samples, or assess repeatability. The comparison of tissue preparation protocols by single groups has been carried

out periodically (Gemperline, Rawson, & Li, 2014; Martin-Lorenzo et al., 2014; Seeley, Oppenheimer, Mi, Chaurand, & Caprioli, 2008) and are important in enabling methodological standardization. The validity of these comparisons must then be proven across different instrumentation and institutions. Multicenter studies are a vital step in this process and are beginning to be carried out by groups such as those of McDonnell, Walch, and Schmitt (Dekker et al., 2014). Key to a better understanding of a technique is a well-characterized reference sample, a particularly challenging requirement in many MSI application areas. Studies pursuing this aim include the recent development of a lateral resolution standard. Development and sharing of test and reference standards and materials will importantly allow the MSI community to benchmark and report their performance (Passarelli et al., 2014). Benchmarking and comparison to other analytical techniques also provide an important level of confirmation for MALDI MSI, examples of which include comparison with liquid chromatography tandem mass spectrometry (LC-MS/MS) (Hankin & Murphy, 2010; Takai, Tanaka, Inazawa, & Saji, 2012), desorption electrospray ionization (DESI) MSI (Eberlin et al., 2011), infrared matrix-assisted laser desorption electrospray ionization (IR-MALDESI) MSI (Barry, Groseclose, Robichaud, Castellino, & Muddiman, 2015), LESA MS (Swales et al., 2014), SIMS imaging, Raman imaging (Bocklitz et al., 2013), magnetic resonance imaging (Sinha et al., 2008), autoradiography (Hsieh et al., 2006; Solon, Schweitzer, Stoeckli, & Prideaux, 2010; Takai et al., 2012), histochemistry (Oppenheimer, Mi, Sanders, & Caprioli, 2010), and immunohistochemistry (Acquadro et al., 2014; Klein et al., 2014). The automated registration of MSI data sets to histology and on-line histological "atlases" has also recently been explored (Abdelmoula, Carreira, et al., 2014; Abdelmoula, Škrášková, et al., 2014; Verbeeck et al., 2014).

Within a field with such a variety of variables and sample types, the effective communication of information relating to the experimental process is particularly necessary. A recent publication entitled "Discussion point: reporting guidelines for MSI" (McDonnell et al., 2015) seeks to set out a framework to ensure the appropriate reporting of the "metadata" associated with MSI workflows. Furthermore, the common data format imzML (Race, Styles, & Bunch, 2012; Schramm et al., 2012) for MSI was recently introduced to better enable sharing and comparison of imaging data from multiple platforms within multiple software packages. New software tools have recently been developed, which allows researchers to evaluate and compare preprocessing and postprocessing (e.g., multivariate analysis) methods and to

process large data from multiple mass spectrometer types. The need for measurements to be repeatable and reliable led to a VAMAS (Versailles Project on Advanced Materials and Standards) interlaboratory study. Participants from 20 different laboratories were provided with a DESI protocol and two samples: a thin film of Rhodamine B and a piece of double-sided tape (both deposited on glass). Repeatability of absolute intensity of Rhodamine B was assessed along with the consistency of size and shape of erosion craters. While some DESI experimental setups gave consistent and repeatable results (repeatability <20%), others were considerably worse and crater sizes varied due to inconsistency of spray and stage movement (Gurdak et al., 2014). Recent reports reconsider spray parameters for tissue imaging: Abbassi-Ghadi et al. reported that through the use of optimal DESI spray and geometry parameters, highly reproducible spectral profiles from a complex, biologically relevant sample could be obtained (Abbassi-Ghadi et al., 2015).

As MSI is used more widely in drug discovery, further research in metrology will be needed to underpin the technique development and to support academic and industrial research. Establishing this reliable framework with standardized approaches will be an important step in using the techniques more frequently in clinical research and in support of regulatory submissions in drug development.

18. CONCLUSION

For maximal effective impact in aiding oncology drug discovery, MSI will need to be used in combination with a range of bioanalysis and molecular technologies that allow researchers to understand the structure within the tumor microenvironment, tumor metabolism, efficacy of therapeutics, and measurements of the phenotypic features of tumors, all of which feeding into both diagnostic and prognostic data. However, MSI can be embedded within all areas of drug discovery. Some researchers are using MSI techniques to aid early lead identification, though most applications still apply MSI to in vivo efficacy and safety studies. However, increasingly MSI applications are being explored in the clinic and it can be assumed that in the near future MSI may well have a role in hospital pathology departments. The challenges of developing new, effective, and safe oncology medicines remain considerable. However, MSI encompasses a formidable array of technologies that are bringing insight and understanding to the molecular maelstrom in oncology, biology, drug discovery, and treatment.

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