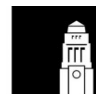

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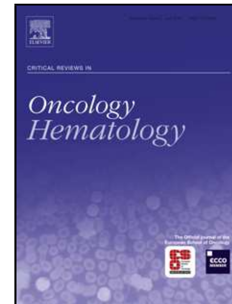

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Precision pharmacology: Mass spectrometry imaging and pharmacokinetic drug resistance

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1. **Highlights:**

   1. Pharmacokinetic resistance is a consequence of a drug not being delivered at sufficient concentration to, or with adequate distribution in, a tumour. The aberrant tumour microenvironment and vasculature influence drug delivery throughout the tumour; specific obstacles to drug distribution such as the blood-brain-barrier may further limit drug delivery.
2. Anticancer drugs also cause significant toxicity, again as a result of distribution to, and within, normal tissues. This will affect the nature and severity of the side effects that patients experience.
3. Drug distribution and penetration through tumours and normal tissues may influence selection of the optimal candidate(s) during drug development as well as affecting the preferred dose and schedule of drug administration in clinical practice.
4. MALDI-MSI is a technique for imaging the distribution of anti-cancer drugs and their metabolites within tumours that can be used in drug development to:

4.1. Characterize drug distribution within the body to identify anticipated toxicities or target organs

4.2. Define drug distribution within the tumour and its relationship with the tumour microenvironment and drug targets

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4.3. Select the best drug candidate, dose, schedule or combinations based on tumour pharmacokinetic profile
4.4. Facilitate proof-of-concept studies for new drug delivery systems.

Abstract:

Failure of systemic cancer treatment can be, at least in part, due to the drug not being delivered to the tumour at sufficiently high concentration and/or sufficiently homogeneous distribution; this is termed as "pharmacokinetic drug resistance". To understand whether a drug is being adequately delivered to the tumour, "precision pharmacology" techniques are needed. Mass spectrometry imaging (MSI) is a relatively new and complex technique that allows imaging of drug distribution within tissues. In this review we address the applicability of MSI to the study of cancer drug distribution from the bench to the bedside. We address: (i) the role of MSI in pre-clinical studies to characterize anti-cancer drug distribution within the body and the tumour, (ii) the application of MSI in pre-clinical studies to define optimal drug dose or schedule, combinations or new drug delivery systems, and finally (iii) the emerging role of MSI in clinical research.

Keywords: MALDI-MSI, intra-tumoural drug distribution, drug development, pharmacokinetics

1. INTRODUCTION

Drug resistance, both de novo and acquired, is arguably the greatest challenge in oncology for cytotoxic chemotherapy, molecularly targeted agents (MTAs) and potentially also for immunotherapies. In addition to well recognized cellular and molecular mechanisms of resistance, the barriers that separate drug in the plasma from the cancer cell such as the aberrant tumour vasculature, the "alien" microenvironment

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with stroma rich in dysfunctional fibroblasts and extracellular matrix, areas of hypoxia and raised intratumoural fluid pressure all affect the concentration and distribution of drug within a tumour (Minchinton and Tannock, 2006). Failure of treatment due to the inability to deliver sufficient drug to a solid tumour has been termed "pharmacokinetic drug resistance" (Tannock et al., 2002).

Precision medicine can be characterized as "the right treatment, for the right patient, given at the right time"; in the context of cancer drug development and pharmacology, this can perhaps be re-formulated as "the right drug, in the right place, at the right concentration". With developments in matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI), intra-tumoural drug concentrations and distribution can now be studied.

1.1 Pharmacology in oncology drug development

Pharmacology data are typically characterized as pharmacokinetic (i.e. what the body does to the drug) and/or pharmacodynamic (i.e. what the drug does to the body). The latter has become increasingly important in modern drug development, involving imaging or the collection of blood, tumour or other surrogate tissues for novel biochemical or genetic analyses. By contrast, pharmacokinetic studies have remained focused on measurements of drug (and metabolite) concentrations, usually in plasma, albeit with increasingly sensitive assays. Remarkably little is, however, generally known about drug concentrations in the tumour (or other extravascular, normal tissues) and even less about how a drug is distributed within a tumour.

In pre-clinical studies the IC_{50} is the concentration of a drug required to achieve half its maximum effect; for cytotoxics this relates to cytotoxicity but for molecularly targeted therapies the IC_{50} reflects binding to the specific target. These data are generated in vitro, with varying durations of drug exposure, bearing little if any resemblance to plasma pharmacokinetics in vivo or clinically, and not taking account of important factors such as protein binding of the drug. Nevertheless, comparisons are frequently made between the IC_{50} of a drug and plasma concentrations in patients and conclusions drawn as to whether a drug is more or less likely to be effective clinically.

1.2. Intra-tumoural pharmacokinetics

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The study of intra-tumoural drug concentrations and distribution is challenging. Tumour drug concentrations have been measured, albeit infrequently, in pre-clinical models or patient samples, but tumour disaggregation for LC-MS/MS analysis precludes generating information on drug distribution within the tumour. Whole body autoradiography (WBA), positron emission tomography (PET) and fluorescence microscopy (FM) have all been used to study drug distribution in pre-clinical models and occasionally in patients. These methods have the advantage of being less invasive than biopsy based assays, but both WBA and PET have low spatial resolution and require radiolabeled drug. FM allows the study of drug distribution at a cellular level but is limited to drugs that fluoresce naturally, unless a fluorescent label can be introduced; drug labelling can, however, be challenging since images may not differentiate between the drug and its metabolites and it can be argued that labelled drug may not behave in the same manner as the unlabeled compound (Cobice et al., 2015).

1.2.1. Potential of MALDI-MSI in drug distribution studies

Whilst many immunohistochemistry, autoradiography and spectroscopic methods are used routinely in pharmacology research, the application of MALDI-MSI is still relatively new. In MALDI-MSI images are created by irradiating the surface of a section of biological tissue, which has been coated with an energy absorbing matrix, with a UV laser. Ions ejected from the surface are subjected to mass spectrometric (MS) analysis and images generated by plotting the intensity of specific m/z values with positional information as a grey or colour scale (Swales et al., 2019). One of the key features of MALDI-MSI that makes its use appealing is the ability to detect and study the distribution of multiple compounds simultaneously without the need for labelling. Additionally, by using accurate mass measurement and tandem MS analysis, molecules can be identified directly on tissue sections. The initial proof of concept study demonstrated the MALDI imaging of proteins in biological tissue more than 20 years ago (Caprioli, Farmer and Gile, 1997); since then, however, MALD-MSI has been applied to the analysis of a wide range of pharmaceutical compounds in situations ranging from whole animal sections to drug eluting stents and 3D tissue models (Prideaux, Staab and Stoeckli, 2010)(Swales et al., 2019)(Huang et al., 2012)(Russo, Lewis, et al., 2018). MALD-MSI can also relate the distribution of compounds to tumour morphology using conventional histology because MALDI-MSI does not disrupt or damage the sample. Limitations of MALDI-MSI include the requirement for

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sample preparation and it only being applicable to molecules that can be ionized, although on-tissue derivatization has been employed to facilitate ionization. MALDI-MSI also generates huge amounts of data requiring complex informatics resources for processing and analysis (Fuso Nerini et al., 2014)(Morosi, Zucchetti, et al., 2013). Until recently, spatial resolution was insufficient to study intra-tumoural drug distribution but resolution of 10-30µm is now routinely achieved with MALDI-MSI making such studies feasible. Finally, MALDI-MSI had previously been only semi-quantitative, but with recent advances some studies are now quantifying drug concentrations (Castellino, 2012)(Takai et al., 2012)(Russo, Bricklebank, et al., 2018).

To minimize the risk of failure later in cancer drug development there is a strong argument for undertaking proof-of-principle studies early to demonstrate that the active drug is delivered to the tumour in vivo, and in patients, at the necessary concentration; this is especially true for novel drug delivery systems. For example, several clinical trials were undertaken with early generation polymer-based pro-drugs before a proof-of-principle pharmacokinetic study showed that MAG-CPT did not deliver camptothecin preferentially to the tumour in patients (Sarapa et al., 2003). In contrast, once Phase I trials with the oral fluoropyrimidine pro-drug capecitabine had been completed and demonstrated that circulating plasma levels of the parent compound and inactive metabolites were high but those of 5-fluorouracil (5-FU) very low (Mackean et al., 1998), a proof-of-principle tumour/tissue pharmacokinetic study demonstrated that 5-FU concentrations were 21-fold higher in tumour than plasma and, more importantly, 3-fold higher in tumour than adjacent normal tissue (Schüller et al., 2000).

We review the use of MALDI-MSI for analysis of the distribution of anti-cancer drugs in preclinical and clinical drug development, (Schöne, Hö and Walch, 2013)(Weaver, Hummon and Amanda, 2013). First, however, we give a brief summary of the technical aspects of MALDI-MSI to facilitate understanding the pre-clinical and clinical data; more detailed reviews of these technical aspects have been covered recently (Swales et al., 2019).

1.2.2 Practicalities of MALDI-MSI

Sample preparation is the crucial first step towards success in imaging an analyte. Samples are usually embedded in gelatin as MALDI-MSI works with cryotissues, which are usually sectioned at 10-12µm. Indeed, paraffin-embedding tissues makes it

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impossible to ionize the sample properly. Parameters such as matrix selection and tissue-specific ion suppression have a significant impact on the sensitivity of analyte detection sensitivity. The type of matrix needs to be optimized for each analyte, which can be for positive or negative ionization, and then applied by spraying or spotting across the whole sample to extract the analyte from the upper layers of the tissue section. Once the sample is inside the MALDI-MSI spectrometer, the region of interest is selected for the laser beam to scan the tissue section. The matrix absorbs most of the energy of the irradiated laser leading to soft ionization of the analyte that allows the ionization of large molecules such as peptides or proteins. Each laser beam generates analyte ions that are separated by their time of flight (TOF) and mass-to-charge values (m/z), i.e. by their "size" and "charge". Ionized analytes are detected by the mass spectrometer from each laser beam and the mass spectrum recorded. Each laser beam contains, therefore, the mass spectrum from analytes derived from that region of interest. Finally to obtain image of the analyte of interest, each analyte's m/z is selected and the software translates the signal into a coloured pixel image. This creates a "map" of the compound within the tissue. Tissue signals from different analytes can be later superimposed to create a composite image. Low mass ions from the matrix or endogenous compounds present in the biological tissue can, however, interfere with the target analytes. To avoid this interference, tandem mass spectrometry strategies (MS/MS) or high resolution mass spectrometry such as Orbitrap or Fournier transform ion cyclotron resonance (FTICR) can be applied (Schöne, Hö and Walch, 2013)(Römpp and Spengler, 2013). (Figure 1).

2. PRECLINICAL STUDIES

2.1. Characterizing anti-cancer drug distribution within the body and/or organs

MALDI-MSI has been used to better understand anticancer drug distribution within the body and within specific organs with respect to potential toxicities.

Whole body imaging of the microtubule targeted cytotoxic vinblastine in mice showed distribution mainly to the liver, kidney and tissue surrounding the gastrointestinal tract (Trim et al., 2008). Interestingly, however, the principal toxicities of vinblastine are

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myelosuppression and neuropathy; gastrointestinal toxicity is also common, reflecting biliary excretion (35%). One technical aspect worthy of comment is that vinblastine imaging was subject to interference from other endogenous ions, especially in the renal cortex. To address this, the authors used MALDI-MSI-MS/MS mode to increase specificity; images were also compared with whole body autoradiography. When irinotecan (CPT-11) distribution was imaged in a mouse colorectal cancer model, high levels of drug and its active metabolite SN38 were detected in the intestinal tract and liver. CPT-11 is metabolized to SN-38 mainly in the liver and diarrhoea is, along with myelosuppression, the principle toxicity (Buck et al., 2015). Here again there was a high level of background noise when analysing SN38 with a MALDI-time-of-flight (TOF) MSI instrument, which could be improved with FTICR-MS. Although technical aspects are not the focus of this review, it is important to recognize the limitations of the technique that is being used and to select the most appropriate instrument and methodology.

MALDI-MSI can also be useful to study drug distribution within a specific organ. A study of imatinib and ifosfamide distribution successfully localized both drugs within the mouse kidney. Images of several phospholipids in the mouse kidney by MALDI-MSI corresponded well to histological features of the kidney stained by haematoxylin-eosin. Composite images of phospholipids, heme (a cofactor of hemoglobin used to delineate the lumen of the blood vessels) and imatinib were performed by MALDI-MSI; imatinib localized to the outer medulla, whereas ifosfamide accumulated in the inner medullary region (Römpp et al., 2011). Dasatinib also accumulated in the renal medulla of mouse kidney (Schulz et al., 2013). Another preclinical study with oxaliplatin, given by HIPEC (Heated IntraoPErative Chemotherapy) in rats, showed oxaliplatin and its metabolites were present almost exclusively in the renal cortex, not penetrating more deeply (Bouslimani et al., 2010), although data on the duration of drug exposure were not reported. These studies demonstrate the limitations of knowing only to which organs a drug distributes, rather than where within a heterogeneous organ that accumulation occurs.

Drug distribution is particularly important with respect to drugs that need to penetrate the intact blood brain barrier (BBB) to have their desired effect; by contrast, for other drugs it is desirable that they be excluded by the BBB to avoid toxicities. In the context of treating primary or metastatic brain tumours, it can be argued that drugs should be

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excluded by the intact BBB but able to cross the disrupted blood-brain tumour barrier. Concentrations in the cerebrospinal fluid (CSF) have been used as a surrogate for drug penetration into the brain but this represents only drug that has crossed the choroidal plexus i.e. the blood-CSF barrier (Hawkins and Davis, 2005). By contrast, MALDI-MSI showed that irinotecan penetrated the blood CSF barrier but not the intact BBB in normal brain (Buck et al., 2015).

Distribution of the Wee1 inhibitor MK-1775 was studied in glioblastoma orthotropic and heterotopic mouse xenografts using a MALDI-MSI TOF/TOF instrument. MK-1775 MALDI-MSI images were generated for both MK-1775 and placebo (i.e. negative controls) animals with taken at the times of peak drug concentrations in the brain and blood. MK-1775 showed antitumour activity, as single agent or in combination with temozolamide, in the heterotopic model but not in the orthotropic model. Using MALDI-MSI, only 5% of the drug distributed into the normal brain but MK-1775 distributed to both glioblastoma models; distribution was, however, more heterogeneous in the orthotropic tumours. This may explain, at least in part, the lack of activity in that model. Orthotropic brain tumours models may not, however, accurately represent the physiologic BBB in this setting as they do not have the intrinsic tumour vasculature and microenvironment. The alternative genetically engineered mouse model (GEMM) might be more representative of the physiological BBB, but such models are much more costly and have other limitations (Pokorny et al., 2015). Similar heterogenic distribution has been described with lapatinib in a model of breast cancer brain metastasis using autoradiography. In this model, brain-seeking 231-BR-HER2 cells were inoculated into the left heart ventricle; after 3-6 weeks brain metastases had developed and the animals had neurological symptoms. ^{14}C-lapatinib was then administered orally or intravenously. Although brain metastases had 7 to 9-fold higher concentrations of lapatinib than normal brain, mean lapatinib concentration in the brain metastases was only 10 - 20% of that at other sites of metastasis (Taskar et al., 2012). Lapatinib is active against brain metastasis in patients with HER2 positive disease but that activity is limited, which may be in part due to relatively low intra-tumoural drug concentrations (Petrelli et al., 2017).

A more complex study imaged BKM120 (a pan-class I PI3K inhibitor) and RAF265 (a RAF serine-threonine kinase inhibitor) simultaneously with heme in healthy mice and an orthotropic mouse model of glioblastoma. BKM120 drug signal did not co-locate

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with heme in the brain of the healthy mouse, indicating that, although separation between heme and the drug was not uniform, BKM120 penetrated the brain parenchyma. On the other hand, RAF265 brain distribution co-located with heme in the aberrant tumour vasculature but was not detected in normal brain parenchyma, indicating limited penetration through the normal BBB. Images of BKM120, RAF265 and heme were performed by MALDI-MSI TOF/TOF, with the MALDI-MSI heme images validated by co-registration with fluorescence microscopy images of fluorescein and FTIC, two widely accepted vascular markers; 3-D models were reconstructed from MS and optical images (Liu et al., 2013).

Finally, erlotinib and its metabolites were also imaged in a model of orthotopic glioma but using MALDI-FTICR to enhance sensitivity and specificity. Around the tumour margins, where there is local disruption of the blood-tumour barrier, there was a high intensity of heme signal while erlotinib was homogenously distributed independently of the heme signal indicating that at least some drug had escaped from the blood vessels (Liu et al., 2013).

These examples show that improved MALDI-MSI technology can answer very specific questions about drug distribution, focusing on healthy tissues. If replicated in pre-clinical models of cancer, this has the potential to inform drug development, improving the therapeutic window by reducing toxicity and increasing efficacy.

2.2. MALDI-MSI studies of intra-tumoural drug distribution

As discussed above, relatively little is known about the intra-tumoural drug distribution of most anticancer drugs but with improved spatial resolution it is becoming possible to characterize drug distribution using MALDI-MSI.

To date, in vivo models of drug diffusion and distribution within tumour have been studied most extensively (Connell et al., 2015)(Huber et al., 2014). Such models are, however, complex and costly. Other options, such as injecting drugs directly onto tumour then imaging drug distribution by MALDI-MSI ex vivo complement in vivo models (Vegvari et al., 2013)(Connell et al., 2015) albeit with the limitation of disregarding the aberrant tumour vasculature. In vitro spheroid models also have limitations, specifically disregarding the complex tumour microenvironment and abnormal tumour vasculature, but nevertheless can be useful (Liu, Weaver and

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Hummon, 2013)(LaBonia et al., 2016) and save both time and money in comparison with in vivo models.

2.2.1. In vitro and ex-vivo studies

Spheroids represent a three dimensional tissue culture model, where cancer cells are grown to form a small sphere; as such they are a rudimentary in vitro representation of a tumour mass with a vascularized area represented by the spheroid rim, a necrotic area at the spheroid core and a hypoxic area between the two. All three regions can be identified in slices through the spheroid and studied using MALDI-MSI.
Irinotecan and its key metabolite SN38 have been identified in each of these three regions, localizing the metabolite to the spheroid rim where cells are active and dividing (Liu, Weaver and Hummon, 2013). HCT-116 colon cancer cell line spheroids of approximately 1mm diameter were treated with irinotecan, three regions of interest within each spheroid identified by optical imaging and each MALDI laser spot classified accordingly. Spheroids were first embedded in gelatin, 12µm thick sections transferred onto slides and α-cyano-4-hydroxycinnamic acid matrix used as this showed the less background interference with irinotecan. Irinotecan and SN38 images were generated using an AutoFlex III instrument with external calibration. Results were presented in 2D format with semi-quantitative data along with a colour scale to show drug intensity; both the parent and metabolite were imaged simultaneously. At initial (2 and 6 hour) time points irinotecan was located in the rim and intermediate regions, but in the spheroid core only at the 12 hour time point. Drug distribution throughout the spheroids was achieved after 24 to 48 hours of exposure to irinotecan when central regions had a higher concentration of irinotecan than at the periphery. By contrast, SN-38 and other metabolites were detected only after 72 hours of exposure, and the signal was more intense in the outer rim of the spheroids than at the core. Figure 2A. It is important to emphasize the complexity of this work and the detail of the analysis. The volume of data and the precision of the MALDI-MSI spheroid images achieve to generate drug distribution at multiple time points would be almost impossible in an in vivo experiment. Similar results were reported using a novel three dimensional printed fluidic device to study colorectal cancer spheroids exposed to irinotecan in combination with 5-fluorouracil and folinic acid and imaged by MALDI-MSI TOF/TOF (Labonia et al., 2018).

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Other work using spheroids has compared the penetration of conventional, "free" doxorubicin to an "in-house" liposomal doxorubicin formulation using MALDI-MSI TOF/TOF instruments (Reeder et al., 2016). In this study, spheroid sectioning was performed with 16µm alternating slices, one for MALDI-MSI and the other for fluorescence microscopy analysis as doxorubicin fluoresce at 480nm. The images were overlayed by ImageJ, demonstrating the feasibility of combining the two methodologies; only MALDI-MSI could, however, image and distinguish doxorubicin from three doxorubicin metabolites (7-deoxydoxorubicinone, 7- deoxydoxorubicinolone and doxorubicinone). Spheroids treated with free doxorubicin had doxorubicin in the outer layers of the spheroid by 2 hours and it penetrated throughout the spheroids regions by 12 hours. A similar pattern was observed with liposomal doxorubicin but doxorubicin concentrations were higher in the outer rim than at the core at 12 hours, suggesting slower permeation of the liposomal formulation. No differences were seen in the distribution of doxorubicin metabolites between the two formulations. We have imaged paclitaxel in HCT-116 spheroids exposed to drug for 4 hours (Figure 2B) and shown rapid penetration throughout the spheroid (unpublished data).

Patient derived organoids may represent better the heterogeneity of human cancers than conventional spheroids. Organoids derived from a patient with colorectal cancer exposed to irinotecan showed that the drug initially localized to the edge of the organoid but by 24 hours it had reached the core. The active metabolite SN38 was detected at 24 hours but not at 6 hours, and did not co-locate with irinotecan; both compounds were imaged by MALDI-MSI TOF/TOF (Liu et al., 2017), and broadly supported earlier spheroid studies.

Ex-vivo approaches have also been used with drug applied to human lung and breast cancers. An important landmark was the first human tissue drug imaging by MALDI-MSI with spatial resolution of 30µm. Two first generations, clinically relevant, epidermal growth factor receptor (EGFR) inhibitors, erlotinib and gefitinib, were nanospotted onto sections of 3 different histological sub-types of non-small cell lung cancer using a MALDI LTQ Orbitrap XL mass spectrometer in MS/MS data collection mode. Drug signal intensity was higher in stroma than in tumour tissue for each of the tumour sub-types. The EGFR mutation status of the tumours was not reported so it is unclear whether the absence of such mutations contributed to the lower intratumoural drug concentrations (Marko-varga et al., 2011). Interestingly, in breast cancers exposed

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to tamoxifen drug signal (again identified using a MALDI LTQ Orbitrap XL mass spectrometer) was higher in tumour than in stroma in oestrogen receptor (ER) positive tumours; the opposite was found in ER negative tumours (Végvári et al., 2016).

These in vitro and ex vivo studies show that MALDI-MSI can define biologically significant differences in the penetration and metabolism of anti-cancer drugs but are far removed from the clinical situation. It is, therefore important to understand how MALDI-MSI performs in more relevant in vivo models.

2.2.2. In vivo studies

In vivo studies of intra-tumoural drug penetration and distribution have the principle advantage of representing better drug pharmacokinetics (absorption, distribution, metabolism and excretion), the tumour vasculature and stroma and using a clinically relevant route of drug administration.

An in vivo study of erlotinib distribution in a murine model of spontaneous pancreatic ductal carcinoma showed higher concentrations of the drug in healthy tissues than in the tumour. Genetically engineered murine models have the advantage of having an intrinsic tumour microenvironment and vasculature. Erlotinib was imaged with MALDI-MSI TOF/TOF instrument, with additional images obtained using a MALDI FTICR instrument to differentiate erlotinib and its metabolites. Areas with a higher proportion of atypical glands had a higher peak intensity of erlotinib than the stroma. To prove that differences in peak intensities were not due to ion suppression, “on tissue” spotting of erlotinib was performed as a positive control. Survival of the mice did not correlate with overall erlotinib levels but did appear to correlate with the proportion of erlotinib in the atypical glands and with the percentage of those glands in the tumour (Grüner et al., 2016). A further MALDI-MSI and LC-MS/MS lung cancer murine xenograft study showed more heterogeneous distribution of erlotinib in the tumour, with less drug signal in necrotic areas, compared to normal lung. These findings were supported by MALDI-MSI and LC-MS/MS analysis of tumour resected from a patient with NSCLC and an activating EGFR mutation (Tsubata et al., 2017).

MALDI-MSI can be useful not only to determine where a drug localizes but also how it distributes through a tumour in vivo over time. For example, in mice bearing colorectal cancer xenografts treated with sunitinib daily for 10 days, levels of sunitinib in the

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tumour increased continuously up to day 7. By day 4, high levels of sunitinib's active metabolites were homogeneously distributed at the center of the xenografts. Similar levels and distribution of sunitinib were demonstrated when the drug was directly deposited onto the tissue surface ex vivo as described above. Temporal distribution of sunitinib and its metabolites was obtained using an orbitrap instrument. (Connell et al., 2015).

Drug distribution within tumour can be correlated with the vasculature by imaging a vessel marker such as CD31 in adjacent tissue sections, using immunohistochemistry or immunofluorescence, or by imaging heme with MALDI-MSI in the same section. Huber et al. (Huber et al., 2014) used immunohistochemistry to image CD31 and MALDI-MSI TOF/TOF to image erlotinib and afatinib in lung cancer xenografts, and sorafenib in a sarcoma xenograft model. Consecutive sections used for immunohistochemistry and MALDI-MSI where then superimposed with digital image analysis. Tumour regions associated with high drug levels correlated with areas of high vascularization, rich in small vessels. Furthermore, differential diffusion patterns were seen for the two drugs. Erlotinib concentration decreased in a linear fashion from the vessel up to a distance of 100µm then remained constant, whereas afatinib distribution plateaued 20µm from the blood vessel with concentrations then decreasing up to 140µm from the vessel (Huber et al., 2014).

Likewise, the presence of drugs can also be correlated with their targets using either MALDI-MSI or techniques such as immunohistochemistry or immunofluorescence to image a drug's receptor. Studies of EGFR inhibitors described above (Vegvari et al., 2013)(Tsubata et al., 2017) did not report the mutational status of the tumours, which could potentially influence drug distribution. However, a recent study of the anaplastic lymphoma kinase (ALK) inhibitor alectinib in ALK amplified and wild type neuroblastoma xenografts found that drug distribution was heterogeneous and appeared not to be influenced by ALK status with alectinib imaged by MALDI-MSI MS/MS. In this experiment D8-alectinib (i.e. deuterated alectinib) was added to the matrix in order to correct possible ion suppression from endogenous tissue components or the matrix and, therefore, to verify the heterogeneity of alectinib distribution (Ryu et al., 2018). In another study using a colorectal xenograft model, composite images showed that some areas of the tumour with higher VEGFR2 expression had higher sunitinib concentrations. Sunitinib images were obtained using MALDI Orbitrap with VEGFR2

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and other sunitinib targets imaged by immunofluorescence in serial sections (Torok et al., 2015).

Another potential use for MALDI-MSI drug penetration studies may be in choosing between candidate lead compounds in pre-clinical drug development. Wide variability was found in the intra-tumoural distribution of a series of five anti-angiogenic receptor tyrosine kinase inhibitors in two murine colorectal cancer xenograft models (Torok et al., 2017). Motesanib, pazopanib, sorafenib, sunitinib and valatanib were imaged using a MALDI-MSI Orbitrap mass analyzer. In one model only sunitinib demonstrated clear biological activity. Interestingly, sunitinib also achieved the highest, and most homogeneous, intra-tumoural concentrations of the five inhibitors. In the other model a second inhibitor, vatalanib was detected at homogeneously high concentrations; again, there was a reduction in both tumour growth and angiogenesis.

These studies are two dimensional representations of a three dimensional tumour. Recently, however, paclitaxel distribution and concentrations have been described in a xenograft model of malignant pleural effusion using serial two dimensional MALDI-MSI TOF/TOF data to construct a three dimensional model with drug easily detected at the edge of the tumour but little if any paclitaxel in much of the xenograft, especially in necrotic or fibrotic areas (Giordano et al., 2016).

Taken together, these in vivo studies show that it is possible to study drug distribution with MALDI-MSI and correlate drug distribution with tumour structure or with drug targets. The next question is if, and how, this information might be integrated into pre-clinical drug development.

2.3. Application of MALDI-MSI to pre-clinical oncology studies

To measure drug concentrations with LC-MS/MS the tissue sample is homogenized, so all structural information is lost and the spatial distribution of drug within a tumour is not known. MALDI-MSI has the potential to establish proof-of-principle when testing different drug schedules, or drug delivery systems, and can be applied to both novel and established anticancer drugs.

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Dose and treatment schedule, along with modification of the tumour microenvironment and novel drug delivery systems, can all influence the uptake and distribution of a drug (Minchinton and Tannock, 2006); regions of the tumour not exposed to optimal drug concentrations may then develop drug resistance leading to treatment failure (Tannock et al., 2002). Clinically, the goal is to maximize tumour drug delivery and efficacy without exacerbating toxicity. Drug distribution within a tumour may also be clinically relevant; although, one might expect that higher drug concentrations in a tumour would lead to greater efficacy, "false positive" results may occur if the drug is not evenly distributed.

2.3.1. Drug dose and schedule

In an example of how dose and schedule may be relevant a human melanoma xenograft model was treated with two different schedules/doses of paclitaxel, either 60mg/kg iv as a single dose or pre-treatment with 8mg/kg/day iv for 5 days and then a final dose of 60mg/kg iv. The daily dose schedule appeared to not only achieve a higher concentration of paclitaxel in the tumours but drug distribution was also more homogenous when imaged by MALDI-MSI. Images here were obtained by a nanoparticle-assisted laser desorption/ionization mass spectrometry imaging technique, which used titanium dioxide (TiO2) nanoparticles matrix suspension to avoid heterogeneous matrix crystallization and to diminish background interference (Morosi, Spinelli, et al., 2013).

2.3.2. Drug combinations

Cesca et al. (Cesca et al., 2016) explored the combination of bevacizumab with paclitaxel in conventional ovarian and colon carcinoma subcutaneous xenografts and in an orthotropic model of ovarian carcinoma. They determined concentrations and distribution of paclitaxel by LC-MS/MS and MALDI-MSI TOF/TOF, respectively; modification of the tumour vasculature after administration of bevacizumab administration was studied immunohistochemically by staining for CD31 and by DCE-MRI. Although the addition of bevacizumab enhanced the efficacy of chemotherapy, the intratumoural concentration of paclitaxel was lower in animals receiving the combination than in those treated with paclitaxel alone. Interestingly, however, the addition of bevacizumab was associated with more homogenous distribution of paclitaxel within the tumour when imaged by MALDI-MSI and slower efflux of drug

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from the tumour which could explain the higher efficacy of the combination; these differences were not seen in normal tissues. Similar effects were seen with another cytotoxic, doxorubicin, suggesting that “normalization” of the tumour vasculature enhanced efficacy. A second study in an EGFR mutant NSCLC xenograft model gave, however, contrasting results (Nishidate et al., 2017). Erlotinib penetrated tumour tissue well, with broad distribution within the tumour when imaged by MALDI-MSI, irrespective of co-administration of bevacizumab and appeared similar in both sensitive and resistant tumours.

The tumour vasculature of animals pre-treated with bevacizumab appears to contain a higher percentage of small vessels (Morosi, Spinelli, et al., 2013) with more permeable endothelium (Yuan, 1998); there is also a correlation between the proportion of small vessels and tumoural drug levels in pre-clinical models (Huber et al., 2014). The microtubule inhibitor cytotoxic agent eribulin has other, non-cytotoxic effects in pre-clinical models including “re-modelling” of tumour vasculature (Funahashi et al., 2014). This effect was confirmed in multiple pre-clinical models, but not with another microtubule inhibitor, vinorelbine (Ito et al., 2017). Interestingly, pre-treatment with eribulin (but not vinorelbine) also increased the accumulation of radio-labelled liposomal doxorubicin in an H1650 non-small cell lung cancer xenografts (Ito et al., 2017); to date, however, MALDI-MSI studies of the impact of eribulin on drug delivery have not been published. The concept that modifying the tumour vasculature, leading to more homogeneous distribution of chemotherapy and greater efficacy, illustrates the potential clinical relevance of drug distribution studies.

Inhibitors of the DNA damage repair enzyme poly-(ADP-ribose) polymerase (PARP) have been widely evaluated clinically, especially in patients with BRCA mutated and/or triple negative breast cancer (TNBC). Intra-tumoural penetration of one PARP inhibitor, veliparib, varied widely between and within TNBC xenografts (Bartelink et al., 2017). When administered with the DNA damaging cytotoxic carboplatin, intratumoural concentrations measured by LC-MS/MS correlated positively with platinum adduct formation, a clinically relevant pharmacodynamic marker. MALDI-MSI images of veliparib showed dose dependent penetration of veliparib into the tumour. This suggests that synergy between a PARP inhibitor and cytotoxic chemotherapy could be maximized by selecting a PARP inhibitor with optimal tumour penetration or by modifying the dose and/or schedule to enhance tumour penetration.

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MALDI-MSI has also been used to image cassette-dosed drugs, where multiple compounds are co-administered to mice bearing xenografts. This reduces the number of animals needed, and provides pharmacokinetic and drug distribution data at an early stage giving confidence that the compound is present at the site of action. Moreover, it is a useful tool for drug-drug pharmacokinetic interaction studies (Swales et al., 2014).

2.3.3. Drug delivery systems

Other strategies that have been adopted to improve drug delivery include new pro-drug delivery systems that selectively release the active "warhead" in the tumour. Surprisingly, there has been little pre-clinical or clinical work to demonstrate that delivery systems such as nano-particles and antibody drug conjugates (ADC) actually do increase intra-tumoural drug concentrations, or improve drug distribution within tumours. Albumin bound paclitaxel, nab-paclitaxel, enhances drug delivery in tumour xenografts compared to paclitaxel but no direct comparison between intra-tumoural and healthy tissue drug concentrations has been published (Desai et al., 2006; Chen et al., 2015). Preclinical studies of trastuzumab emtansine (T-DM1, Kadcyla) demonstrated internalization of the ADC within HER2 positive cancer cells but, intratumoural drug concentrations were not reported (Barok, Joensuu and Isola, 2014; Diessner et al., 2014).

One relevant exception was a pre-clinical study with AQ4N, a pro-drug of a topoisomerase II cytotoxic designed to be activated in hypoxic regions within tumours, which did incorporate MALDI-MSI to study drug distribution in vivo. A proof-of-principle study in mice bearing H460 xenografts demonstrated the release of AQ4, the active metabolite, in areas with low ATP as a marker of hypoxia. MALDI-MSI images of AQ4N, AQ4 and ATP showed little overlap between AQ4N and AQ4 with ATP having similar distribution to the AQ4N (Atkinson et al., 2007). Interestingly a companion early phase, translational clinical study confirmed the release of the active drug (detected by LC-MS/MS) in hypoxic tumour regions identified by Glut-1 staining (Albertella et al., 2008). (Figure 3)

Another study compared conventional paclitaxel and paclitaxel incorporated in a micelle (NK105), designed to exploit the enhanced permeability and retention (EPR) effect that has been well described in relation to altered vasculature in pre-clinical tumour models. Distribution of paclitaxel was studied in a pancreatic cancer xenograft

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model and in normal neural cells after treatment with NK105 or paclitaxel. Images of paclitaxel were obtained using MALDI-MSI with a combination of MS and MS/MS modes. NK105 delivered more paclitaxel to the tumour, and less to neural tissues, than conventional paclitaxel, and was associated with both greater efficacy and less neurotoxicity (Yasunaga et al., 2013).

Finally, Fujiwara et al. (Fujiwara et al., 2016) studied another ADC comprising a monoclonal antibody against anti-human tissue factor, a transmembrane protein strongly expressed in various cancer and tumour vascular endothelia, linked to the highly potent cytotoxic monomethyl auristatin E. Using MALDI-MSI they demonstrated the release of monomethyl auristatin E in a pancreatic xenograft model. The release of the active compound was seen at 3, 24 and 72 hours after administration, with maximal drug accumulation at 24 hours and preferential accumulation in cancer cells rather than stroma. The authors advocated MALDI-MSI as a powerful tool for the design and pre-clinical optimization of ADCs.

In conclusion, MALDI-MSI is a useful pre-clinical tool to investigate the impact of drug schedules and combinations on pharmacokinetic profiles in order to enhance drug delivery to tumours. Moreover, MALDI-MSI is applicable to the investigation of new drug delivery systems as a proof-of-principle technique where the prodrug and active compound can be imaged simultaneously.

3. APPLICATION OF MALDI-MSI IN CLINICAL RESEARCH

Although to date there are limited examples of MALDI-MSI in the clinical setting, it has the potential to be incorporated in early phase/translational clinical trials. The activity of anti-cancer drugs is invariably greater in pre-clinical studies than in clinical trials (in routine practice), due in part to the limitations of in vivo models (Mak, Evaniew and Ghert, 2014). Demonstrating the distribution of a drug, as well as its activity, at an early stage in clinical development would be helpful in driving progression to later stage clinical trials.

We described above ex vivo MALDI-MSI studies of drugs in clinical samples (Végvári et al., 2016), but to date very few studies have explored the role of MALDI-MSI in patients with cancer. Distribution of the EGFR inhibitor erlotinib was studied in material obtained at autopsy from a patient with an EGFR mutated lung cancer.

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MALDI-MSI showed that erlotinib was distributed to normal tissues and tumour in several organs but was not detected in the brain (Noda et al., 2015). Similar studies have been undertaken in tissues from patients with melanoma where vemurafenib drug distribution and BRAF expression have been correlated. BRAF V600E immunohistochemistry positive regions had a high vemurafenib signal when analyzed by MALDI-MSI, while it was undetectable in wild type BRAF tumours. Vemurafenib was also image by MALDI MS/MS mode correlating well the product ions with the precursor ion. (Kwon et al., 2015)(Sugihara et al., 2014).

Preliminary results have been presented from a key phase I clinical trial in which the PARP olaparib was imaged by MALDI-MSI in seven biopsies taken from six patients. The drug signal detected by MALDI-MSI correlated well with the concentration of drug within the tissues when compared with LC-MS/MS data, and the distribution of olaparib was higher in areas of necrosis within the tumour (Shimoi et al., 2014)(Yonemori et al., 2014).

In the clinical setting, MALDI-MSI can also be useful also to understand the efficacy and toxicity of cancer drugs. For example, in one study biopsies were taken from skin and normal tissue in patients with pancreatic cancer treated with gemcitabine and erlotinib who had developed a skin rash. Erlotinib distribution was measured by MALDI-MSI and drug concentration by LC-MS/MS. Erlotinib was detected at higher concentrations in skin affected by the rash than in normal skin. (Nishimura et al., 2018). Figure 4. Another study of patients with gastric/gastro-esophageal junction cancer who received neoadjuvant chemotherapy reported a correlation between higher platinum concentrations, within the resected tumour, and response. Platinum concentrations were measured by LC-MS/MS, platinum distribution in tissue components by imaging mass cytometry and tissue collagen identified by trichrome staining. Surgical specimens from 10 patients were available with tumour and adjacent "normal" tissues collected. High interpatient platinum tumour concentrations were observed which could be explained in part by platinum bound to tissue collagen (Cao, Chang, Cabanero, et al., 2018). A further study with imaging mass cytometry, in patients with colorectal cancer who had completed adjuvant oxaliplatin plus 5-FU (FOLFOX), showed deposition of platinum in skin biopsies from the lower legs up to 65.6 months after completing chemotherapy suggesting a possible mechanism of platinum sensory neuropathy (Cao, Chang, Zhang, et al., 2018).

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CONCLUSIONS

The characterization of drug concentrations, and in particular drug distribution, within tumours or normal tissues is a challenge that the rapidly developing technique of MALDI-MSI has started to address and may have an important role in drug development (Table 1).

MALDI-MSI already has the potential to inform pre-clinical studies by comparing the penetration of candidate molecules into different regions of spheroids in vitro and xenografts in vivo. Similarly, in pre-clinical pharmacological and toxicological studies the presence of active compound at high concentrations in organs such as the kidney may be a concern, but that level of concern may be affected if the localization of drug to a particular region of the kidney could be determined by MALDI-MSI. Pre-clinical efficacy studies use doses, schedules, and combinations of drugs that are often determined empirically with little or no knowledge of tumour and normal tissue drug concentrations and distribution. MALDI-MSI has the potential to inform optimal dosing and/or scheduling of molecularly targeted agents, cytotoxics and immunotherapies to optimize tumour penetration and distribution. However, the area where MALDI-MSI has arguably the most immediate potential role is in the pre-clinical validation of pro-drug delivery systems.

The ultimate aim would be to incorporate MALDI-MSI studies in early clinical trials and recent studies suggest this is now becoming possible. Intra-tumoural drug distribution has been characterized in vivo using MALDI-MSI for a hypoxia activated pro-drug, an antibody drug conjugate, and a nanoparticle but clinical data have yet to be published and there are many more ADCs in development. Indeed, no such clinical proof-of-principle tumour pharmacokinetic data have been published for the clinically important ADC trastuzumab emtansine (Verma et al., 2012)(Krop et al., 2014), which is approved and widely used in the treatment of women with HER2 positive metastatic breast cancer or for nanoparticle albumin–bound paclitaxel (nab-paclitaxel), which is used to treat patients with breast, lung and pancreatic cancers (Rugo et al. 2015; Socinski et al. 2012; Von Hoff et al. 2013).

In addition to the technical challenges for clinical MALDI-MSI studies, another potential limitation is the need for tumour biopsies. Such biopsies are, however, increasingly incorporated within early phase clinical trials either to identify the presence

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of a molecular biomarker or to study PD endpoints and this should not be a major barrier to obtaining tumour biopsies for MALDI-MSI studies either from patients in the expansion cohort(s) of phase I trials or in a subsequent, specific tumour pharmacokinetic study.

Preliminary results from the phase I olaparib trial suggest that we may be on the verge of using MALDI-MSI to deliver precision pharmacology, in other words “the right drug, in the right place, at the right concentration”.

Conflict of Interest and Authorship Conformation Form

Please check the following as appropriate:

- All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.
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Short CV for authors:

Maria Jove

Maria Jove is a medical oncologist with particular interest in drug development and lung cancer. She completed her specialization in medical oncology in 2012 at "Institut Catala d'Oncologia", Barcelona, Spain. Maria worked as a Clinical Fellow in this institute for two years before moving to the UK to take up a position in St James's University Hospital in Leeds as a Clinical Fellow in the Phase 1 unit under the supervision of Professor Chris Twelves. In April 2015 Dr Jove commenced her PhD project, "Intratumoral drug penetration and distribution", with the Pharmacokinetics Group (Lead, Professor Paul Loadman) at the Institute of Cancer Therapeutics (Hon Clinical Director, Professor Twelves) in Bradford, for which she received a grant from the Spanish Medical Oncology Society.

Actually, Dr. Jove is working in the "Institut Català d'Oncologia" in the Phase 1 Unit, where she is leading several clinical trials, and in the lung cancer clinics.

Jade Spencer

Jade Spencer is a Research Assistant at the Institute of Cancer Therapeutics, University of Bradford. Jade gained her BSc degree in Forensics and Medical Science and MSc degree in Toxicology and Safety Pharmacology from Bradford in 2011, and has since worked in the Institute of Cancer Therapeutics investigating the metabolism and mechanism of action of new and established anti-cancer small molecules.
Jade has considerable expertise in chromatographic techniques and mass spectrometry for the analysis of both small molecule drugs and macromolecules agents focussing predominantly on cancer. Jade also has experience in the analysis of Fatty acids and lipid mediators involved in cancer prevention and has substantial collaborations with the University of Leeds. Jade is experienced in a wide range of in vitro and molecular biology techniques used for mechanistic studies.

Malcolm Clench

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Malcolm Clench is Professor of Mass Spectrometry at Sheffield Hallam University. He is Director of the Centre for Mass Spectrometry Imaging. His research interests are in imaging mass spectrometry and its application to projects in drug discovery, ADME, proteomics and metabolomics. He is author/co-author of 117 refereed publications, h-index = 30 (Scopus) and has been Director of Studies for 34 completed PhD students to date.

Paul Loadman

Professor Paul Loadman heads the drug metabolism team at the Institute of Cancer Therapeutics, University of Bradford and has >20years experience studying the pharmacokinetics and metabolism of anti-cancer drugs. The team in Bradford have studied a wide range of clinically available and novel small molecules including antimetabolites, platinating and alkylating agents, peptides and antivascular compounds. Prof Loadman is actively involved in the design, analysis and reporting of pre-clinical and clinical pharmacokinetic studies and acts as a pharmacokinetic and drug metabolism consultant for numerous pharmaceutical and biotechnology companies.

Together with Prof Twelves (Clinical Director,) the DMPK team have established a Quality Management System within the ICT which has enabled a strong involvement in the pharmacokinetic monitoring of small molecules in major clinical trials. Other major clinical collaborations include with Professor Mark Hull at the University of Leeds (The seAFOod Polyp Prevention Trial, www.seafood-trial.co.uk) where the laboratory in Bradford are analyzing a series of bioactive lipid mediators such as ω-3 PUFAs, resolvin E1 and PGE-M in plasma, urine, erythrocytes and rectal mucosa in order to gain insights into the mechanism of action of EPA and aspirin.

Chris Twelves

Dr Chris Twelves is Professor of Clinical Cancer Pharmacology and Oncology and Head of Clinical Cancer Research Groups at the Leeds Cancer Research UK Centre, and Honorary Consultant Medical Oncologist

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He is a medical oncologist with a particular interest in new drug development and clinical pharmacology; his clinical practice has been in colorectal and breast cancer.

His first degree was in Pharmacology and Experimental Medicine. After qualifying as a doctor and training as an oncologist in London he was Senior Lecturer, then Reader, in Medical Oncology in Glasgow at the Beatson Oncology Centre before taking up his current post at the University of Leeds and St James's Institute of Oncology.

Prof Twelves is also Director of the Leeds NIHR Clinical Research Facility Experimental Cancer Medicine Centre in Leeds and has been a member of the Cancer Research UK New Agents Committee and Chair of the New Drug Development group of the EORTC. He has been involved in the development of several important new agents including capecitabine, and eribulin.

Professor Twelves has published over 250 papers in journals including the New England Journal of Medicine, Lancet and Journal of Clinical Oncology and spoken at numerous international meetings. He has also edited, or contributed to, several books.

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Legends

Figure 1. **Typical workflow of a MALDI-MSI experiment.** Matrix coated cryo-sectioned tissue is placed at MADLI-MSI and region of interest is selected. The laser beam irradiates a selected section of a tissue impacting sample at each x and y co-ordinate. Analytes travel according to TOF and m/z to the mass spectrometer where the mass spectrum is recorded for each x and y co-ordinate. Masses of interest are selected to generate images. This figure shows the image of 5'-deoxy-5-fluorouridine (5dFUR), a fluoropyrimidine metabolite, from a tumour section from an MDA-MB-231 xenograft treated with capecitabine (Jove et al., 2018).

Figure 2. **3D Models of drug distribution with MALDI-MSI.** (A). 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 7 consecutive 12 μm slices from a single spheroid in 120 μm vertical intervals (Liu, Weaver and Hummon, 2013). (B). Distribution of Taxol (Green) overlaid on endogenous PC lipid signal (red) in a HCT 116 spheroid after 4h treatment.

Figure 3. **MALDI-MS images showing the distribution of AQ4N, AQ4 and ATP in tumour tissue.** (A) The position of the tumour, visualized by the phosphatidyl choline head group at m/z 184; (B) the distribution of AQ4N ($[M+Na]^+$ (m/z 467)) overlaid against the distribution of AQ4 ($[M+H]^+$ (m/z 413)); and (c) the distribution of ATP $[M-H_3PO_4]$ (m/z 409) overlaid against the distribution of AQ4 $[M+H]^+$ (m/z 413). The signal intensities of all ions imaged have been normalized against respective matrix ions. Controls for each compound can be seen on the right-hand side of the images. In (b) there is little overlap (which would be shown as yellow) between regions containing the prodrug AQ4N (green) and active compound AQ4 (red). Similarly, the overlap in (c) of the distributions of AQ4 and ATP is also found to be quite distinct.

Figure 4. **Representative molecular images of erlotinib distribution in skin rash and adjacent normal skin.** (**A**) Hematoxylin and eosin staining of the adjacent normal skin including epidermis to deep dermis layers, which were concurrently collected at the time of rash biopsy. Scale bar = 500 µm. (**B**) Determination of erlotinib distribution in the normal skin by mass spectrometry imaging. Molecular images were acquired at a step

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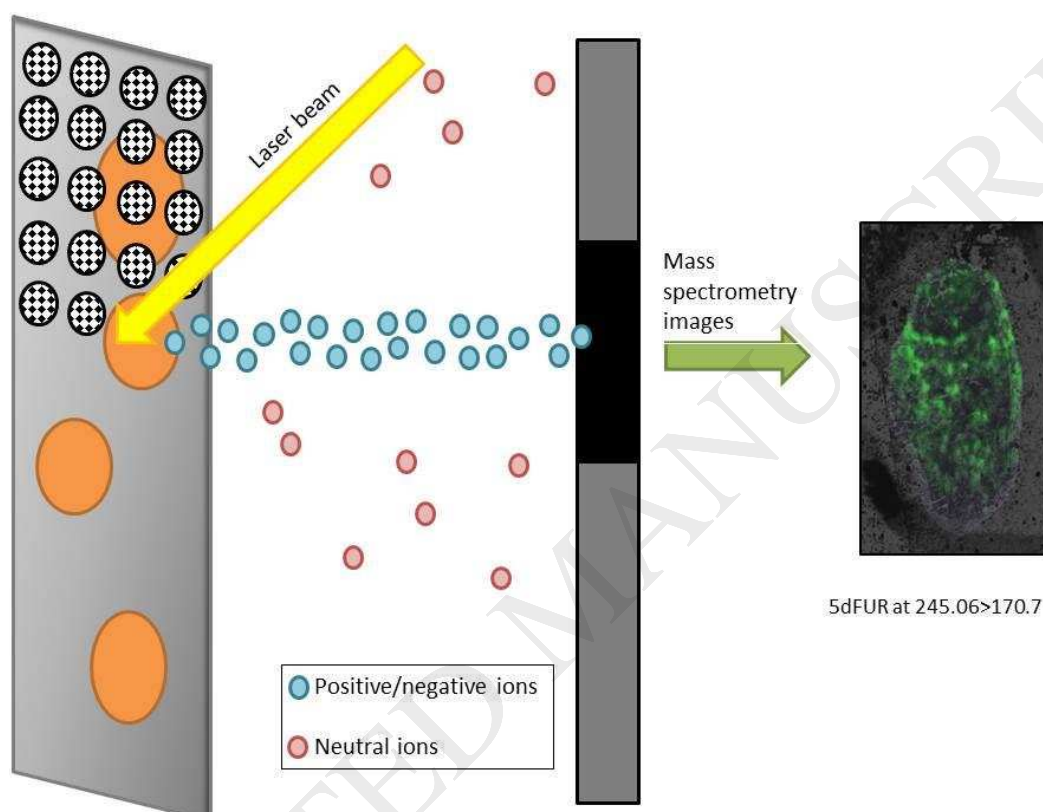
size of 60 μm. Scale bar indicates erlotinib quantity, pg/pixel. (**C**) Hematoxylin and eosin staining of the rash, showing that inflammatory cells infiltrated into the papillary dermis and superficial-reticular dermis. Scale bar = 500 μm. (**D**) Molecular image of erlotinib distribution in the rash, indicating that erlotinib was predominantly localized in the superficial layer of the skin. (Nishimura, M. et al. 2018)



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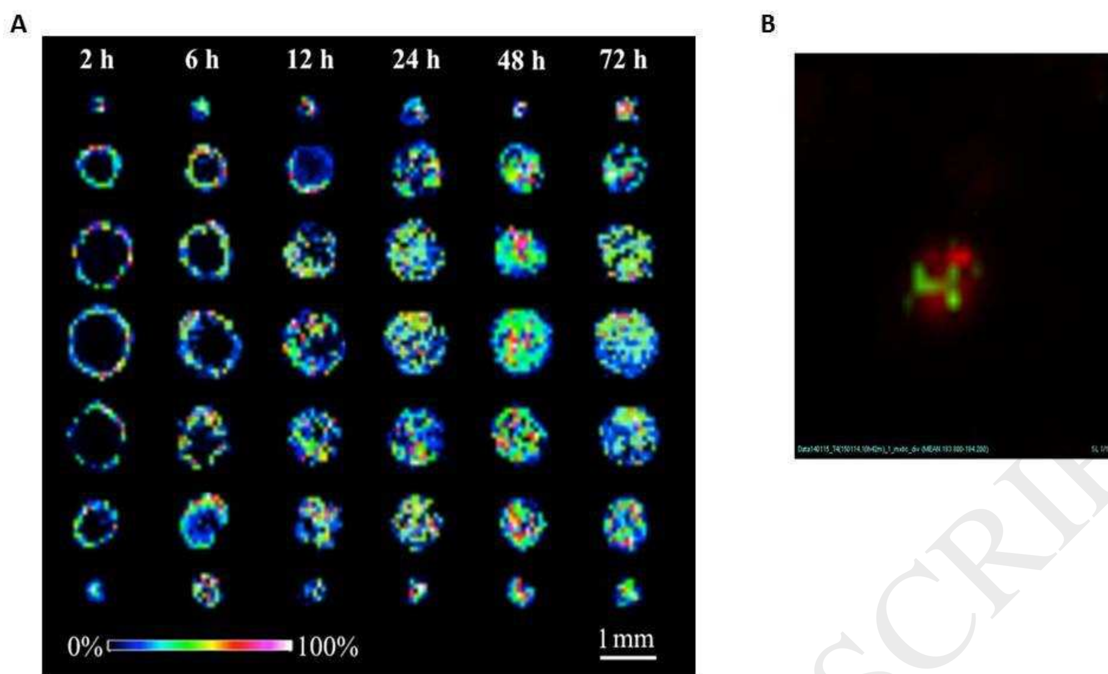

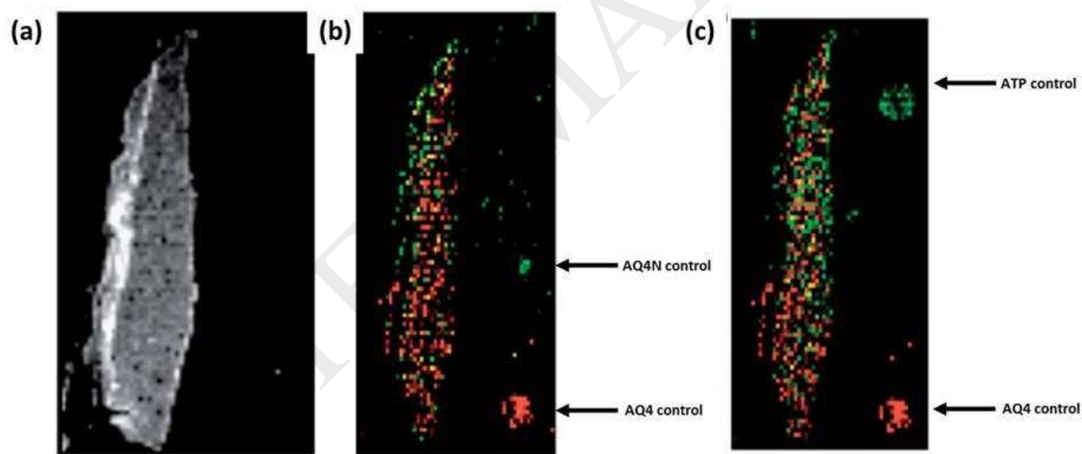

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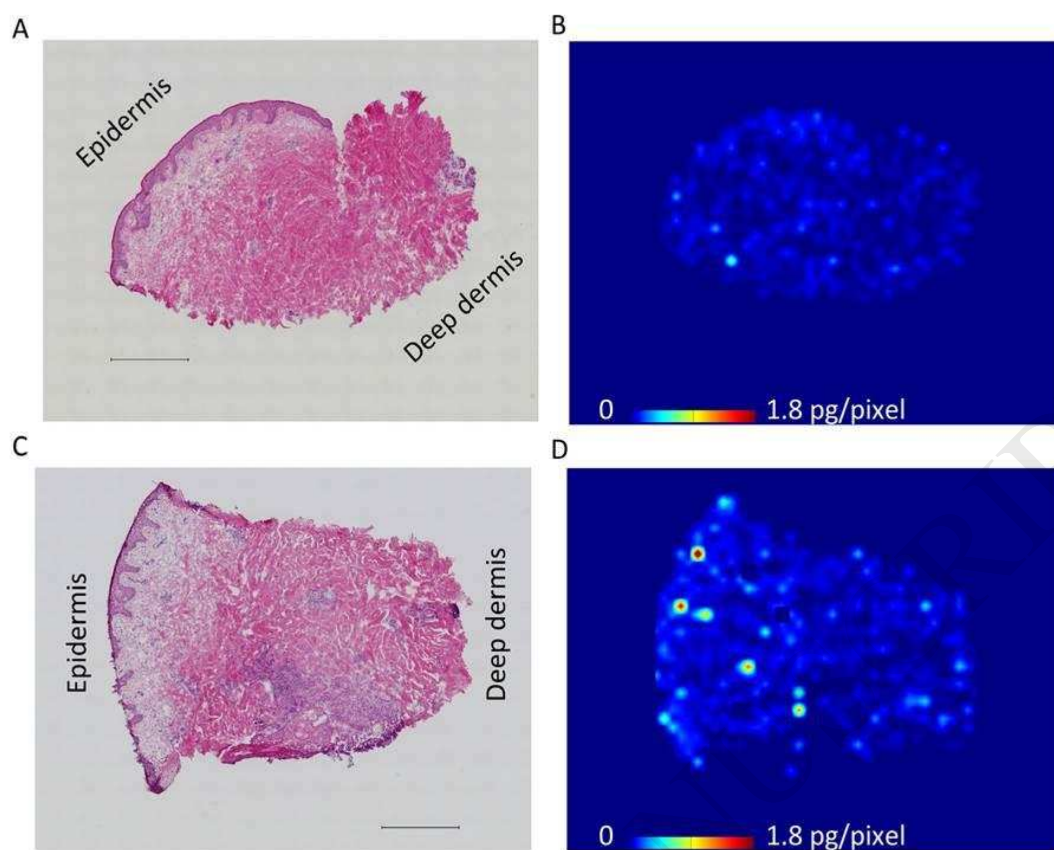

Table 1. **Applicability of MALDI-MSI in drug distribution research**

Applicability of MALDI-MSI in drug distribution research

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Drug distribution within body and organs:
PK studies *in vivo*
- Possible toxicities
- Organ site targets (CNS)

In clinical research:
- Studies of drug distribution from patient's tumour/tissue biopsies or autopsies

Drug distribution within tumour:
Pre-clinical research:
In vitro
- Understanding how the drug moves through solid tissue (3D tissue culture models)

In vivo
- Drug distribution and correlation with tumour structure (vessels, stroma).
- Potential role to study drug interaction with its targets.
- Diffusion of drug through the tumour.

In clinical research:
- Potential use of clinical samples to image drug in patients tumours.

Proof-of-principle studies:
Useful for new drug delivery systems and optimisation of new schedules or drug combinations.
Pre-clinical:
In vivo studies for drug distribution are useful to answer:
- Is the drug being delivered preferentially in the tumour than in other healthy tissues?
- Is there a more homogeneous distribution, better retention or uptake within the tumour? Preclinical proof-of-principle for new drug delivery system, drug combinations or schedules.

Clinical:
- As part of early phase clinical trials to confirm previous preclinical results before moving forward to phase II and III clinical trials.
- Proof-of-principal clinical trials.

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