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Biomedical application of OrbiSIMS in
cancer: identification of anti-cancer agents and
metabolite profiles

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Thesis submitted to the University of Nottingham for the
degree of Doctor of Philosophy

Abstract

Small molecule targeted therapies, which have been widely adopted in cancer treatments, deliver drugs directly to specific targets. Therapeutic efficacy is achieved once the target attains the necessary drug concentration. In the pursuit of novel drug candidates, the use of advanced analytical techniques is essential for enhancing our comprehension of drug concentrations at target sites and aiding in visualizing drug distributions within cells and tissues. Such advancements could potentially amplify the therapeutic efficiency of anticancer drugs. Nevertheless, many existing analytical techniques are hampered by limitations in sensitivity and visualization. This thesis introduces the superior capabilities of orbitrap secondary-ion mass spectrometry (OrbiSIMS), a solution to these challenges. OrbiSIMS facilitates the observation of an extended spectrum of endogenous metabolites, enriching biomolecular data collection. In this study, the OrbiSIMS system was used successfully for the analysis of specific biological matrices, specifically BCR-185R and MCF-7 breast cancer cells and allowed the capture of exhaustive endogenous metabolite data. Notably, the instrument enhanced the detection of lipids and amino acid species and allowed the successful identification of a broad array of metabolites. Moreover, while leveraging the imaging capabilities of OrbiSIMS, we discerned drug distribution patterns at the subcellular level. The insights gained in this study make a considerable contribution to our understanding of drug transport and have significant ramifications for the future of drug development.

Although we found that OrbiSIMS possesses strong capabilities for identifying and visualising biomolecules below the cellular level, its ability to monitor drug levels under different treatment conditions and endogenous responses remains unclear. Therefore, this study evaluated the potential of OrbiSIMS to monitor drug-induced changes in endogenous metabolites and its semi-quantitative efficacy. It focussed on the anti-cancer agents Tamoxifen and Lapatinib and revealed the ability of OrbiSIMS to detect these drugs at minuscule concentrations. The instrument detected these agents at a 2000-fold dilution and an unprecedented discovery was made: the concentration of glutamine and its TCA cycle intermediates reduces in the context of MCF-7 cell metabolism. Additionally, in our research, we noted that eight established biomarkers, specifically from the lipid families phosphocholine (PC) and phosphatidylinositol (PI), responded to different treatment conditions. This discovery

demonstrates the importance of OrbiSIMS in biological monitoring and biomarker identification under different treatment scenarios.

The developed OrbiSIMS workflow was applied to investigate tamoxifen-resistant breast cancer and potential treatment strategies. Using this advanced analytical technique, we examined the responses of tamoxifen-resistant MCF-7/TamR-1 cells to treatments with tamoxifen, dasatinib, or their combination. Our research indicates that leucine is a key element in tamoxifen-resistant cell growth, which supports earlier findings regarding the role of leucine in drug resistance. Remarkably, the tamoxifen-resistant MCF-7/TamR-1 cells lacked increased intracellular tamoxifen intensity, even with dasatinib, which suggests that other resistance mechanisms are occurring. This research provides unparalleled 3D chemical profiles of anti-cancer agents in cancer cells, marking a pioneering use of OrbiSIMS for such analyses. The developed OrbiSIMS system will serve as an exemplar tool for addressing and comprehending the biomedical challenges within cancer research.

Acknowledgments

This thesis could not have been completed without the amazing assistance, support, and encouragement of several friends, family members, and co-workers.

Firstly, I would like to express my gratitude to my PhD supervisors, Dr. Zheyang Zhu, Dr. David Scurr, and Prof. Morgan Alexander, for their patience, inspiration, and knowledge throughout my PhD-related study. Their assistance was invaluable during my research and writing of this thesis especially during the Covid epidemic crisis. I am extremely appreciative of the PhD meetings' discussions, contributions, and occasionally challenging questions, as well as their continuous support. I have been incredibly fortunate to have worked with someone so dedicated and caring.

I would want to thank everyone at the School of Pharmacy who has given their knowledge and experience to this project, especially those in the BSB. I am incredibly appreciative of all the technical assistance I have received. Particularly, Dr. Long Jiang, who introduced me to the field of SIMS analysis, and Dr. Nichola Starr, whose breadth of knowledge, expertise, and willingness to assist has been essential, especially for the data analysis, deserve special recognition. And special thanks to Dr. Stefanie Kern who was SIMS analysis manager based on nmRC building with her kindness help on dealing with my sample and Dr. Gustavo Trindade who gave me support about using the software. In addition, thanks for the help from Dr. Julie Watts who introduced the cryogenic sample preparation method to help me.

I would like to thank all of my friends for their endless support during my hard PhD life, especially Oluwatoyin Ayodele and Dr. Xinuo Li. They have consistently provided me with guidance and support at crucial times, and without them, I would not have reached the end stage of PhD life.

Finally, to my family. My lovely wife, Chenyang who has always supported and encouraged me. My parents, Xiaoyan and Guixing who always support any of my decision and got my back. They never stop encouraging me to strive for excellence and demonstrating their undying faith in me.

List of Abbreviations

AML – Acute Myeloid Leukaemia

DESI – Desorption Electron Spray Ionization

DMD – Duchenne Muscular Dystrophy

DPPC – Dipalmitoyl phosphatidylcholine

EM – Electron Microscope

GCIB – Gas Cluster Ion Beam

LC/MS² – Liquid Chromatography-Tandem Mass Spectrometry

LMIG – Liquid Metal Ion Beam

m/z – Mass to charge ratio

MALDI – Matrix Assisted Laser Desorption Ionization

MSI – Mass Spectrometry Imaging

NAFLD – Non-Alcoholic Fatty Liver Disease

PC – Phosphatidylcholine

PC – Principal components

PC-DFA – Principal Component Discriminant Function Analysis

PCA – Principal component analysis

PE – Phosphatidylethanolamine

SIMS – Secondary Ion Mass Spectrometry

SIMS-MFP – Secondary Ion Mass Spectrometry Molecule prediction

TME – Tumour microenvironment

ToF – Time of Flight

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Chapter 1. General introduction

Cancer is a devastating disease that is responsible for a significant number of deaths worldwide. Numerous treatment strategies have been implemented to address this global issue. Currently, immunotherapy, chemotherapy, and targeted therapies are the most common approaches for treating cancer (1). One of the main problems associated with these treatment approaches is that inefficient amounts of drug molecules reach their intended target(s), most of the drug dose are distributed in off-target sites, resulting in treatment failure and several adverse effects (2). Therefore, it is important to know the anti-cancer agent's levels at the target site. However, some of the analytical approaches that are currently accessible have the drawback of not being able to precisely measure and visualise the levels of these molecules at the target site. Further understanding the drug's levels at targeting site, visualising its location, and drug effects in cells and tissues, are of utmost importance to the pharmaceutical industries in the development of new drug candidates. Hence, to provide new insights into the molecular cancer research, it is essential to explore novel application of advanced analytical techniques emerged latently.

1.1 Therapeutic strategies for cancer

Cancer is a significant health burden, with high mortality rates that makes the search for effective therapeutic strategies imperative. For decades, surgery, radiation therapy, and chemotherapy have been the primary treatment options for cancer. However, with the advancements in our understanding of the molecular mechanisms of cancer, novel therapies such as immunotherapy and nanotechnology have emerged (1).

Immunotherapy is a treatment that harnesses the immune system's power to combat cancer. As cancer originates from normal cells, the immune system may not always recognize it as foreign. This makes it challenging for the immune system to target cancer cells. Immunotherapy can increase or modify the functioning of the immune system (3-6), enabling it to identify and destroy cancer cells. Additionally, nanotechnology has been applied to anti-cancer treatment, enhancing the pharmacokinetics, and reducing the systemic toxicity of chemotherapy by selectively delivering anticancer drugs to tumour tissues (6-10).

Immunotherapy presents challenges in penetrating cells due to the large size of monoclonal antibodies used (4-6). Small molecules, in contrast, have the potential to directly target specific proteins within cancer cells, making them a promising alternative to monoclonal antibodies (11-12). Therefore, using nanotechnology for small molecules as a strategy for cancer treatment seems to be a promising approach. However, the use of nanotechnology to transform drug molecules into nanoparticles, while aiding in reaching the target site, requires time-consuming development processes and extensive training, thus presenting limitations (9). Nevertheless, small molecule targeted drugs offer advantages in pharmacokinetics, affordability, patient compliance, drug storage, and transportation, making them worthy of further research (12).

1.2 Cancer treatment with small molecule agents

Small molecules have the ability to penetrate cells, making them a promising option for anticancer agents that target dysregulated signalling pathways and inactivate kinases during carcinogenesis (12). Depending on their mechanism and target, small molecule anticancer agents can be broadly categorized as chemotherapy agents or targeted therapeutic agents.

1.2.1 Chemotherapy

Traditionally, chemotherapy drugs have been used to treat cancer by preventing cell division and DNA replication. Many of these drugs also target the microtubule dynamics of the mitotic spindle, leading to cell death (13). While early anticancer drugs such as platinum compounds, nucleoside analogues, topoisomerase inhibitors, and vinca alkaloids are still in use today and have been shown to increase the chance of cure and slightly extend survival time in patients with paediatric leukaemia and testicular cancer (14), although they have many negative side effects. One of the most significant drawbacks is that chemotherapy is not targeted therapy (13), meaning that while the drugs can kill cancer cells, they can also harm healthy cells throughout the body, including those in the stomach, blood, and skin. Therefore, there is an urgent need to explore alternative cancer treatments that can specifically target cancer cells while minimizing harm to healthy cells.

1.2.2 Molecular-targeted therapy

Due to the many drawbacks of chemotherapy, targeted therapy has been the focus of recent research efforts (15). Several therapies, including molecular therapy (16-18), anti-angiogenic therapy (19-21), immunotherapy (22-25), signal transduction therapy (26-28), and nucleic acid-based therapy (29-32), have gained increasing public interest. These new treatments involve specific targets for cancer diseases, and their anti-cancer effects arise from different pathways, including inhibition of proliferation, induction of cell apoptosis, inhibition of metastasis, regulation of immune function, and reversal of multidrug resistance (33-42). Researchers are exploring these pathways to develop more effective and safer treatments for cancer patients.

In accordance with the concept of targeted therapy, researchers have developed anti-cancer drugs that target cancer molecules with greater precision (43-44). Cancer cells exhibit altered genetic material during the process of cell generation, which is distinct from normal cells, and these modifications are necessary for the tumour to sustain growth and maintenance (45). Consequently, these changes in genetic material may lead to the identification of oncogenes or oncogenic proteins that are produced through gene fusion, overexpression, acquired or functional mutations (45). The resulting oncogenes or cancer proteins are mainly involved in various signalling pathways and activities, such as mTOR, MAPK signalling pathways, and cancer metabolism (46). These pathways are critical for the differentiation and proliferation of cancer cells. For instance, the mTOR signalling pathway is activated in most tumours and regulates gene transcription and protein synthesis to control cell proliferation and differentiation (47-49). Furthermore, cancer cells possess a different metabolism than healthy cells to support their abnormal activities and nutritional requirements. Hence, the target of cancer protein or cancer gene can be referred to as a tumour target (46). Targeting tumour proteins or gene targets has the potential to treat patients with various malignant tumours, making it a promising new direction in cancer treatment. The use of this approach has the advantage of being more precise and effective, minimizing harm to healthy cells, and reducing the negative side effects of traditional chemotherapy.

1.2.3 Challenges and limitation in cancer treatment

In recent years, there have been significant advancements in cancer treatment, particularly in the area of molecularly targeted therapy. This approach targets specific molecules involved in cancer growth and spread, resulting in a more precise and effective method of treatment (50-51). Consequently, there has been a marked improvement in the life expectancy and quality of life for patients with cancer. Despite these achievements, there are still several challenges that need to be addressed before molecularly targeted therapy can be widely employed in the treatment of cancer.

One of the main challenges is the limited number of available therapeutic targets, many of which are difficult to target directly or have low therapeutic value (52-53). Additionally, tumours often have multiple molecular abnormalities, making it challenging to identify the most effective target (54-57).

Another obstacle is the emergence of treatment resistance, where tumours can adapt and change in response to treatment, rendering the therapy ineffective (58-63). To overcome this challenge, researchers are exploring new strategies by targeting multiple pathways simultaneously or disrupt drug resistance processes. It is also crucial to emphasize the importance of drug concentrations in tissue or cells for effective cancer treatment. The therapeutic window, or the range of drug concentrations required to achieve a therapeutic effect without inducing toxicity, is essential for successful cancer treatment (64-68). Therefore, therapeutic drug monitoring is necessary to ensure that drug concentrations remain within the therapeutic window, improving the efficacy of the treatment and reducing the likelihood of the body developing resistance to the medication.

1.3 Anti-cancer drugs present in cells and tissues

The concentration of drugs in tumour tissue or cancer cells is one of the most important factors affecting the efficacy of anticancer drugs. Measuring drug concentration in tissues or cells is crucial for optimizing cancer treatment efficacy (69-72). Drug concentration can provide valuable information about drug exposure, pharmacokinetics, and pharmacodynamics (72). This knowledge is crucial for

understanding how effective a drug is, how much toxicity it causes, and what level of resistance it may face.

1.3.1 Determination of drug levels and visualisation of drug location

Preclinical studies are crucial in the drug development process as they evaluate the safety and efficacy of new drugs before administering them to humans (73). In these studies, researchers use animal models to determine the appropriate dosage and administration route for the drug (74-75). Measuring drug concentration in animal tissues and cells is an important tool to achieve this objective. Preclinical studies also offer an opportunity to study how drugs are distributed and metabolized in different tissues and organs, including cancer (76-77). This information is essential for assessing the effectiveness of drugs that target cancer cells while preventing off-target effects on healthy tissues. Therefore, monitoring drug levels in cell or tissue is critical to evaluate whether the drug is effectively penetrated and delivered into the tumour cell or tissue that achieves therapeutic levels.

1.3.2 Analytical methodologies

Measurement of drug concentration at the target site is crucial to evaluate the effectiveness and safety of a medicine (78-80). Various methods, such as sample preparation and analytical techniques, are employed for this purpose.

1.3.2.1 Sample preparation

The preparation of biological samples is a challenging process that can significantly affect the presence of bioassay and genotoxic contaminants (81-83). As a result, sample preparation plays a crucial role in determining drug levels and providing accurate and reproducible experimental results.

Ultracentrifugation is commonly utilised to separate different components of a cell and extract drugs from tissues or cells during the sample preparation process.

Following separation and extraction, several analytical techniques, such as chromatography and mass spectrometry, are employed to examine the level of extracted samples (84-86). These techniques enable the measurement of the quantity of a drug, and their suitability for various purposes is dependent on their sensitivity, specificity, and accuracy levels (87).

1.3.2.1.1 Analytical ultracentrifugation

Analytical ultracentrifugation (AUC) represents a powerful laboratory technique employed to investigate the physicochemical properties of macromolecules, such as proteins and nucleic acids, in solution (88). By subjecting samples to high centrifugal forces within an ultracentrifuge, AUC facilitates the evaluation of sedimentation and diffusion rates of particles, thereby providing valuable insights into molecular weight, size, shape, and molecular interactions (89-91). This method boasts significant applications across various domains, including biochemistry, biophysics, and molecular biology, as it enables the extraction of samples from diverse cellular components, such as membrane, cytoplasm, and nucleus (92-94). Moreover, AUC facilitates the determination of drug concentrations within these components and plays a pivotal role in investigating biomolecular interactions and protein folding (95-97). Despite its numerous advantages, AUC exhibits certain drawbacks, encompassing high costs and limited accessibility of requisite equipment, stringent sample preparation requirements, limited sensitivity for low molecular weight solutes or weak interactions, labour-intensive and time-consuming experiments, complex data analysis, and low-throughput capabilities (98-100).

1.3.2.2 Chromatographic methods

High-performance liquid chromatography (HPLC) is a widely used analytical method to isolate, identify, and quantify complex mixtures (101). This method employs differential techniques to divide sample components into a stationary phase and a mobile phase. The mobile phase, a liquid or gas, flows through the chromatography column, while the stationary phase is a substance packed into the column. By modifying the composition of the mobile phase, the elements of the mixture can be separated based on their physical and chemical properties (102-103). AUC, a crucial step in sample preparation, facilitates the separation of different components of a cell and the extraction of drugs from tissues or cells. The extracted samples can then be analysed using HPLC. However, HPLC may not provide sufficient information about the sample's composition. To overcome this limitation, liquid chromatography-mass spectrometry (LC-MS) has been developed as a complementary technique to HPLC. LC-MS combines the separation ability of HPLC, following AUC for sample

preparation, with the detection and identification ability of mass spectrometry (104-106).

LC-MS is commonly utilised for analysing *in vitro* and *in vivo* samples to determine the concentration of a drug that has entered cancer cells or tissues, through assessing the amount of a drug absorbed by cancer cells (107). In addition to studying potential anticancer drugs, LC-MS analysis of biological samples has been extensively demonstrated to be useful for detecting and quantifying endogenous metabolites, thus making it a valuable technology for metabolomics research (108-113).

Although LC-MS is a powerful tool for determining the concentration of specific molecules, there are still limitations to its application in evaluating anticancer drugs. One of the major concerns is the time-consuming and complex extraction process required to extract all substances of interest from the biological matrix for liquid chromatography phase analysis (109). This process often entails the use of ultracentrifugation, which is accompanied by disadvantages such as the need for expensive equipment and a heightened susceptibility to errors if not performed correctly. Inadequate execution of the extraction process may result in contamination of the chromatography analysis.

Furthermore, the nature of sample preparation for chromatography analysis, including the use of ultracentrifugation, cannot provide imaging capabilities (104-105), which are crucial for revealing the spatial distribution information of compounds in cancer cell or tissue, particularly for compounds investigated in preclinical studies. Thus, continued advancements in analytical methods are necessary to gain new insights and understanding in the field of anticancer drug research.

1.3.3 Mass spectrometry imaging

The integration of mass spectrometry with imaging (MSI) technologies is a widely accepted approach for analysing the localisation of drugs in biological materials. The use of advanced analysers, such as the time-of-flight (ToF) analyser, allows for multiple analyses and provides the benefits of label-free operation. By simultaneously analysing drug molecules and complex biological environments, this technique

enables the examination of the relationship between drugs and endogenous response (114-116). As a result, matrix-assisted laser desorption ionisation (MALDI) and desorption electron spray ionization (DESI) methods, which are part of the MSI technique, are commonly used in biomedicine. These methods can identify large molecules, such as proteins and peptides, over a broad range of masses (117-120).

1.3.3.1 MALDI

Matrix-assisted laser desorption/ionization (MALDI) analysis is a technique that uses a matrix compound to cover the sample, and a laser beam to detect the sample, causing molecules to desorb and ionize from the surface (121). The resulting ions are then sent to a mass analyser to generate a chemical map of the sample. Figure 1.1 provides an example of MALDI, illustrating its imaging capabilities by revealing specific histological characteristics at different spatial scales. The first two images display coronal sections of a mouse brain with phospholipids (indicated in red and green) that detail the striatum, cerebellum, and corpus callosum. The third image shows a section of the mouse's intestinal tract. The histology data provided by the phospholipids (red) can be used to determine the localization of medicinal molecules (green) (120, 122-124).

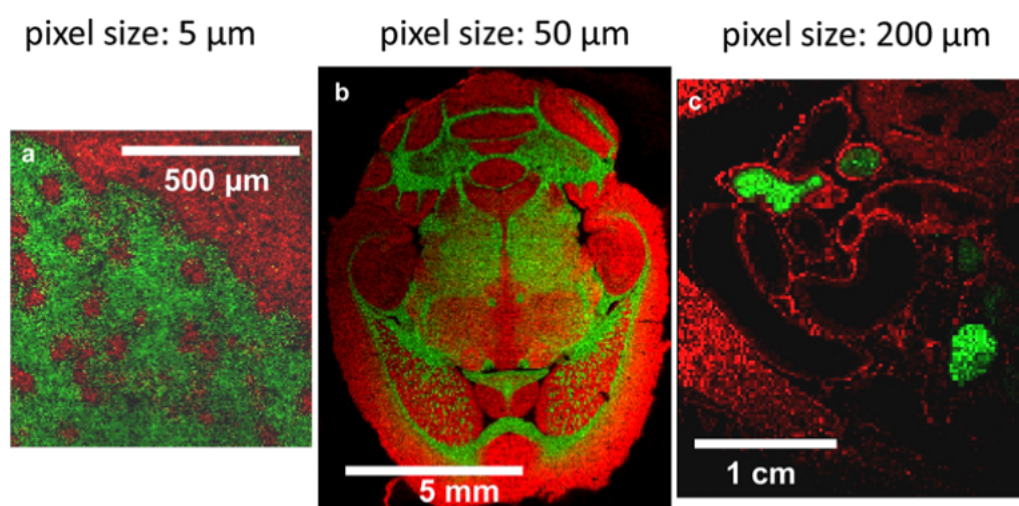


Figure 1. 1 MALDI image example of phospholipids (green and red) on mouse brain section. A) Brain section with 170x170 pixel. B) Image of horizontal mouse brain section. C) Intestinal tract image of rat brain section. (Reprinted with permission from reference [123])

Despite its ability to detect large molecules such as lipids and proteins, MALDI imaging has limited sensitivity to small molecular weight molecules ($m/z < 1000$). This is due to the matrix absorbing the laser energy needed for ionization, potentially affecting the intensity of small molecules and making their identification challenging (124). As a result, when it comes to analysing anti-cancer agents, MALDI imaging is less suitable than DESI, since most anti-cancer agent molecules have a molecular weight smaller than 1000.

1.3.3.2 DESI

DESI utilizes charged droplets, generated through electrospray under atmospheric pressure, to ionize samples for subsequent analysis by a mass analyser (125-126). The charged droplets are created by electro spraying with pneumatic assistance and directed towards the sample surface of interest (125). The analytes present in the sample are extracted into a solvent film formed on the surface, and microdroplets containing the analyte molecules are formed upon subsequent droplet collisions. These microdroplets are then drawn into the mass spectrometer for analysis (126). DESI is a method that is relatively easy to implement with regards to both instrument characteristics and sample preparation. Unlike MALDI, which requires vacuum conditions, DESI experiments can be conducted without vacuum. This unique feature has led to an increase in the use of DESI in biological research (127). For example, DESI has been used for 2D imaging of lipids, offering 3D reconstruction of the mouse brain. The resulting reconstructed 3D model provides extensive insights into the relationship between the corpus callosum's lateral structure and the distribution of lipids in the brain (127-128).

Despite its accessibility in terms of sample preparation and equipment, DESI has limitations in achieving high spatial resolution due to its inability to focus its spray effectively, resulting in a maximum pixel size of 50 micrometres (129-130). This spatial resolution is significantly lower than the microscale achievable with MALDI. Thus, DESI may not be an optimal choice for analysing targets at the cellular level.

1.3.4 Emerged analytical techniques

As previously mentioned, advanced MSI technology shows a promise in the field of biology as it eliminates the need for additional sample labelling. Specialised instruments with high resolution, mass accuracy, and spatial resolution are required to obtain comprehensive data when applying MSI techniques for biomedical research (131). Secondary ion mass spectrometry (SIMS) is an MSI technique that meets these requirements. SIMS enables high-resolution imaging of biological samples with excellent sensitivity, making it a valuable tool for studying biological processes and disease mechanisms.

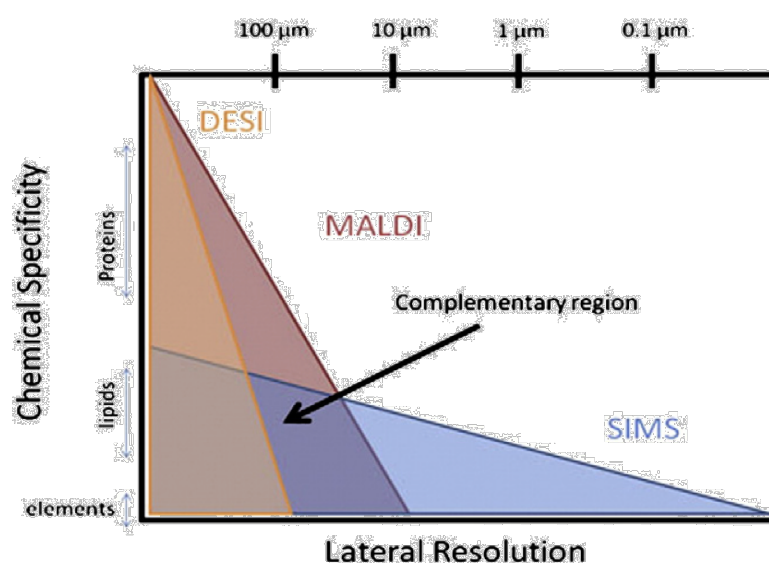


Figure 1. 2 The diagram illustrates the differences between three MSI techniques: DESI, MALDI and SIMS. The specific target to of each technique based on the mass range. (Reprinted with permission from reference [132])

In Figure 1.2, a comparison of the detection range of SIMS, MALDI, and DESI is presented. MALDI and DESI can detect high-mass molecules while also being capable of mapping analyte distributions. In contrast, SIMS provides the highest spatial resolution and three-dimensional information, but it is limited to detecting small molecules. Given the goal of exploring the effect of anti-cancer agents and endogenous metabolite response in this project, which falls within the mass detection range of SIMS imaging, it may be a suitable MSI analysis technique to use.

1.4 SIMS

As a type of mass spectrometry technique, SIMS was first proposed as a characterisation tool in the 1970s. The original prototype of SIMS used a single-atomic argon ion beam and a quadrupole analyser to investigate the adsorption of carbon monoxide on metal single-crystal surfaces (131-134). In the 1980s, synthetic polymers were characterised using argon and xenon ion beams with quadrupole mass analysers (135). Over time, spatial resolution was improved to achieve 100-nanometre resolution (136).

By the late 1980s, SIMS instruments with gallium ion beams and ToF analysers were specifically developed for biological samples (136-137). The first biological application of SIMS was used to analyse the lipid content of protozoan membranes. Since then, it has been widely used to collect lipid information on various diseases, including Fabry disease and Duchenne muscular dystrophy, due to its ability to detect high-abundance lipids and other small biomolecules (138-139). The widespread use of this instrument in biological research is due to its high sensitivity and selectivity in detecting and characterising biomolecules.

Secondary ion mass spectrometry (SIMS) is a surface analysis technique that operates under ultra-high vacuum conditions. The technique involves bombarding the sample surface with a primary ion beam, such as Bi^{3+} , Ar_n^+ , and Cs^+ ions from different sources, to generate the analysed ions. The energy of the primary ion beam can be controlled between 1 to 50 kV. The resulting collisions between the primary ions and the atoms and molecules of the sample cause a sequential impact called collision cascading. This process generates mostly neutral atoms but also believed to produce molecules. The charged ions produced are then extracted into a mass spectrometer for chemical analysis of the surface. The SIMS technique provides a 'chemical image' of the analyte, as illustrated in Figure 1.3 (134).

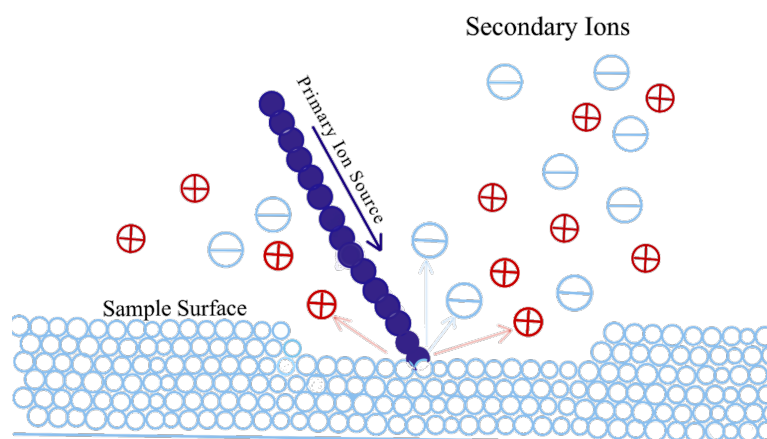


Figure 1. 3 Schematic illustration of ionisation process in SIMS. Different source of primary ion beam impacted on sample's surface and causing desorption of multiple species due to collision cascade. Only a small number of species emitted are charged, while the majority are neutral atoms or molecules (136).

The use of liquid metal ion guns (LMIGs), such as Bi^{3+} , can significantly increase ion yield in surface analysis techniques. However, their application can lead to severe damage to the sample, particularly to the subsurface, making the analysis limited to the top surface. On the other hand, multi-atomic ion beams with larger cluster sizes, such as C_{60}^{+} and gas cluster ion beams (GCIBs), can reduce the damage caused to the sample and even cause minimal damage. Therefore, combining different ion beams can also overcome the static limitations of surface analysis techniques, like SIMS, and improve the sensitivity of the analysis, enabling the evaluation of a broader range of materials.

1.4.1 SIMS methodologies

When the primary ion beam impacts the surface, a collision cascade occurs, which leads to the emission of secondary ions from the sample's surface. The energy transferred from the ions to the atoms of the sample during the collision cascade causes the emission of secondary ions, some of which become desorbed ions. Each secondary ion produced is either a molecular ion or a fragment of the sample's molecular structure, which appears as an ion peak in the spectrum (140-141). Based on this mechanism, SIMS has two basic modes: static and dynamic.

1.4.1.1 Static SIMS

In the static mode of SIMS, the primary ion dose is limited to the static limit, which is equal to or less than 10^{13} impacts per cm^2 . The primary ion gun only impacts less than 1% of the sample's topmost layer of ions under this condition (Figure 1.4). This minimizes the damage caused to the sample while limiting the area of desorption to 10 nm^2 per primary ion impact. Because only the top surface is consumed in this mode, it is suitable for surface analysis. If the evaluation's purpose is surface analysis, the static mode ensures that the sputtered secondary ions are produced from an area that has never been impacted (136, 140-141).

1.4.1.2 Dynamic SIMS

The dynamic mode of SIMS is different from the static mode as it can study the subsurface information of the sample. In dynamic SIMS, a dual-beam method is used that includes the primary ion beam for analysis and the sputtering beam to study the depth profile of the sample. This mode is named dynamic SIMS because as the analysis progresses, the secondary ions released from the surface continuously retreat into the bulk material. Dynamic SIMS is typically used to determine the depth distribution of the target analyte, with depth resolution between tens of nanometres and sub-nanometres. It can also be used to analyse the bulk composition of trace elements to ascertain depth dispersion. Therefore, the dynamic mode of SIMS can be used to plot 3D chemical information and destructive impacts under high-energy primary ion beam irradiation (140-142).

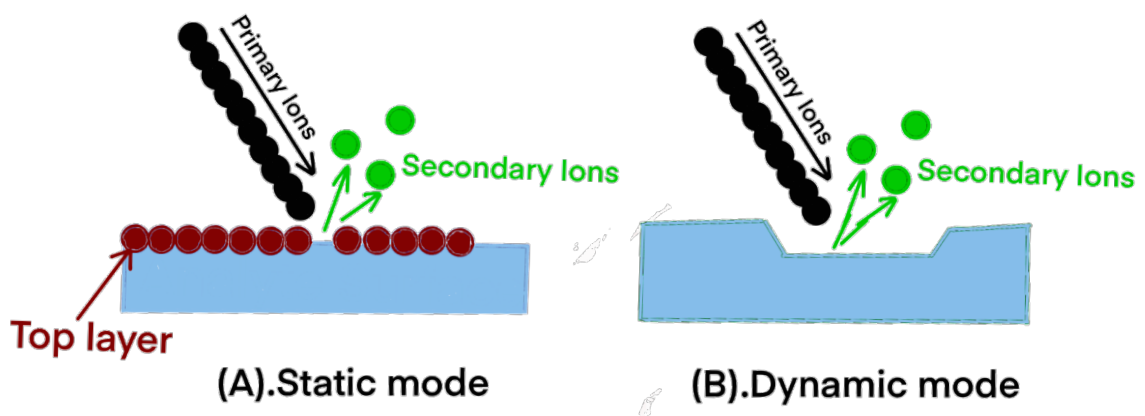


Figure 1. 4 Two analysis modes of SIMS. (A). Static mode offers information regarding the sample's surface atomic layer. (B). Dynamic mode employs high primary ion dosage to remove material to gather 3D information, resulting in the

development of a crater (136).

1.4.2 Sputtering and ionisation efficiency

Initially, the SIMS technique was used for surface analysis in the late 1960s.

However, its poor ion yield and low imaging quality hindered its effectiveness. To overcome these challenges, researchers started using cluster ion beams such as Bi_3^+ , C_{60}^+ , H_nO_n^+ , and GCIBs, which greatly improved sensitivity. The polyatomic beams' clusters enable energy to spread across all atoms, reducing surface damage and ion fragmentation. Consequently, the analysis can cover intact molecules rather than just fragments. The sputtering yield, which refers to the average number of atoms produced from the target per impact particle, is correlated with the primary ion beam's energy and type (134, 144-145).

In Figure 1.5, molecular dynamics simulations conducted on a computer illustrate the results of ion beam impacts from various sources. The simulation was performed at a kinetic energy of 15 keV. As demonstrated in the figure, Ga and Au_3 possess the greatest penetration power into the surface top layer, resulting in more damage. Furthermore, Ga and Au_3 produce the lowest secondary ion yields.

beam impacts appear similar, the damage caused by GCIBs is comparatively minimal. This could be attributed to the lower kinetic energy per particle in the cluster, as well as the fact that gas cluster metal ion beams generally have lower ionisation probabilities. As a result, Ar₈₇₂ interacts with sample atoms more gently than C₆₀, resulting in less material fragmentation. It should also be noted that the ion yield produced by C₆₀ impact is much higher than that produced by GCIBs, as only a few ions are generated by the latter (144).

In SIMS analysis, ionisation plays a vital role alongside the sputtering of ions. This is because the production of secondary ions in SIMS analysis depends on both the primary ion beam's impact on the analyte surface and the ionisation of sputtered ions. The ion yield is a crucial factor that can significantly impact SIMS ionisation. Typically, the ionisation probability in SIMS analysis is less than 10^{-5} , resulting in a low ion yield of less than 0.01 from a $1\text{ }\mu\text{m}^2$ analysis area under static mode SIMS (146). As illustrated in Figure 1.5, an increase in cluster size can result in an increase in ion yield. However, previous studies have shown that increasing the cluster size may not always lead to an increase in ionisation efficiency. This is because the impact energy and ionisation efficiency may decrease if the beam energy does not increase proportionately (146). Therefore, while an increase in sputtering production can be expected with an increase in cluster size, the ionisation efficiency may not necessarily follow the same trend.

1.4.2.1 Matrix effect

Besides the impact of the primary ion beam, the local chemical environment of the analyte also plays a crucial role in influencing ionisation efficiency in SIMS analysis. The local chemical environment can have a significant impact on the target ionisation efficiency, which is known as the matrix effect, due to SIMS's high sensitivity (147). The complex chemical environment of the analyte can cause changes in the ion-enhancing or suppressing effect, making the matrix effect particularly relevant for biological samples. These samples are highly heterogeneous, comprising lipids, proteins, endogenous metabolites, and nucleic acids (147). To ensure precise outcomes, it is necessary to mitigate the matrix effect in SIMS analysis.

The most commonly observed ionisation mechanism in the SIMS system is proton exchange. However, highly ionised molecules can inhibit the ionisation of less ionised molecules, thereby exacerbating the matrix effect (148). Previous studies have proposed several methods to reduce the matrix effect, such as sample pre-treatment with gold coating and the utilisation of water polyatomic beams as the analysis beam. By reducing the matrix effect, it may be possible to enhance the ionisation efficiency, which is a crucial factor in SIMS analysis (149).

To gather accurate analyte information in SIMS analysis, it is crucial to enhance the ionisation efficiency. Insufficient ionisation efficiency can lead to a reduction in the amount of analyte information obtained. Laser post-ionisation is a technique that can be used to increase secondary ion production by irradiating the plume just above the sample, where sputtered neutrals from the sample can be ionised. However, the efficiency and durability of laser beams still require improvement, as the ionisation efficiency can differ among samples, and not all species can be ionised by the laser beam (150).

Enhancing primary ion beams is the most common approach to augment ionisation efficiency, as compared to using lasers. The optimal analysis beam should generate the maximum number of secondary ions while minimising the loss of biological molecular information. SIMS instrumentation, which involves a primary ion beam coupled with various analysers, is critical for obtaining molecule information (151).

1.4.3 SIMS instrumentation

SIMS instrumentation offers many configurations, particularly through the use of diverse primary ion guns and sputtering beams combined with different mass analysers. In this thesis, the primary focus will be on two SIMS systems: the conventional ToF-SIMS system and the innovative OrbiSIMS (133). While ToF-SIMS is a commonly used form of SIMS system, and OrbiSIMS is a novel system that was developed in 2017 (133), ToF-SIMS is currently more widely used than OrbiSIMS.

1.4.3.1 ToF-SIMS

In the ToF-SIMS instrumentation, a dual-beam system is used to ionize the sample, consisting of the primary ion beam (analysis beam) and sputtering beams (flood gun), as shown in Figure 1.6. The ToF reflectron mass analysers are also installed in the instrument, which act as low-pass filters. The reflectron voltage can be adjusted to change the acceptance of the analysers, with a higher reflectron voltage allowing more ions to be transmitted. The strength of the electric field above the sample surface is essential for extracting secondary ions, which should be high enough to extract ions while preventing the ion beam from being distorted. The ToF-SIMS system is widely used due to its ability to achieve high-resolution and high-mass accuracy.

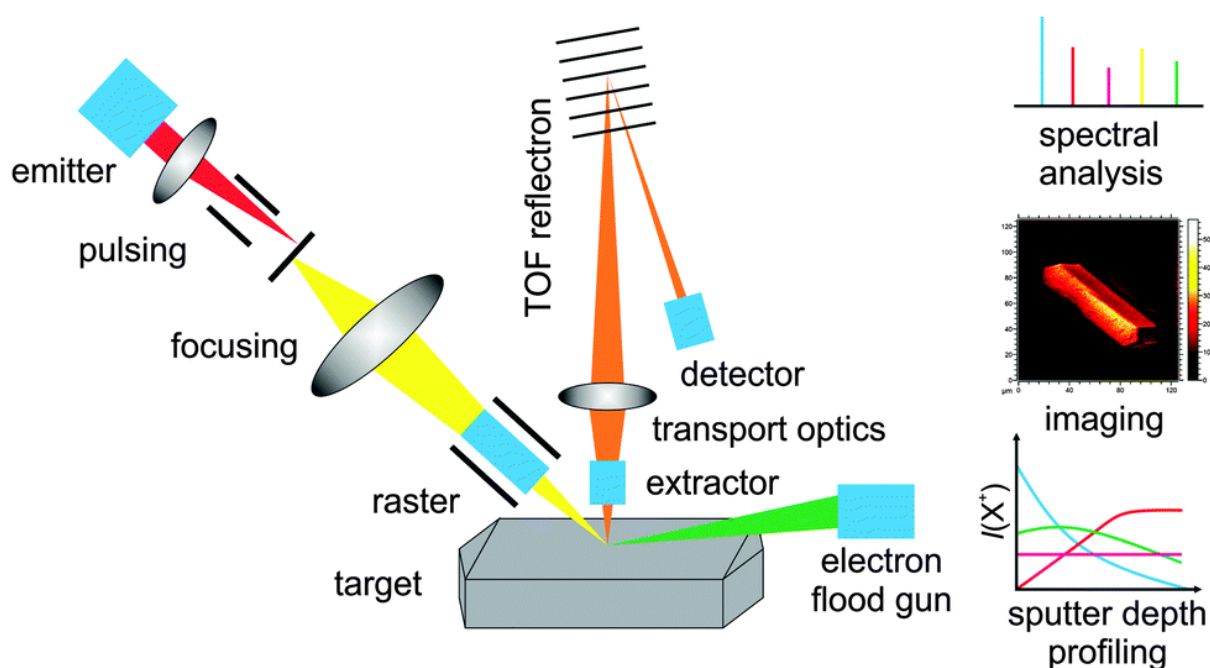


Figure 1. 6 Schematic illustration of ToF-SIMS instrumentation. The basic components of ToF-SIMS are target plate, the primary ion beam, the electron flood gun, extractor, transport optics, time of flight analyser and detector. (Reprinted with permission from reference [152]))

In addition, during the scanning of the analyte surface with the primary ion beam, the dynamic emittance technique is implemented to decrease the scattering of secondary ions in the ToF analysers. The reflectron installed in the ToF analysers serve as a means for energy concentration, ensuring that the discharged ions with minor energy variations can have uniform flight times (152).

In ToF-SIMS analysis, the mass-to-charge (m/z) ratio is determined by the ToF analyser, which calculates the particles' "ToF" in nanoseconds from the detector's impact time (153). After the primary ion beam strikes the sample surface, secondary ions are generated and directed towards the detector through a flight path. The ion optics guide the secondary ions to the mass analyser, where their m/z value is determined and displayed as individual peaks on the spectrum (153).

The IONTOF 5 system is known for its significant advantages, including a spatial resolution of 60 nm on samples and a pixel frequency or repetition rate of 50 kHz (153). However, achieving good mass resolution requires the use of bunch mode for the primary ion gun. This means that the primary ions must be condensed into an ion packet below a specific number of nanoseconds (131). It is crucial to limit the width of the primary ion pulse to a maximum of 600 ps for the optimised experiment as it can greatly affect the mass resolution. Although the mass resolution for SIMS analysis is approximately 10,000, which is lower for gathering more accurate biological information, it is still a crucial consideration (152-154).

1.4.3.2 OrbiSIMS

The OrbiSIMS technology holds tremendous promise in the realm of producing more precise outcomes by accurately identifying biomolecules from the sample. This innovative instrument amalgamates the advantages of both analysers, including the high spatial resolution and rapid speed of the ToF analyser, along with the high mass resolution and tandem MS capability of the Orbitrap analyser (155-159). The Orbitrap mass analyser, first introduced by Alexander Makarov in 2000, has since made substantial contributions to MS-based research owing to its exceptional mass resolution and accuracy (155-159).

The Orbitrap mass analyser is a powerful tool that employs a distinctive electrostatic field with orbital trapping to determine the mass-to-charge ratio of ions. Unlike the ToF analyser, which relies on the measurement of the time required for an ion to travel a certain distance, the Orbitrap analyser induces harmonic oscillation of ions along the field's axis. The oscillation of ions generates a current that can be measured to determine their mass-to-charge ratio. The obtained data are further processed using

Fourier transform algorithms before being presented in the spectra. The Orbitrap analyser has gained significant recognition in mass spectrometry-based research due to its superior mass resolution and accuracy. Its ability to provide precise and reliable data has been instrumental in expanding our understanding of complex biological systems.

In Figure 1.7, it can be observed that the Orbitrap analyser is composed of a spindle-shaped electrode positioned at the centre and a barrel-shaped electrode arranged along its axis (155). The Orbitrap analyser presents a number of benefits, including an outstanding mass resolution of 240,000, which is notably higher than that of the ToF analyser. The Orbitrap has a larger trapping volume that facilitates the confinement of a greater number of secondary ions in the trap, thus reducing space charge effects. Furthermore, the Orbitrap allows for precise mass identification, with an error rate of less than 2 ppm and a high dynamic range greater than 5,000 within a single scan (155).

In a 2017 study, the OrbiSIMS instrument was investigated for its ability to map endogenous and exogenous molecules in cells and tissues by combining two sources of primary ions (LMIG and GCIB) and two MS analysers (ToF and Orbitrap) (133). By utilizing the high mass resolving power and accuracy of the Orbitrap in combination with the speed of the ToF analyser, metabolic information at the cellular level was evaluated. The OrbiSIMS instrument offers a spatial resolution of 1.34 μM for organic molecules and 172 nm for inorganic molecules and has a mass resolving power of 240,000 at m/z 200. The instrument has revealed metabolic changes, such as a linear correlation between intact lipid and increased drug concentration (133), and the metabolome changes between different phenotypes of macrophages. The OrbiSIMS surpasses the ToF-SIMS system in its capabilities for exploring metabolites at the cellular level due to its high mass resolving power.

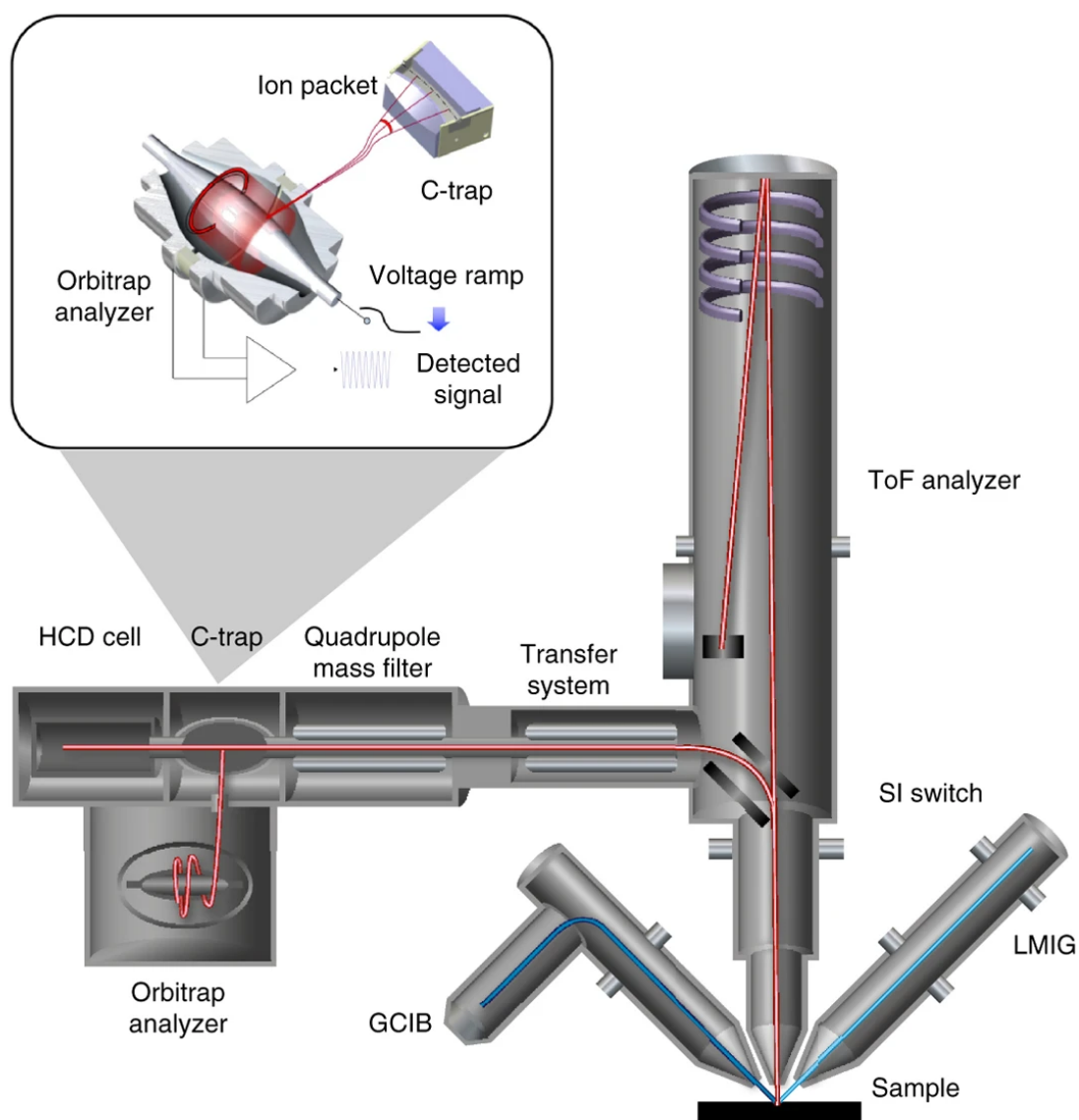


Figure 1. 7 Schematic illustration of novel 3D OrbiSIMS instrumentation. The novel hybrid instrumentation combines the Orbitrap and ToF analyser both. The Orbitrap mass analyser is a spindle where ions are orbited due to dynamic electrostatic field, and dual set up of primary ion beam GCIB and LMIG provides flexibility of analysis. And the SI switch allows altered between different analysers. (Reprinted with permission from reference [133])

1.4.4 Data analysis of SIMS

The data analysis of SIMS is an essential aspect of the experiment, in addition to the advanced instrumentation used. The method of SIMS data analysis is dependent on the experiment's objectives, as highlighted by research (134). The data analysis process can be divided into two categories, namely targeted and untargeted analyses, depending on the specific objectives of the experiment.

1.4.4.1 Targeted analysis

Targeted analysis is a crucial aspect of SIMS data analysis, which involves mapping and quantifying known metabolites. The primary objective of this approach is to identify and determine the bio-distribution of drugs in cells and tissues to achieve the therapeutic goal. By analysing the bio-distribution of pharmaceutical compounds, PK activities of drugs can be revealed, as indicated by previous research (134, 160).

1.4.4.2 Untargeted analysis

In the field of SIMS analysis, untargeted analysis differs from targeted analysis in that it aims to provide a comprehensive profile of metabolite information. While the experimental setup for untargeted analysis requires less preparation, data analysis is more extensive. One of the significant applications of untargeted SIMS data analysis is for studying diseases and discovering biomarkers (161). By analysing untargeted data, it is possible to identify variations in unknown ions that could contribute to identifying molecular modifications during disease progression. While the identity of these ions remains unknown, they could provide valuable information about diseases based on the symptoms and testing.

To achieve the untargeted goals of SIMS analysis, statistical methods are required to analyse the complex and detailed datasets produced by advanced instrumentation with high mass resolution and sensitivity. Statistical analyses must be conducted to reduce the complexity of these datasets (134, 162-164). Multivariate analysis is a widely used method in the SIMS community as it allows for the processing of large datasets, which is a common characteristic of SIMS data. This thesis will primarily concentrate on principal component analysis (PCA), which is one of the frequently used multivariate techniques.

1.4.4.2.1 PCA

As a type of multivariate analysis, principal component analysis (PCA) is a statistical method used to analyse variance and identify strong patterns in data collection. It reduces dimensionality with little data loss, making it a useful tool in SIMS analysis where the results are multivariate. Each SIMS analysis yields a number of spectra with varied mass channels and intensities that can be categorised according to differences in spectra. Researchers typically use PCA to analyse these variables statistically and identify patterns (165-166).

Figure 1.8 illustrates the fundamental principle of PCA in geometric projection form. The figure contains a set of data points from a 3D dataset, and the three orthogonal PCs are drawn based on the variance within the dataset to explain the direction of the most variance. The greater the dispersion of the data points in reference to the PCs, the greater the variance. However, the PCs are not randomly drawn on a line; rather, they are calculated using the eigenvectors and eigenvalues. The eigenvectors indicate the direction of the major components, while the eigenvalues indicate the variance of the dataset. Figure 1.8 presents three eigenvectors and three eigenvalues, one for each PC. PCA is a commonly used multivariate technique in the SIMS community due to its ability to handle large datasets.

Additionally, the use of PCA analysis in conjunction with SIMS data can aid in simplifying the raw data by reducing the number of dimensions, making it more comprehensible and simpler to analyse (165-168). This method can also aid in identifying the most important eigenvalues and representative variables of an experiment. By rearranging and rotating the axes to the required number of dimensions, the higher eigenvalues can be displayed. Then, the observations can be projected onto a new axis called a factor, with the projection of the factor onto the initial variable referred to as a loading, and the observations projected onto the factor being scores. Loadings are a useful method for detecting crucial signals in PCA results that lead to the differentiation of various sample groups. As a result, over the years, PCA has been an invaluable tool for enhancing the spatial interpretation of numerous peaks by producing a few easily interpretable score images. The subsequent results chapter of this thesis will primarily focus on utilizing PCA analysis to distinguish between different groups (167-168).

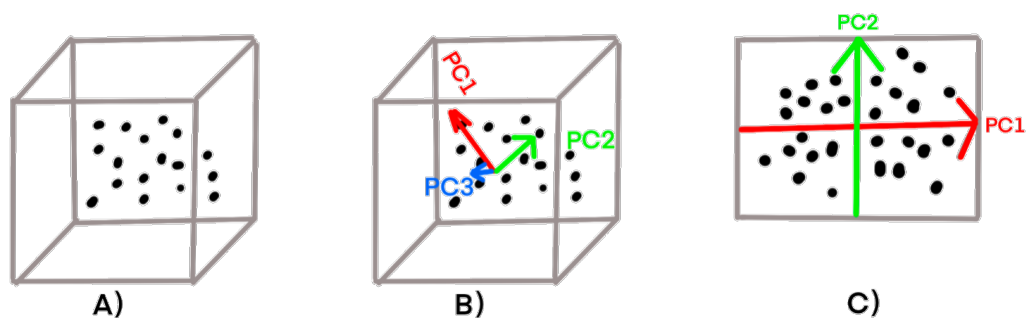


Figure 1. 8 Schematic illustration of PCA. A) three-dimensional data set. B) three principal components based on the variance. C) Two-dimensional data points of first two principal components.

1.5 Biomedical application of SIMS

1.5.1 ToF-SIMS

Many studies have demonstrated the usefulness of ToF-SIMS in analysing biological components (168-172). In addition to endogenous metabolites, ToF-SIMS can also assess the effects of anticancer drugs on biological matrices. For instance, cisplatin and oxaliplatin have been examined in cells and tissues using ToF-SIMS (173-174). Furthermore, ToF-SIMS can detect these compounds at subcellular levels, and their distribution varies between different cell types (173-177). ToF-SIMS has also been employed to explore the interaction of anticancer drugs with endogenous cellular components such as metabolites and lipids, to develop more effective drugs (178-180). Despite the potential utility of this technique in the study of anticancer drugs, it has not received much attention in this field, and research on this topic is limited.

As discussed in section 1.4.2, the gas cluster ion beam technique has significantly improved SIMS' ability to study biological materials, owing to its high mass resolution and chemical specificity, which can discriminate complicated structures in such materials. The use of cluster sources reduces surface degradation and fragmentation, allowing for the detection of intact biomolecules and the identification of compounds. These advantages have revolutionised bioanalysis, providing chemical profiles and images with exceptional spatial and depth resolution of complex structures such as biological tissues and cells. This technique has the potential to significantly enhance drug delivery studies by enabling 3D distribution analysis of

foreign substances within biological tissue, which was previously limited to 2D surface analysis of vertical tissue slices. The availability of advanced OrbiSIMS has increased the likelihood of success in this endeavour.

1.5.2 OrbiSIMS

In the preceding section, it was noted that the 3D OrbiSIMS represents a hybrid mass analyser that combines the strengths of high-speed, high-quality resolution ToF imaging with the tandem mass spectrometry capability and high mass accuracy of the Q-Exactive HF Orbitrap analyser, while being equipped with a micro-focused gas cluster ion beam as standard equipment (133). The 3D OrbiSIMS boasts a spatial resolution for molecular imaging of less than 2 micrometres across the full width at half maximum (133). Notably, it features a novel liquid metal ion beam Bi³⁺, which is able to image inorganic and fragment ions at a half-maximal spatial resolution of 200 nanometres due to its liquid metal composition. These capabilities render the 3D OrbiSIMS an exceptionally powerful and versatile instrument (133).

In 2017, OrbiSIMS was introduced and employed to identify and map 74 lipids in the corpus callosum region of the mouse brain, including information on their subcellular localization. Furthermore, the technique was found to map moderate levels of a range of endogenous metabolites, such as neurotransmitters like gamma-aminobutyric acid, dopamine, and serotonin, in the stratum oriens region (133). The OrbiSIMS was also used for conducting metabolic profiling of individual macrophage NR8383 cells exposed to varying levels of amiodarone, owing to its high spatial resolution and low fragmentation (133). The results of this study suggest a correlation between the accumulation of amiodarone and the increase of phospholipids and cholesterol in the bloodstream.

The OrbiSIMS technique has emerged as a promising label-free biomedical imaging method for next-generation analytical analyses. Its applications have been reported in various fields, such as neuroscience, microbiology, cell biology, and dermatology. For instance, the OrbiSIMS technique has been employed to investigate neurotransmitters in mouse brain slices and amiodarone ions in macrophages (133). Additionally, it has been used to assess the impact of antimicrobial treatment on structural changes in

biofilms (181) and to identify drugs in diverse areas of brain tissue (182). The technique has also been successfully utilised for direct cell analysis to characterise different phenotypes and observe molecular biomarker changes at the cellular level, thereby enhancing our understanding of immune disease response and implanted medical device interactions (183). Furthermore, the 3D OrbiSIMS profiling analysis has enabled the tracking of the penetration of a peptide (Pal-GHK) through ex vivo human skin at values below 100 ppm, which are commercially relevant (184). This exemplifies the exceptional capacity of OrbiSIMS to obtain highly specific chemical compositions from low-concentration samples.

The current biomedical applications of OrbiSIMS demonstrate its immense potential in the field of biomedical research. However, its ability to precisely locate anticancer medications at the cellular level remains unknown. Moreover, although OrbiSIMS is believed to be more sensitive than ToF-SIMS IV, no definitive evidence supports this claim. Hence, the objective of this study is to investigate whether OrbiSIMS can provide information on anticancer agents and altered endogenous metabolites in biological matrices, as well as facilitate significant biomedical research. This research will contribute new insights to the understanding of the analytical capabilities of OrbiSIMS, which may aid in the development of novel analytical techniques with improved sensitivity and specificity.

1.6 Aims and objectives

By employing both established and novel analytical techniques, this thesis strives to address the current challenges in anticancer drug research, as outlined in the preceding sections. The initial phase of the project will concentrate on examining a selected panel of anticancer drugs in biological matrices (human liver tissue and cancer cells) to determine whether OrbiSIMS offers superior capabilities for detecting biomolecules in biological matrices compared to ToF-SIMS. The second phase of the project will focus on the development and application of the OrbiSIMS technique to concurrently investigate the cellular response of endogenous metabolites to test drugs under various treatment conditions. These works aim to establish a suitable workflow for studying biomolecules, including anticancer drugs and endogenous metabolites that respond to drug treatment, with imaging work providing detailed localisation.

Ultimately, the developed workflow has been applied to investigate tamoxifen-resistant breast cancer and its potential treatment strategies, serving as an exemplar case in addressing and comprehending the genuine biomedical challenges within cancer research. The development of anticancer pharmaceutical products requires analytical techniques that yield more precise information about drug concentrations at the cellular level and the target site. Currently, no alternative instrument rivals OrbiSIMS in terms of its advantages, which include simple sample preparation, high chemical specificity, and mass resolution with lateral and axial resolution. In summary, this project seeks to capitalize on these benefits to advance cancer research, with the hope that OrbiSIMS chemical specificity and imaging capabilities will offer new insights into the field.

Chapter 2. Materials and Methods

2.1 Materials

The breast cancer cell lines MCF-7 were generously provided by Kanouthar Bouzinab at the Centre for Biomolecular Sciences (CBS) building, University of Nottingham, UK. The tamoxifen-resistant cell lines MCF-7/TamR-1 were acquired from ATCC (ATCC, CRL-3435).

Rosewell Park Memorial Institute (RPIMI) 1640, fetal bovine serum (FBS), penicillin (10,000 units), streptomycin (10 mg/mL) solution, trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA), high glucose Dulbecco's Modified Eagle Medium (DMEM), 0.4% trypan blue solution, 10 M ammonium formate solution, and bovine liver homogenate BCR-185R (trace elements) were obtained from Sigma Aldrich, UK. VWR Chemical, UK, supplied the 96-well plates. 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) and the Nunc Lab Tek II chamber slide system were procured from Thermo Fisher, UK. Glass hemacytometers, dimethyl sulfoxide (DMSO, HPLC grade), phosphate-buffered saline (PBS) tablets, and parafilm were acquired from Fisher Scientific. Leica provided the 3mm gold-coated copper flat carriers and 3mm gold-coated flat disks.

4-Hydroxytamoxifen (4-OHT) (HPLC grade) was obtained from Hellobio, UK. Capecitabine, dasatinib, daunorubicin hydrochloride, docetaxel, epirubicin, gefitinib, lapatinib, pictilisib, quizartinib, sorafenib, tamoxifen, tegafur, temozolomide, venetoclax, and vismodegib, all of HPLC grade, were procured from Sigma Aldrich, UK.

2.2 Chemical solution preparation

2.2.1 Chemical stock solution

The stock solution for each compound under investigation was prepared by dissolving the compound in dimethyl sulfoxide (DMSO), resulting in a final concentration of 10 M. Subsequently, the stock solution was utilized for spiking into the biological sample.

2.2.2 Pure compound for SIMS analysis

Fifteen pure compounds were solubilized in DMSO (10 mM), with a droplet of each compound placed on a circular glass substrate. Subsequently, the samples were lyophilized using a freeze dryer (Benchtop Freeze Dryer, Biopharma).

2.3 Cell sample collection and preparation

2.3.1 Cell culture and maintenance

The human breast cancer cell line MCF-7 and its tamoxifen-resistant derivative MCF-7/TamR-1 were maintained under specific culture conditions. MCF-7 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma, R8758-500ML), supplemented with 10% fetal bovine serum (Sigma, 12103C-500ML) and 1% Penicillin-Streptomycin (Sigma, P4333-100ML). Upon reaching 70-80% confluence in T75 cell culture flasks, cells were washed once with trypsin-EDTA, detached, and then suspended using an appropriate volume of fresh trypsin-EDTA. They were then incubated at 37°C with a 5% CO₂ atmosphere for about 5 minutes. Following detachment, cells were collected by adding fresh medium and transferred to a 15ml Falcon tube. Cell pellets were obtained by centrifugation for 5 minutes at 1000 rpm at room temperature, before being resuspended in fresh RPMI 1640 medium for further experimentation or transferred to a new T75 flask for further passage. In parallel, the MCF-7/TamR-1 cells were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) (ThermoFisher, 11965-092) supplemented similarly, but with the addition of 1 µM of 4-hydroxytamoxifen (Hellobio, HB2508) included in the DMEM to sustain tamoxifen resistance. The maintenance method for MCF-7/TamR-1 was largely similar to that of MCF-7, ensuring comparability between the cell lines.

2.3.2 Cell counting

For subsequent experiments, cell numbers were determined prior to experimentation. Cell pellets were resuspended in 1 mL of fresh medium, and a 20 μ L aliquot of the cell suspension was transferred to an Eppendorf tube and mixed with 80 μ L of 0.4% trypan blue solution (Sigma, T8154-20ML). Subsequently, 100 μ L of the cell-trypan blue mixture (1:4 ratio) was used to fill a glass hemacytometer (Sigma, Bright-line, Z359629). Cell counts were performed under a 10X microscope, with only unstained (live) cells included in the count from the corner set of 16 squares. The average number of live cells from the corner set of 16 squares was then multiplied by 10,000, followed by an additional multiplication by 5 to account for the five-fold dilution with trypan blue. The $C_1V_1=C_2V_2$ equation was then used to determine the required volume to be taken from the 1 mL suspension.

2.3.3 Cell sample preparation for SIMS analysis

MCF-7 and MCF-7/TamR-1 cells ($0.5 \times 10^5/\text{mL}$) were separately seeded on the glass substrate of the Nunc Lab-Tek II Chamber slide system, featuring 8 wells. The chamber was incubated for 3 hours at 37°C with 5% CO₂, allowing the cells to adhere to the slides. Following the 3-hour incubation, depends on the purpose of experiment, the cell media was replaced with fresh media containing different concentrations of drugs and incubated for different time at 37°C with 5% CO₂. For the different treatment time condition, the MCF-7 cell was individually incubated with IC₅₀ value of tamoxifen (10 μ M) and lapatinib (15 μ M) on the time range of 10s; 0.5 hr; 1 hr; 1.5 hr; 2 hr. For the different concentration treatment condition, the MCF-7 cell was individually incubated with different concentration of tamoxifen (0.005 μ M; 0.01 μ M; 0.05 μ M; 0.1 μ M; 0.5 μ M; 1 μ M; 5 μ M; 10 μ M) and lapatinib (0.01 μ M; 0.05 μ M; 0.1 μ M; 0.5 μ M; 1 μ M; 5 μ M; 10 μ M; 15 μ M) for 2 hrs. The MCF-7/TamR-1 cells were individually incubated with tamoxifen (10 μ M), different concentration of dasatinib (0; 5 μ M; 10 μ M; 20 μ M; 30 μ M) for 2 hrs. After incubation, the medium was aspirated, and the cell-seeded substrates in each well were washed three times with 150 mM ammonium solution (Sigma 78314). This washing process minimized salt interference from the cell culture medium and prevented cell rupture, which would have occurred if water had been used. The ammonium formate removed salt interference without damaging the cells and did not leave residues that might interfere

with the signal. Following washing, the solution was removed, and the samples were lyophilized using a freeze dryer (Benchtop Freeze Dryer, Biopharma). The dried samples were sealed with Parafilm (Parafilm®, Sigma, P7793) and stored at -80°C until SIMS analysis. Prior to SIMS experimentation, the samples were desiccated at room temperature in a vacuum to equilibrate them to room temperature. The purpose of dehydration was to avoid cell disruption during the analysis, which was conducted under a high vacuum environment.

2.4 Tissue sample preparation

2.4.1 Tissue homogenate sample

The BCR-185R powder, a standard lyophilized bovine liver tissue, was initially suspended in deionized water. Following suspension, the sample was centrifuged at 7,500 rpm for five minutes, and the supernatant was subsequently removed. To eliminate salt interference from the biological tissue sample, the powder was washed three times with 0.1 M ammonium formate (pH 7.4) through centrifugation at 7,500 rpm for five minutes. Finally, the washed BCR-185R powder was resuspended in deionized water, resulting in a final concentration of 0.1 g/mL.

2.4.2 Drug doped tissue homogenate sample

Initially, the prepared chemical stock solution was introduced into the tissue sample to achieve a final compound concentration of 167 µg/mL. The tissue sample, now containing the compound, was spotted onto a clean glass substrate, and lyophilized for approximately one hour. Subsequently, the sample was stored at -80°C to prevent degradation for future experiments.

2.4.3 The frozen hydrated sample

The drug-doped tissue solutions were meticulously dispensed into the 3mm gold-coated copper flat carriers and covered with 3mm disks. The samples were then high-pressure frozen using the Leica EM ICE system. The frozen samples were mounted on a copper block at the Leica EM VCM station, which was pre-cooled with liquid nitrogen. The mounted block was transferred to the pre-connected Leica EM VCT500 shuttle to maintain cryogenic conditions for the samples. Subsequently, the shuttle was connected to the OrbiSIMS load lock, and the copper block containing the

samples was inserted into the pre-cooled sample holder within the instrument. Finally, the samples were moved to the main chamber in preparation for analysis.

2.5 Cytotoxicity assay

The MTT assay was employed as a cytotoxicity assessment, as MTT is a yellow tetrazole that can react with living cell mitochondria, reducing it to insoluble formazan crystals that deposit inside the cells. Dead cells, on the other hand, do not produce formazan. The amount of MTT produced can reflect cellular activity levels, making the MTT assay a popular method for evaluating cytotoxicity.

Initially, MCF-7 and MCF-7/TamR-1 cell lines were seeded at a density of 5×10^3 cells per well in 100 μ L volume into 96-well plates (Greiner CellStar®, M0437) and incubated at 37°C with 5% CO₂ overnight. Subsequently, the medium was replaced with medium containing various compound concentrations. Plates were then incubated for 48 hours at 37°C with 5% CO₂. Following treatment, 25 μ L of MTT solution (5 mg/mL in PBS) was added to each well, and the plates were covered with aluminium foil to protect from light exposure. The plates were incubated for 2.5 hours at 37°C with 5% CO₂, after which 100 μ L of lysing buffer was added to each well and incubated overnight at 37°C. The following day, the optical densities at a wavelength of 490 nm were measured using a Tecan plate reader. Wells containing only medium, MTT, and lysing buffer, but no cells, were considered the control group.

2.6 Analytical section

2.6.1 SIMS analysis of pure compounds

A bismuth liquid metal ion gun (LMIG) was utilized for static analysis, employing Bi³⁺ clusters as the primary ion beam source. The ToF-SIMS experiment was conducted on an IONTOF IV instrument (ION-TOF, GmbH, Germany). The primary ion beam energy was set at 25 keV Bi³⁺, with both positive and negative polarities acquired for 90 seconds (15 scans). The pulsed Bi³⁺ target current was approximately 0.1 pA, and a post-acceleration energy of 10 keV was applied. The primary ion dose density was maintained below 1×10^{12} ions per square centimetre to ensure analysis within the static limit. Spectra were collected from a 1.2 x 1.2 mm area using a square

raster of 256 x 256 pixels; the patch length of the image was 0.3 mm, and the Bi³⁺ beam size was roughly 3 µm. The primary ion beam was directed at the sample at a 45° angle, and the primary Bismuth LMIG featured a focused beam size of 1-2 µm in HC-BU mode.

For the majority of samples, data was acquired at a resolution of 100 pixels/mm spanning a 4 mm x 4 mm area, in both positive and negative polarities. Utilizing the macro raster stage function and a random raster pattern, each 4 mm x 4 mm square was scanned. A total of 64 separate 0.5 mm x 0.5 mm patches were examined, with each patch undergoing 15 scans. For the primary ion beam focusing mode and the homogenized bovine liver reference samples, data was obtained from 15 scans of a 250 µm × 250 µm patch, at a resolution of 256 × 256 pixels.

2.6.2 ToF-SIMS depth profile analysis

Experiments were conducted using either the ION ToF IV or the 3D OrbiSIMS instrument (both by ION-TOF, GMBH, Germany). The freeze-dried sample was placed onto glass and introduced into the chamber under vacuum conditions. All experiments were performed at room temperature.

The dynamic mode of ToF-SIMS was operated in dual beam mode, utilizing a liquid metal ion beam (LMIG) Bi³⁺ and a gas cluster ion beam (GCIB) with Ar clusters. A Bi³⁺ LMIG beam served as the primary ion beam, with settings applied as described in the previous section, except for using a 128 × 128-pixel resolution. An argon GCIB was employed as the sputter beam. The sputter beam settings depended on various factors, such as beam energy, cluster beam size, and target current. The optimized setting in this project utilized a 15 keV Ar₁₇₀₀ beam with a 10 nA target current. A non-interlaced sputter mode was employed, consisting of a single cycle with 1 frame per sputtering, a 0.5 s pause, and 1 frame per analysis. The experiment was performed at the centre of a 300 µm x 300 µm square, creating a 100 µm x 100 µm crater size with 256 x 256 pixels and 1 shot per pixel.

2.6.3 OrbiSIMS depth profile analysis

The instrument was managed using SurfaceLab 7.1 software, and a 20 KeV Ar⁺ ion beam was employed for mass calibration with a current of approximately 20 pA. Calibration was conducted for both positive and negative modes on a silver (Ag) sample. To enhance ion transmission, the helium gas pressure from the OrbiSIMS was adjusted from low to high during calibration. The calibration procedure was executed through the Orbitrap analyser, and it was regulated by the Q Exactive HF page. For the OrbiSIMS analysis, the depth profile was carried out in both positive and negative polarities for 600s (250 scans). The Orbitrap analyser's mass resolution was set to 240,000. The experiment was performed using a single GCIB 20 keV Ar³⁰⁰⁰ beam, and the analysed crater size was 400 µm x 400 µm. Mass spectra data were gathered within the range of 75–1125 Da.

2.6.3.1 Cryogenic-SIMS analysis

The mounted block which loaded with sample was attached to the shuttle to maintain the sample in cryogenic condition and loaded into the OrbiSIMS airlock chamber and the copper block with samples inserted to the pre-cooled sample holder in instrument. The temperature of this chamber was pre-cooled to – 80 °C by using liquid nitrogen over a 10-minute period. The airlock starts to work, and the pressure was monitored. Once the airlock pressure had reached 1×10^{-6} mbar, the cryostates was slide into the precooled main chamber to be analysed. Depth profile analysis was then conducted using the settings described in section 2.3.5, ensuring that the temperature was maintained at < - 80 °C.

2.6.4 OrbiSIMS imaging work

The experiment was conducted utilizing an OrbiSIMS mass spectrometer in dual beam mode. An electron gun was employed for charge compensation, and the instrument was operated in high mass resolution imaging mode.

The imaging mode incorporated dual beams, with a 60 keV Bi ion beam as the analysis beam and a 10 keV Ar ion beam for sputtering in a non-interlaced mode. The approximate spatial resolution was 0.25 µm, with the fast-imaging mode enabled for the primary ion beams. To enhance mass resolving power, pulsed delayed extraction

was applied to the secondary ions. The analysis was carried out in positive mode, covering a 150 x 150 μm area and 256 x 256 pixels. During the analysis process, the cells were entirely consumed by the ion beams for the image presented in this thesis. Moreover, the delayed extraction mode was implemented for all image work in which the high mass resolution mode is mentioned. Image analysis was conducted using SurfaceLab software, and data processing involved defining the region of interest (ROI) and managing the dataset to obtain the ion intensity of the peak of interest.

2.7 Data analysis

Data from ToF-SIMS and OrbiSIMS experiments were analysed using SurfaceLab 7 software (ION-TOF, GMBH, Germany). In the ToF-SIMS experiment, spectra data were obtained from the static analysis after GCIB sputtering, with the positive and negative spectra calibrated using H^+ , CH_3^+ , C_2H_5^+ , C_3H_7^+ and CH_2^- , C_2H^- , C_3H^- , C_4H^- peaks. For the OrbiSIMS experiment, spectra were collected and summed from the 600s GCIB analysis. Once the peaks of interest had been identified, they were added and normalized to the total ion intensities.

2.7.1 Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.0. Prior to the analyses, the D'Agostino-Pearson test was conducted to verify if the data matrix was generated from a normally distributed population, allowing for parametric analysis. To determine whether a statistically significant difference existed between two groups of data, an unpaired Welch's unequal variance t-test was performed on the entire dataset. P values less than 0.05 were considered statistically significant and denoted by an asterisk symbol. The following notation was used: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

2.7.2 Multivariate data analysis (MVA)

Multivariate analysis (MVA) strategies were employed to manage the extensive and complex datasets generated by this investigation. The objectives of this approach were to reduce overall data processing time, expedite data interpretation, and enhance the confidence associated with the interpretations. Initially, the SIMS depth profile spectra were pre-processed using SurfaceLab software (ION-TOF GMBH). A new

peak list was generated using the automatic search tool with the following parameters: a minimum peak area of 2000 and a signal-to-noise ratio (s/n) of 3. These parameters were selected to minimize interference from background noise. Subsequently, a merged ion peak list was created by combining data from both the control and treatment groups. Finally, the consolidated ion peak list was saved in .txt format for export.

The complex and extensive SIMS datasets were further processed using simsMVA software (<http://mvatools.com>), which combines multiple MVA tools to offer flexibility in handling various samples, large and sparse datasets, and data types typically generated by SIMS spectrometers. This software also includes various data visualization tools and provides a series of data pre-processing steps, such as scaling, smoothing, normalization, and mean centering, essential for effective MVA analysis.

The mass spectrometry mode in simsMVA was updated with the pre-exported .txt file. The imported dataset was first normalized based on total ion intensity before conducting principal component analysis (PCA). The data were then Poisson-scaled and mean-centred before organization. This method scales all variables based on the square root of each variable's average value. If the primary noise source is proportional to the signal's square root, this approach can effectively adjust all variables to the same noise level. Finally, PCA was performed to obtain information on the number of components and loading plots representing the data. This information was considered for the untargeted objective of SIMS depth profile analysis. It is well-known that using PCA to reduce the dimensionality of SIMS raw data can be advantageous, as it simplifies and facilitates interpretation while increasing the analysis's value by focusing on fewer aspects.

2.7.3 SIMS-MFP analysis

SIMS-MFP software was employed to conduct a more in-depth search for compound classes present in the data. This software is specifically designed for SIMS data and utilizes molecular formula matching, initially described by Max et al. (210). It was applied to perform molecular formula prediction on the positive ion depth profile 3D OrbiSIMS dataset obtained from high-pressure freeze-dried breast cancer cell

samples. After executing a peak search using SurfaceLab software (IONTOF, GmbH) with a minimum count of 70,000, a dataset comprising 11,000 distinct ion signals was generated.

Compounds containing $C_nH_nO_2$ (fatty acids), $C_nH_nO_6$ (triglycerides), $C_nH_nO_nN_1$ (ceramides), $C_nH_nO_nS_1$ (sulphates), $C_{<6}H_{<20}N_{<4}O_{<3}$ (amino acids), and $C_nH_nO_nN_nP_1$ (phospholipids) were investigated, where n represents any integer value. Assignments were derived from the output data tables with a mass deviation of less than 2 ppm. These assignments were then validated through comparison with existing literature.

Chapter 3. Molecule profiling studies by SIMS

3.1 Introduction

As delineated in the literature review section, the OrbiSIMS instrument presents significant potential for molecular profiling studies in biological matrices, owing to its exceptional resolving power and spatial resolution capabilities. OrbiSIMS has emerged as a promising label-free imaging technique for biomedical analysis, having successfully detected neurotransmitters in mouse brain slices and amiodarone ions in macrophages (133).

Although OrbiSIMS exhibits superior sensitivity compared to traditional ToF-SIMS IV, attributable to the utilization of distinct primary ion beam sources and the acquisition of more extensive biomolecular information, its capacity to accurately analyse complex biological matrices remains to be elusive. In particular, the ability of OrbiSIMS to localize anti-cancer drug molecules at the cellular level is yet to be ascertained, despite of their known intracellular targeting mechanisms. Prior research has investigated the detection of drug molecules in brain tissue sections (133); however, such investigations may not adequately represent the intricacies of the tumour microenvironment, potentially constraining their applicability. Consequently, it is crucial to assess the proficiency of OrbiSIMS in detecting anti-cancer drug molecules at the cellular level to attain a more comprehensive understanding of their efficacy.

Within the SIMS community, sensitivity is a significant challenge. One of the primary concerns for the pharmaceutical industry is to determine the sensitivity of the SIMS method to different drug molecules and to assess the feasibility of predicting detectability using general principles. Several factors influence sensitivity, including measurement precision, sample size, and the matrix effect (134). The matrix effect, in particular, impacts ionization efficiency due to the local chemical environment. Therefore, a deep understanding of the fundamental processes of SIMS is essential to navigate the challenges presented by various samples.

3.2 Chapter aims

The primary aim of this chapter is to contrast the capabilities of OrbiSIMS and ToF-SIMS in analysing biomolecules within biological matrices. The study will scrutinise numerous aspects of the biological matrix, encompassing the heterogeneity of endogenous metabolites and small molecules of selected anti-cancer drug molecules, to thoroughly assess the potential of OrbiSIMS analysis in biological matrices.

Upon OrbiSIMS analysis in biological matrices, this chapter intends to ascertain whether a correlation exists between the physical properties of compounds and their ionization efficiency, thereby enabling the prediction of compounds amenable to SIMS analysis. Prior research has implied a potential association between the logP value of a compound and its ionization efficiency for SIMS analysis, which will be examined in this study. A panel of up to 15 anti-cancer medications will be chosen based on their logP values to demonstrate the extensive applicability of OrbiSIMS.

To probe the mechanisms underlying the effects of various anti-cancer medications, the study also evaluated the capacity of OrbiSIMS to analyse endogenous metabolites in MCF-7 breast cancer cells. Investigating these features in future cross-comparison studies will substantiate that the biomolecular analysis provided by SIMS corresponds to morphologically significant breast cancer alterations.

Finally, to employ OrbiSIMS for sample cross-comparison and physiological property investigation, several critical experimental variables must be addressed. This study will explore the influence of diverse sample preparation techniques on the endogenous metabolite composition and concentration of small-molecule medications in homogenised liver sample BCR-185R, with the aim of determining the optimal preparation method for this project.

3.3 Results and discussion

3.3.1 Reference spectrum acquisition of compound interest

To determine the presence of a drug in biological samples, it is crucial to first analyse and identify the standard sample of the drug. Subsequently, this method was employed to select the representative peak of the drug.

The ion peak of each pure compound was examined in both positive and negative ion modes prior to assessing the 15 compounds in the biological materials. ToF-SIMS analysis in static mode generated positive ion spectral data within the m/z 1–900 range. Spectra were obtained from the first layer of a pure chemical sample.

Table 1 The list of 15 anti-cancer agent ions identified by ToF-SIMS

Drug	Polarity	m/z	Pure compound	Within the homogenate
Capecitabine	Negative	358.3424	$[M-H]^-$	Not identified
Dasatinib	Positive	489.0181		$[M+H]^+$
		347.2934		$C_{15}H_{19}N_6O_2S^+$
Daunorubicin	Negative	338.2042		$C_{19}H_{14}O_6^{2-}$
Docetaxel	Negative	806.8717		$[M-H]^-$
Epirubicin	Negative	338.2042		$C_{19}H_{14}O_6^{2-}$
Gefitinib	Positive	447.9098		$[M+H]^+$
		360.9403		$C_{18}H_{16}ClFN_3O_2^+$
		346.3028		$C_{17}H_{14}ClFN_3O_2^+$
		332.3320		$C_{16}H_{12}ClFN_3O_2^+$
		318.1833		$C_{15}H_{10}ClFN_3O_2^+$
		302.4027		$C_{15}H_{10}ClFN_3O^+$
Lapatinib	Positive	581.0581		$[M]^+$
		458.3018		$C_{26}H_{18}ClFN_3O_2^+$
Pictilisib	Positive	514.6498		$[M+H]^+$
		434.9753		$C_{22}H_{24}N_7OS^+$
		350.2894		$C_{18}H_{16}N_5OS^+$
		264.4720		$C_{14}H_8N_4S^{2+}$

Quizartinib	Positive	560.6798	[M+H] ⁺	
		421.1048	C ₂₂ H ₂₁ N ₄ O ₃ S ⁺	
		264.2447	C ₁₅ H ₈ N ₂ O _S ⁺	
Sorafenib	Positive	465.8259	[M+H] ⁺	
		270.8302	C ₁₄ H ₁₂ N ₃ O ₃ ⁺	
Tamoxifen	Positive	372.5159	[M+H] ⁺	
Tegafur	Negative	199.1588	[M-H] ⁻	Not identified
Temozolomide	Positive	195.1584	[M+H] ⁺	Not identified
Venetoclax	Negative	867.4322	[M-H] ⁻	
Vismodegib	Positive	422.3078	[M+H] ⁺	[M+H] ⁺

Table 1 lists the ions of each compound, and to compare the intensities of different compounds, it is necessary to standardise the intensities. Ion yield was defined as the relative intensity used to produce the signal and was normalized to total ion counts. A majority of the ions of the 15 compounds were detected in positive ion mode and identified by [M+H]⁺ or specific fragments. For sorafenib, [M+H]⁺ and [M-H]⁻ could be identified under both positive and negative ion modes; however, the positive ion displayed a greater relative intensity than the negative ions, suggesting that positive ions may be considered as representative peaks for subsequent research (Table 1).

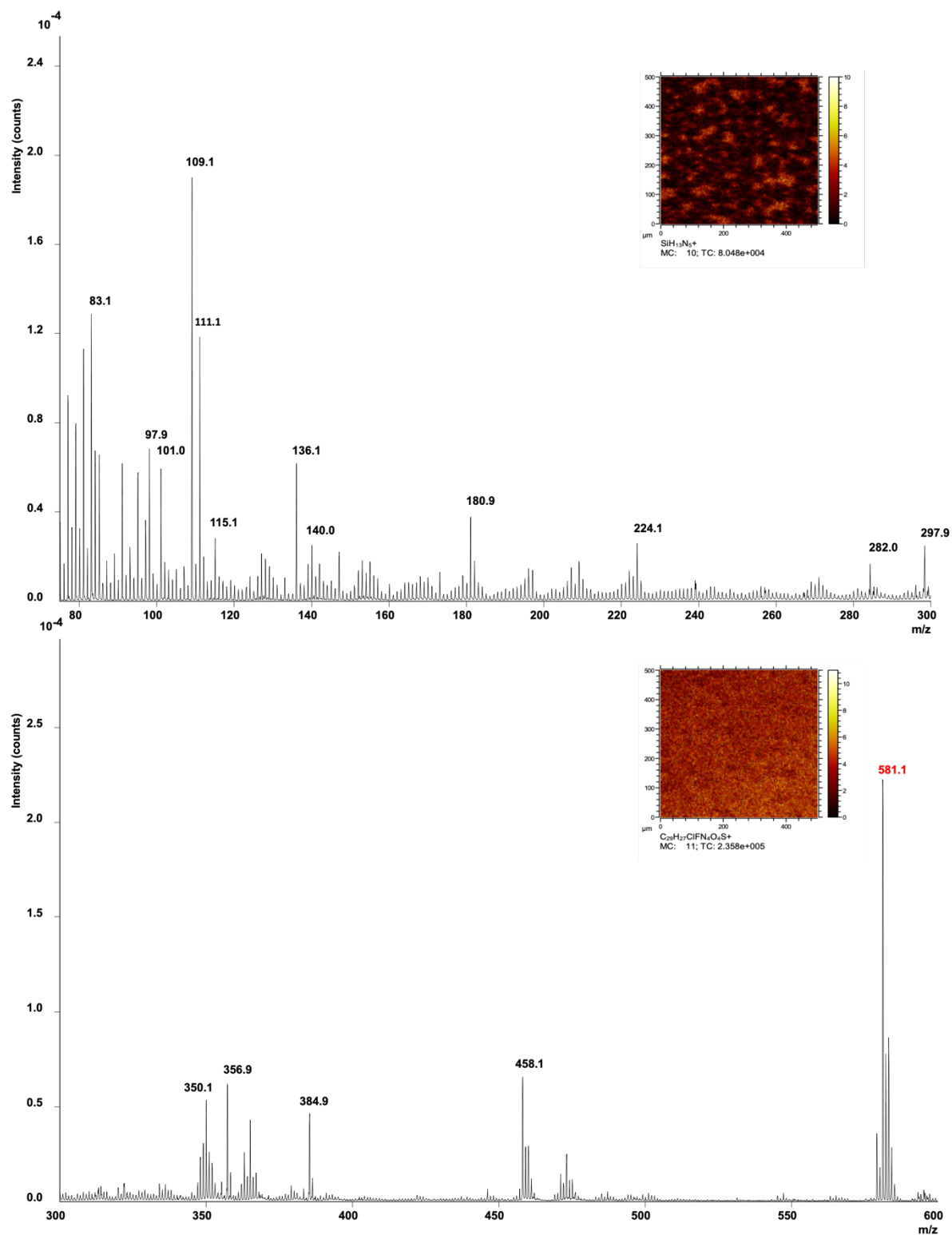


Figure 3. 1 Representative ToF-SIMS analysis of pure lapatinib ion spectrum and image. The spectrum was required from positive ion mode. The mass ranged from m/z 75-600. A) m/z 75-300; B) m/z 300-600. The significant ion peaks were labelled with mass and the protonated $[\text{}^{13}\text{C}_1\text{M}+\text{H}]^+$ was identified by positive ion mode and marked in red colour. The spectra are normalized to the total ion intensity.

Figure 3.1 presents a representative spectrum of pure compounds, specifically derived from pure lapatinib. As illustrated in the figure, a common pattern was observed in the spectrum, with most identified ions possessing masses between 75 and 300 m/z, including fragmented ions such as CH_3^+ (m/z 15.02), C_2H_5^+ (m/z 29.03), and C_3H_7^+ (m/z 43.07). The empirical formula for lapatinib is $\text{C}_{29}\text{H}_{26}\text{ClFN}_4\text{O}_4\text{S}$, corresponding to a relative molecular mass of 581.1 g/mol. The protonated ion $[\text{}^{13}\text{C}_1\text{M}+\text{H}]^+$ was identified at m/z 581.1, indicating that lapatinib could be detected in this experiment. This demonstrates the potential for lapatinib detection in SIMS experiments, as diagnostic peaks of lapatinib were observed at the same peak (185) in prior LC/MS experiments, despite the limited SIMS experiment reports. Furthermore, the molecule image of lapatinib in the figure exhibits a strong signal from the area containing the pure compound, providing indirect evidence for the existence of the lapatinib peak. Other prominent peaks in the positive spectra, such as sodium ions at m/z 23, were identified, with many lower mass fragments comprising hydrocarbons with masses ranging from m/z 1 to 50. This is attributable to the nature of the LMIG, as more fragment ions could be produced, as discussed in the literature review.

In addition to lapatinib, Figure 3.2 presents the positive ion ToF-SIMS spectra of tamoxifen. Tamoxifen has a relative molecular mass of 371.515 g/mol. Several peaks previously reported by Vegvari (2016) were observed in positive ion MALDI spectra, with similar ion peaks, including m/z 129, m/z 152, and m/z 165, also detected (186). Positively charged precursor ions of the tamoxifen ion $[\text{M}+\text{H}]^+$ were readily discernible in the positive-ion spectrum at m/z 372.9, with distinct and substantial peaks. Similar to lapatinib, the small mass area contained numerous fragment ions ranging from m/z 1–50. Therefore, tamoxifen molecules can also be identified using SIMS.

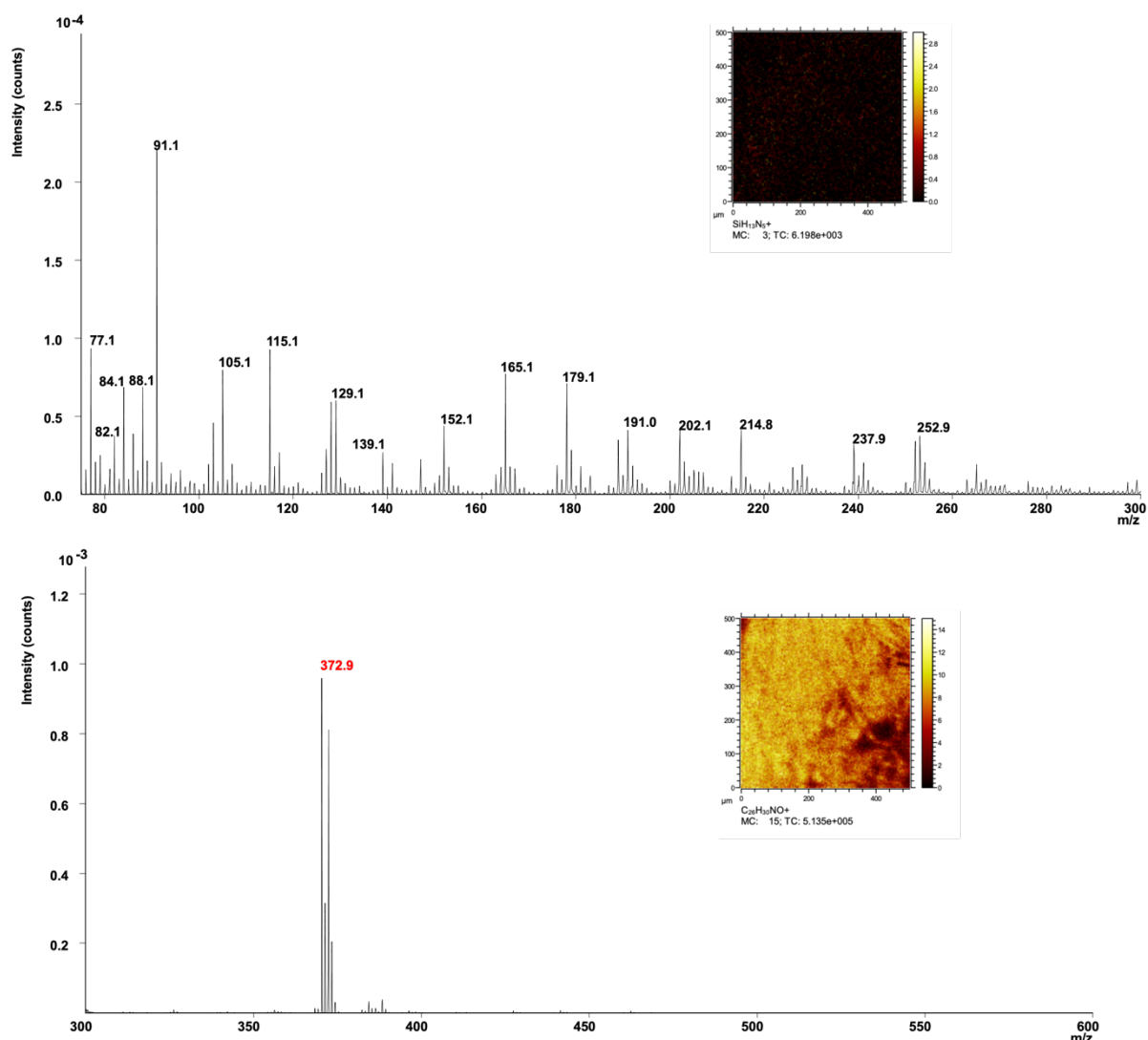


Figure 3. 2 Representative ToF-SIMS analysis of pure tamoxifen ion spectrum and image. The spectrum was required from positive ion mode. The mass ranged from m/z 75-600. A) m/z 75-300; B) m/z 300-600. The significant ion peaks were labelled with mass and the protonated $[M+H]^+$ was identified by the positive ion mode and marked in the red colour. The spectra are normalized to the total ion intensity.

In addition to the ion peaks from the pure compounds, the spectrum also exhibits contamination peaks. Despite purchasing premium raw materials, a small number of early samples obtained from the laboratory's supply were contaminated. This contamination was attributed to silicones with linear structures, as evidenced by the silicon ion peaks. Moreover, Vickerman reported cyclic silicone ions at m/z 207 and 281 (136). The source of the silicones was not investigated; however, it is likely that

they originated from the manufacturing process. Furthermore, contamination signals may have arisen from the glass substrate. Figure 3.1 and 3.2 illustrates that the selected $\text{SiH}_{13}\text{N}_5^+$ signal is clearly visible from the base glass substrate. Owing to an error in sample preparation, a pure compound may have been contaminated during the drying process, exposing the glass substrate through these voids. Silicone contamination highlights the challenges encountered when conducting ‘real-world’ experiments.

In summary, pure compounds exemplified by tamoxifen and lapatinib, were detected in SIMS analysis. Similar observations were made in the analysis of the other thirteen compounds (Table I). Consequently, the representative peaks of these compounds identified using SIMS can be applied to subsequent studies.

3.3.2 Direct profiling of biomolecules using the SIMS systems

3.3.2.1 Biomolecule profiling studies of tissue homogenate

As previously stated, standard peaks for the fifteen drugs were identified for the pure drug using SIMS experimental analysis. However, it remains unclear whether these fifteen drugs can be detected in biological samples. Thus, this study aims to investigate the applicability of the SIMS system for analysing biological samples and to determine if the fifteen anti-cancer drugs can be detected in such samples.

Homogenised bovine liver tissue was directly analysed using a fast-imaging data gathering mode $\text{Bi}3^+$ with a ToF analyser (ToF-SIMS VI) and an optimised mode for gathering biomolecule information using a GCIB with an Orbitrap analyser (OrbiSIMS). This comparison demonstrated that the OrbiSIMS instrument is superior for obtaining molecular data from biological matrices.

Figure S1 presents a representative spectrum of the $\text{Bi}3^+$ couple, as measured by the ToF analyser. As the LMIG was employed as the analysis beam in the ToF-SIMS analysis, a majority of mass peaks appeared in the first spectral range, which is comparable to the pattern observed in the mass spectra generated from the pure drug analysis. However, identifying intact endogenous molecule ion peaks during ToF-SIMS analysis is challenging because of the large molecular weight of most intact

endogenous metabolites, often exceeding 400 Da. In contrast, OrbiSIMS analysis employed the GCIB in conjunction with the Orbitrap analyser, enabling the formation of secondary ions as intact, less-fragmented molecules. Consequently, OrbiSIMS analysis identified more peaks in the large m/z region, specifically between 400 and 900 m/z , where a majority of intact molecules are located (Figure S2).

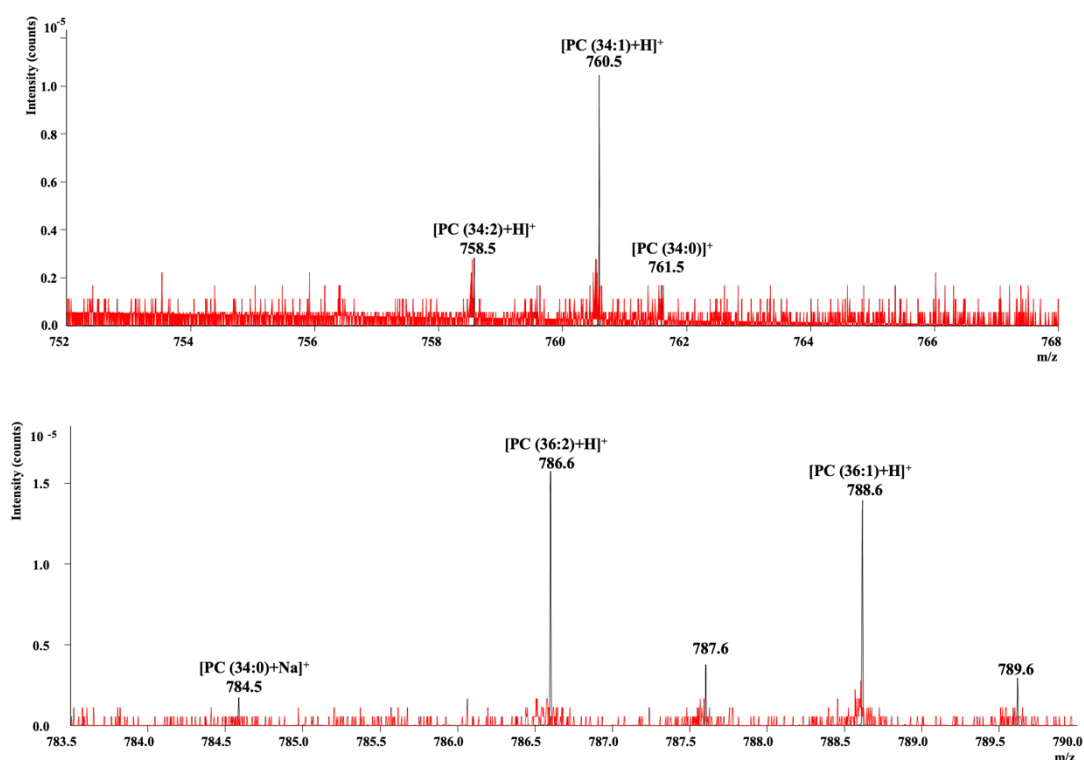


Figure 3. 3 Representative SIMS analysis of pure tamoxifen ion spectrum and image. The spectrum was required from positive ion mode. The mass ranged from m/z 75-600. A) m/z 75-300; B) m/z 300-600. The significant ion peaks were labelled with mass and the protonated $[M+H]^+$ was identified by the positive ion mode and marked in the red colour. The spectra are normalized to the total ion intensity.

Application of the large cluster ion beam considerably increased the relative intensity of OrbiSIMS, enabling the detection of more detailed biochemical data, such as lipids, which are crucial for energy storage and constitute the major components of cell membranes. Over the past few decades, lipids have emerged as potential biomarkers for cancer diagnosis, and their functions have become increasingly well understood. OrbiSIMS detected a greater number of phosphatidylcholine (PC) species than ToF-SIMS, as illustrated in Figure 3.3. Overlaid peaks in the spectrum represent signals

from various PC species. The spectrum demonstrates that these endogenous lipid signals are absent from the ToF-SIMS spectrum (red); however, they present as sharp peaks in the OrbiSIMS spectrum (black). This indicates that OrbiSIMS possesses superior resolving ability, allowing for the exploration of more biological information. In addition to lipids, OrbiSIMS identified more metabolites in the biological matrix than ToF-SIMS (Figure 3.4), with 114 species observed in OrbiSIMS and 24 species observed in LMIG Bi_3^+ -coupled ToF-SIMS. The details of these endogenous metabolite data are provided in the Appendix (Table S1).

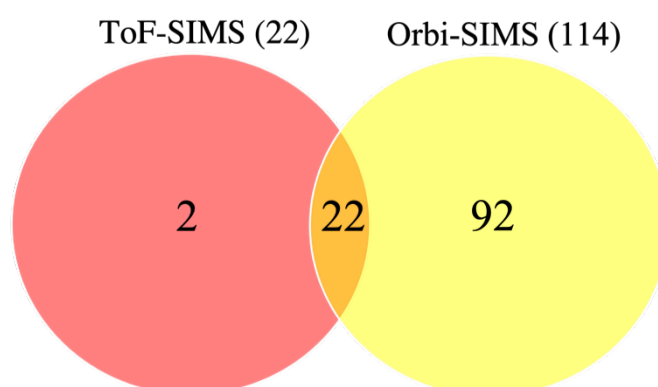


Figure 3. 4 Venn Diagram comparison number of the endogenous metabolite which observed in BCR-185R.

In summary, both ToF-SIMS and OrbiSIMS can be used to study endogenous metabolites of biological matrices. However, LMIG, which is employed in ToF-SIMS, generates a considerable number of fragmented peaks, rendering the detection of intact endogenous molecules, which generally possess higher masses in ToF-SIMS analysis, difficult. In contrast, OrbiSIMS can detect more intact endogenous molecules using the GCIB and the Orbitrap analyser. Therefore, OrbiSIMS is more suitable for the analysis of biological samples and drugs.

3.3.2.2 Identification of anti-cancer agents within biological matrix

Although endogenous metabolites and lipids are readily identifiable via OrbiSIMS analysis, it remains unclear whether OrbiSIMS can detect drug molecules. As the intracellular location of the target site for anti-cancer agents necessitates the transfer of drug molecules from the extracellular to the intracellular space, it is crucial to

investigate drug activity at the disease site (69-72). Consequently, identifying drug molecules within a biological matrix is a critical prerequisite.

Figure 3.5 demonstrates that in ToF-SIMS analysis, seven out of the fifteen drug candidates were identified, whereas in the OrbiSIMS analysis, eight out of the fifteen drug candidates were detected. Compared to ToF-SIMS analysis, the relative intensity from OrbiSIMS displays a range of enhancements with sharper and clearer ion peaks. Notably, lapatinib was not detected using ToF-SIMS; however, it was detected using OrbiSIMS, indicating the suitability of OrbiSIMS for analysing exogenous compounds, such as drug molecules.

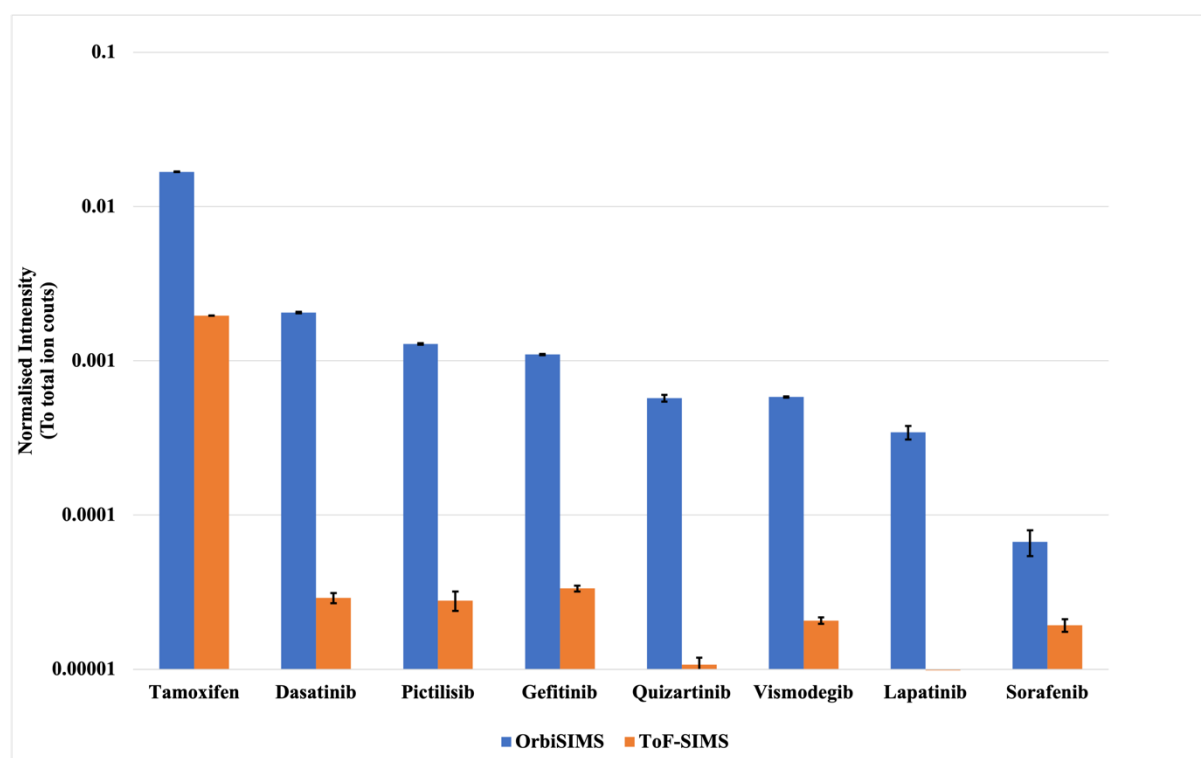


Figure 3. 5 Ion intensity of active compounds detected in OrbiSIMS and ToF-SIMS. The intensity of $[M+H]^+$ ion was counted and normalised to the total ion counts. The bar chart reflects normalized ion intensity (to total ion counts) values obtained from three separate experiments (mean $\pm$ s.d). Blots are representative of the data sets (N=3).

Although OrbiSIMS failed to detect all of the anti-cancer agents, the ion spectra of eight detected compounds showed substantial differences between the ToF and OrbiSIMS systems. Figure 3.6 presents the superimposed spectra of the eight drugs identified using BCR-185R: tamoxifen, quizartinib, dasatinib, sorafenib, gefitinib, lapatinib, vismodegib, and pictilisib. The spectra of the eight drugs from OrbiSIMS

analysis (black) exhibited distinct and more intense peaks than the spectrum from ToF-SIMS analysis (red). This can be attributed to the use of the GCIB as the analysis beam in OrbiSIMS and its pairing with the sophisticated Orbitrap analyser. Makarov introduced the Orbitrap analyser in 2000, initially achieving mass resolutions in the range of 10^4 – 10^5 , which enabled the identification of metabolism through lipidomic analysis (155–159). Subsequent advancements over the past decade have increased the mass resolution power of the analyser to 240,000 and intensified the analysis (133).

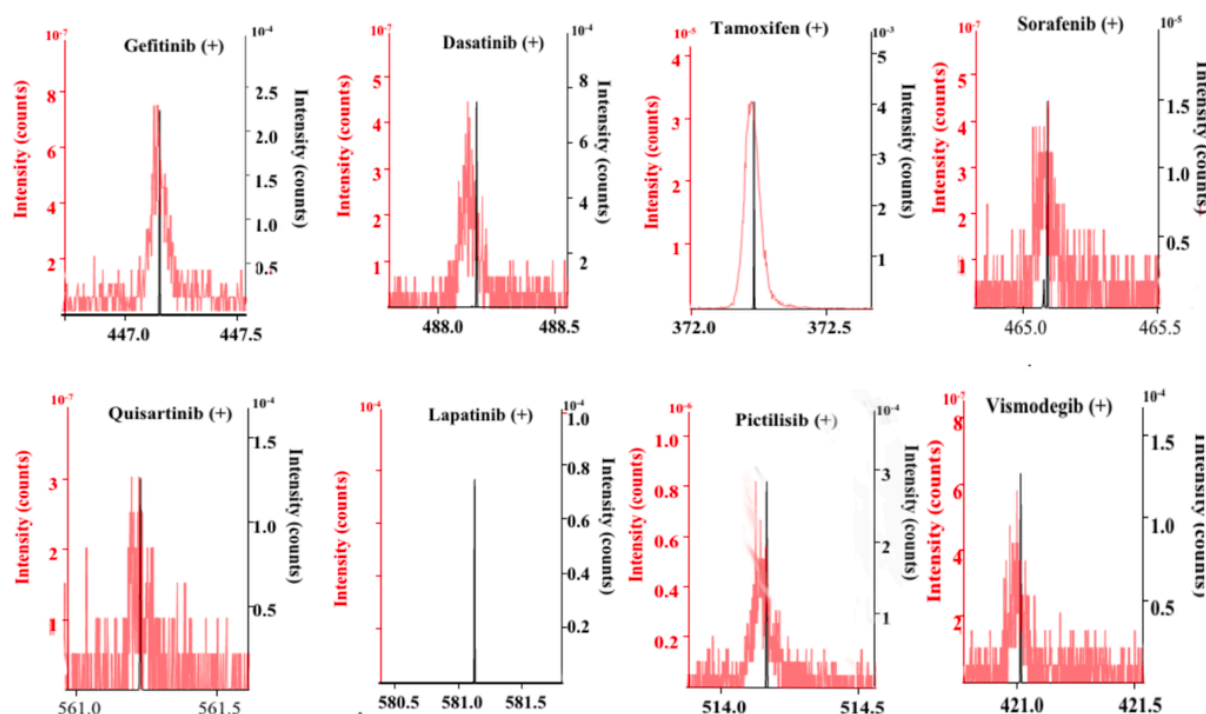


Figure 3. 6 ToF-SIMS and OrbiSIMS spectrum of representative active compounds. OrbiSIMS spectrum is shown as black colour and ToF-SIMS spectrum is shown as red colour.

When using OrbiSIMS to analyse drug molecules within a biological matrix, the inability to detect certain drug ions or their fragments may stem from the low ionisation potential of the compound, substantial matrix effects, or suppression of drug signals by other molecules due to the complex chemical environment. However, the molecular signals of these 15 compounds were clearly detected when analysed as pure compounds (Table 1), suggesting that matrix suppression effects might have been responsible for the failure to detect the signals of seven compounds in the reference tissue sample. At present, the cause of signal suppression for these chemicals remains unknown, warranting further research, including examination of a

larger number of chemical substances. Nevertheless, this provides strong evidence that the reference sample can potentially be used to quantify matrix effects.

The type of analysis beam employed in SIMS experiments can considerably influence the analysis and is affected by analytical factors such as spatial resolution and secondary ion yield. The ToF-SIMS device discussed in this chapter employed an LMIG derived from Bi_3^+ primary ions as the analysis beam. Although the application of LMIG offers the highest spatial resolution, its high-energy content results in severe fragmentation and destruction of the analyte. In contrast, the analytical beam used in OrbiSIMS is a GCIB source, such as Ar^+ . Using an Ar^+ cluster primary ion increases collisional damage and enhances secondary ion yield. Despite possessing the same kinetic energy as Bi_3^+ , the kinetic energy carried by the Ar^+ cluster was distributed evenly among each constituent particle, resulting in reduced sample fragmentation and a higher probability of intact molecule identification when using an Ar^+ cluster. This potentially explains why lapatinib was detected using OrbiSIMS and not using ToF-SIMS.

In summary, while both ToF-SIMS and OrbiSIMS can be employed for the analysis of endogenous metabolites in biological matrices, OrbiSIMS is more suitable for detecting intact molecules owing to the application of GCIB and the Orbitrap analyser. The ability to detect more drug molecules and show clearer and more distinct ion peaks demonstrates the advantages of OrbiSIMS for analysing exogenous compounds such as drug molecules. Further research is necessary to understand the factors influencing signal suppression and to optimise the application of OrbiSIMS for the analysis of a broader range of chemical substances in biological samples.

3.3.2.3 Predicted ionisation of compounds by its physical-chemical properties

In the preceding section, the potential of Secondary Ion Mass Spectrometry (SIMS) for identifying anti-cancer agents within biological matrices was underscored. Nonetheless, the prediction of molecular detectability using Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) and Orbitrap Secondary Ion Mass Spectrometry (OrbiSIMS) poses a significant challenge, particularly within intricate biological matrices such as cells and tissues. Furthermore, it is critical to note that in the prior

section (section 3.3.2.2), the experimental results indicated that seven substances remained unidentified. This finding has potentially substantial implications for the progression of drug development. The current section thus evaluates the physicochemical attributes of these compounds to ascertain whether any could be utilized to predict the ionisation efficiency of ToF-SIMS.

Various criteria could be employed to predict and identify compound behaviours in future research during the development of medicinal compounds, including the partition coefficient ($\log P$) (187-189), distribution coefficient ($\log D$) (190-191), and acidic dissociation (pK_a) (192). Luc's (2016) work suggests a potential relationship between the $\log P$ value of a molecule and the ionisation efficiency in SIMS analysis (193). The $\log P$ value represents the partition coefficient of the compound, which is proportional to the concentration of compounds in two distinct solvents and is primarily governed by the lipophilicity of the compound in the solvent (194).

Theoretically, a high $\log P$ value indicates that a substance is more lipophilic, which may affect its bioavailability (195-196). If the target of a compound is intracellular and reaches the target region, the drug molecule must be able to pass through the cell membrane to be effective. The cell membrane, predominantly composed of bilayer lipids including cholesterol and phosphocholine, may allow more molecules to pass through and reach the target if the substance is lipophilic (197).

Table 2 List of 15 anti-cancer agents with their logP values and molecular weight

Name of Drugs	MW	logP (ALOGPS)	logP (ChemAxon)
Capecitabine	358.3424	1.17	0.77
Dasatinib	488.0181	2.77	3.82
Daunorubicin	527.5210	1.68	1.73
Docetaxel	807.7717	2.59	2.92
Epirubicin	543.5201	1.41	0.92
Gefitinib	446.9021	4.02	3.75
Lapatinib	581.0581	5.18	4.64
Pictilisib	513.6218	2.24	2.46
Quizartinib	560.7102	5.13	5.16
Sorafenib	464.8251	4.12	4.34
Tamoxifen	371.5247	5.93	6.35
Tegafur	200.1692	-0.09	0.024
Temozolomide	194.1512	-1	-0.28
Venetoclax	868.4427	6.92	6.76
Vismodegib	421.2927	4.22	3.93

To explore whether log P values could be used to predict SIMS study outcomes, 15 compounds were analysed. As shown in Table 2, the molecular weight and log P values of each compound were obtained from the Drug Bank and specified by the programs ChemAxon and ALOGPS. ToF-SIMS analysis was performed, and the normalised ion intensity in relation to log P was displayed, identifying seven compounds in BCR-185R.

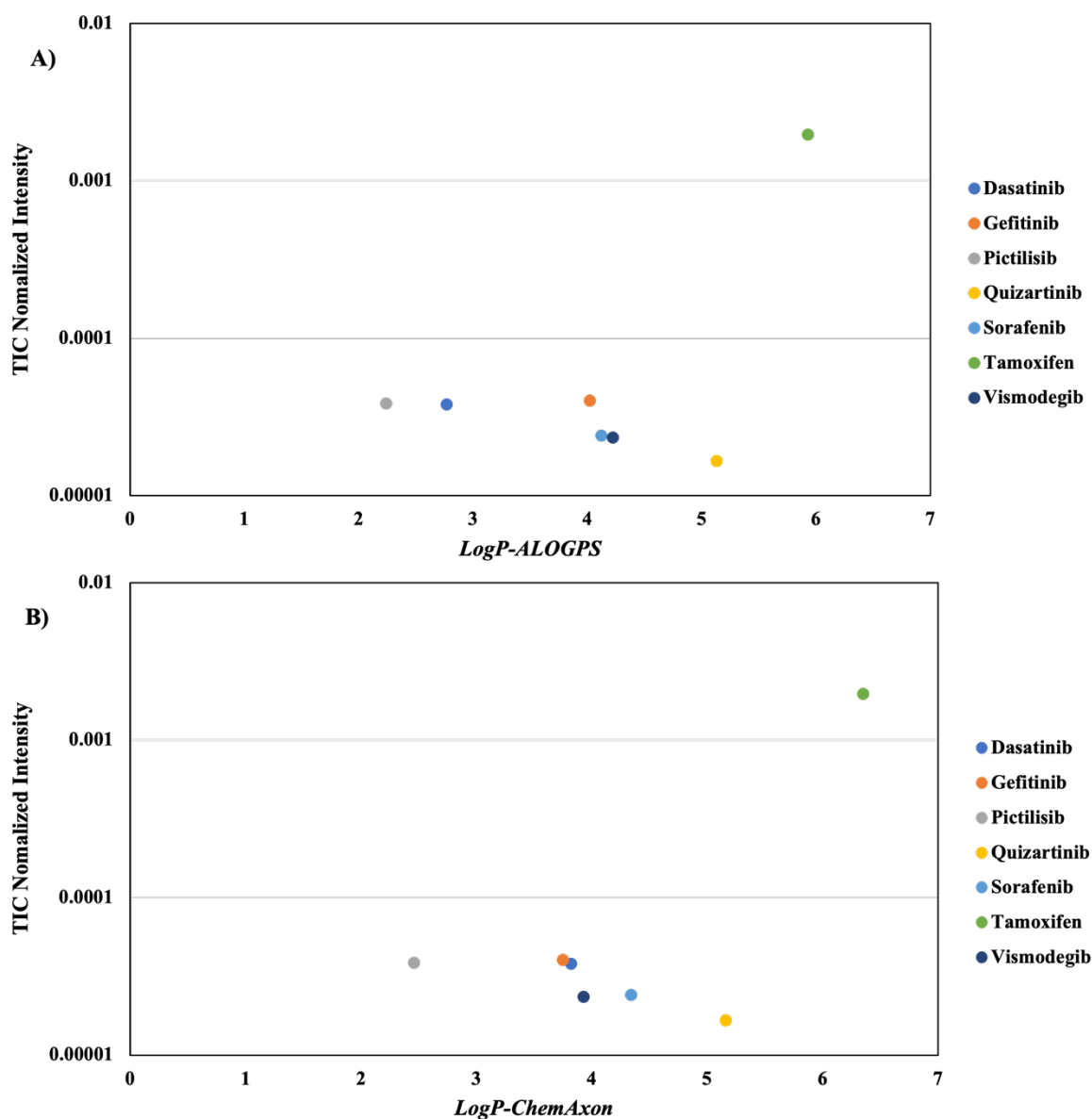


Figure 3. 7 The normalised ion intensity vs log P for positive secondary ions of drugs in the biological matrix BCR-185R by ToF-SIMS analysis: (a) logP retrieved from ChemAxon, (b) logP retrieved from ALOGPS. The ion intensity was normalised to total ion counts.

As shown in Figure 3.7, tamoxifen exhibited the highest intensity, whereas the remaining compounds showed low intensities. As demonstrated by the equation $Y_{\text{Intensity}} = 0.386\log P - 6.029$, with an R^2 of 0.3693, the relationship between log P and relative intensity was illogical (Figure 3.7), contradicting that of Luc's study, which indicates a positive correlation between ion intensity and log P values.

After plotting the ion intensity from TOF-SIMS analysis against the log P value, the relationship between log P and ion intensity from OrbiSIMS analysis was also investigated. Figure 3.8 clearly demonstrates that despite a considerable increase in ion intensity observed in the OrbiSIMS experiment, no correlation was found between log P values and ion yields for each identified molecule, with a similar pattern emerging when compared to the ToF-SIMS study.

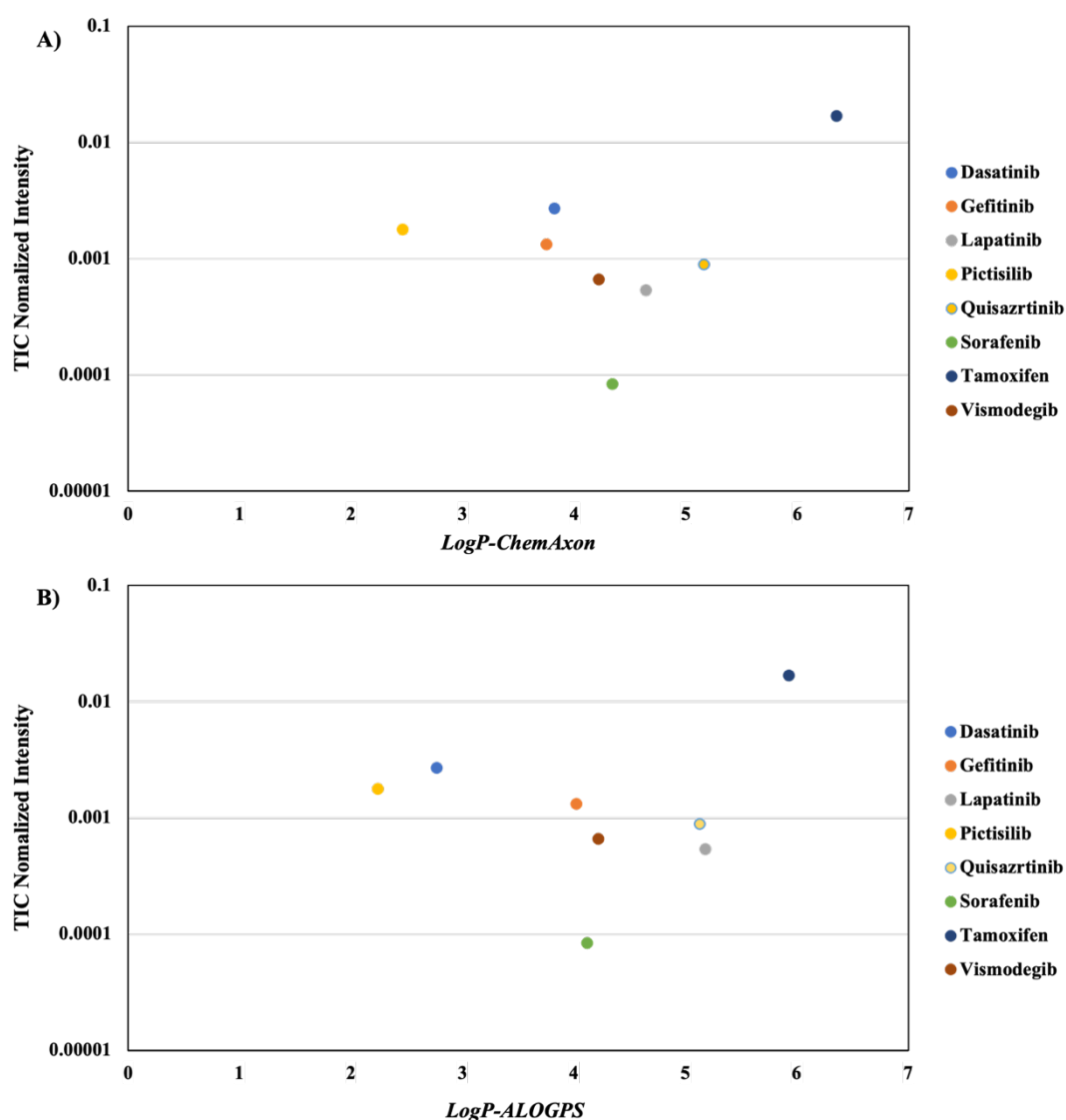


Figure 3. 8 The normalised ion intensity vs Log P for positive secondary ions of drugs in the biological matrix BCR-185R by OrbiSIMS analysis: (a) logP retrieved from ChemAxon, (b) logP retrieved from ALOGPS. The ion intensity was normalised to total ion counts.

In addition to examining the correlation between log P and ion intensity, the relationship between the log D value of a pharmaceutical compound and its intensity was further explored. This is because log D, as opposed to log P, describes the distribution of the compound in all possible forms at a given pH (190-191). Figure 3.9 displays the correlation between a constant log D and the compound OrbiSIMS signal in the tissue homogenate. This correlation presented the same result as the signals plotted against the log P-value, in which no significant association was identified.

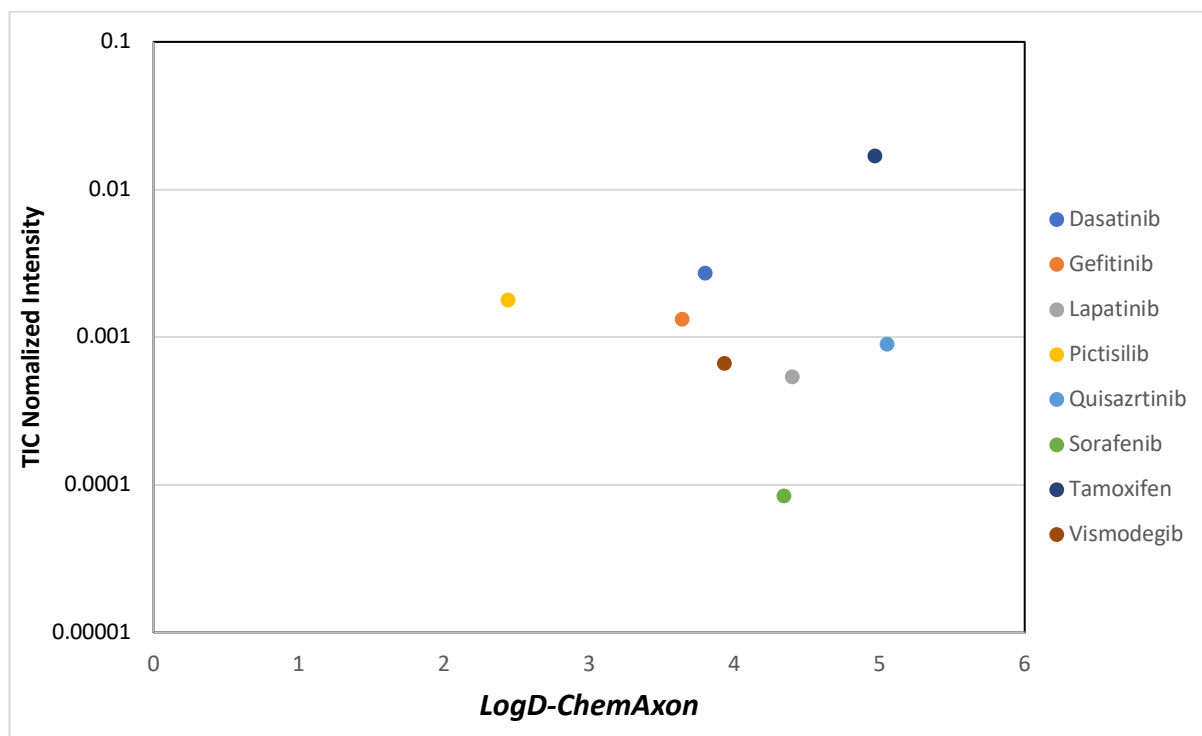


Figure 3. 9 The normalised ion intensity vs Log D (pH:7.4) for positive secondary ions of drugs in the biological matrix BCR-185R by OrbiSIMS analysis; log D retrieved from ChemAxon. The ion intensity was normalised to total ion counts.

Generally, the log D and log P partition coefficients are employed to quantify lipophilicity. The partition coefficient (log P) is defined as a measure of the solubility of a compound in two immiscible solvents (commonly octan-1-ol and water) and represents the lipophilicity of neutral substances (198). Most pharmaceutical substances are weak acids or bases, implying that at least one hydrogen atom can form a negatively or positively charged anion or cation. Log D accounts for both neutral and charged forms of the molecule, rendering it a more accurate predictor of the

properties of pharmaceutical substances. It is widely accepted that cellular pH is neutral (ranging between 7.0 and 7.4) (199), and numerous studies employing various methodologies have provided insights into this topic. Some studies have used complementary techniques, such as confocal and electron microscopy, to address this issue (200-201). Furthermore, several studies have shown that a pH imbalance can contribute to cancer progression (202-203). Certain organelles, including mitochondria with a pH of approximately 8 and secretory and endocytic organelles with pH values ranging from 4.7–6.7, exhibit distinct pH environments (199). As most pharmaceutical substances are weak acids or bases, log D may offer a more accurate correlation than log P when analysing compounds within cells using ToF-SIMS because of pH variation. In general, using log P and log D values could potentially serve as a suitable approach for predicting the ionisation of pharmaceutical compounds since log D is effectively value of log P with consideration of pH value. However, log P has typically been employed more frequently owing to the availability of numerous software packages that provide computation algorithms for this parameter, facilitating its calculation.

Ionisation is crucial for mass spectrometry, and numerous ionisation techniques are available. Electrospray ionisation (ESI) is arguably the most well-known and well understood method (204). The relative ionisation efficiency can be determined by comparing the ionisation of two compounds, one test and one standard, and subsequently ranking the compounds on a logarithmic scale using a mathematical model. This approach was tested on ten chemical compounds using an ion trap mass spectrometer in ESI (205-206), revealing that the absolute ionisation efficiency primarily depends on the formation of the sodium adduct. However, this mathematical method has only been applied to single-proton species (206). The experiment was expanded to include 62 chemically diverse compounds, resulting in a difference of six orders of magnitude. Researchers identified the pKa and molecular volume of the molecule as the two most influential factors, whereas the log P hydrophobicity measurement was not statistically significant. In contrast, experiments conducted in methanol and acetonitrile demonstrated a correlation between the ionisation efficiency and log P. These findings begin to illuminate the ionisation of small molecules, although the ToF-SIMS ionisation process may differ considerably (206). However,

the ionisation mechanism and effectiveness of ToF-SIMS remain poorly understood, particularly in biological matrices.

Vorng et al. conducted the most comprehensive study to date, analysing 20 pharmacological substances with varying physicochemical properties in a biological matrix. Notably, lyophilised bovine liver tissue served as a biological model, and the concentration of the drug used was 167 g/mL, which exceeded the therapeutic doses (193); 9 of the 20 drugs assessed displayed a protonated signal in positive ion mode, 4 exhibited a deprotonated signal in negative ion mode, and 7 showed no signal (193). Most drugs detected in positive ion mode contained a tertiary amine, and a linear relationship was observed between the ion production of the drug and the log P value. The ionisation efficiency of drugs in matrices considerably decreased and varies considerably between positive and negative modes when comparing the yield of pure compounds to that of compounds in a biological system (193).

The presence of the tissue homogenate likely influenced the ionisation efficacy of the drug, and the remaining unexplained ionic signal may have been suppressed by a complex process. To examine this phenomenon, 15 pure pharmaceuticals were placed separately on a clean glass substrate and analysed using ToF-SIMS, considering that ion signal suppression could result from interference from the biological matrix or the matrix effect. In Figure 3.10, the normalised ion intensity of the 15 compounds is plotted against their ChemAxon-calculated log P values.

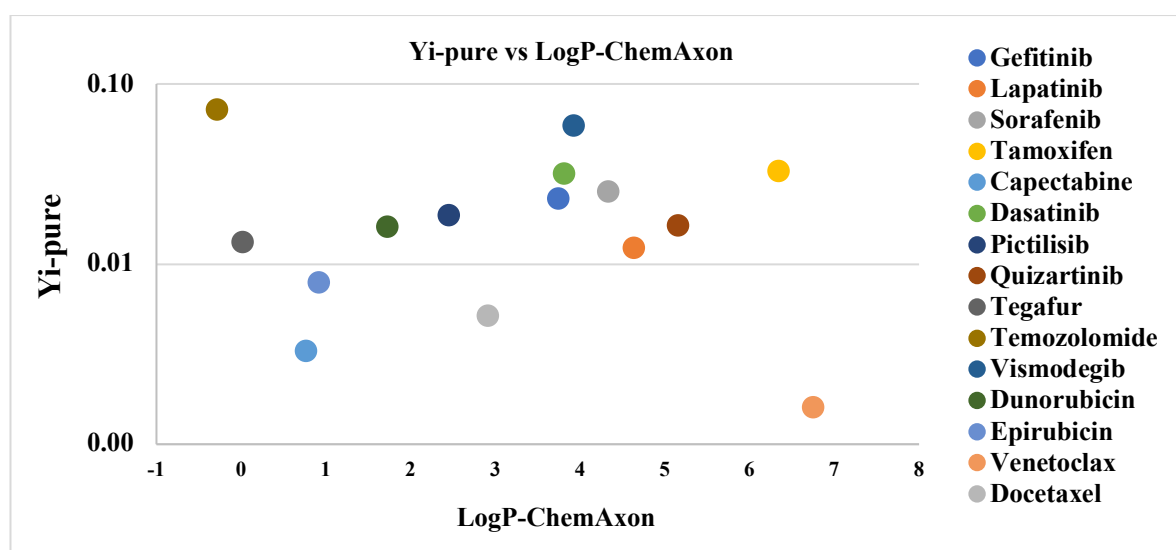


Figure 3. 10 The normalised ion intensity vs Log P (retrieved from ChemAxon) for positive secondary ions of pure drugs by ToF-SIMS analysis. The ion intensity was normalised to total ion counts.

As shown in the Figure 3.10, the results for the pure chemicals paralleled those of the tissue experiment, with no correlation between log P and ion intensity identified using SIMS analysis. Nonetheless, all 15 pure analyte molecule ions were detected, suggesting that a noteworthy matrix effect may account for the inability to identify the seven chemicals within the biological matrix using OrbiSIMS.

Moreover, it should be emphasised that the sample was repeatedly rinsed with an ammonium formate solution (pH 7) before being freeze-dried and analysed using SIMS during sample preparation. Ion intensity, a critical factor in SIMS analysis that depends on the ionising efficiency of the analysis target, is enhanced by washing the sample with an ammonium formate solution, which reduces salt interference. Various studies have shown that washing the sample with ammonium formate during sample preparation increases the ion intensity and reduces salt interference from biological materials (193, 207). Specifically, when using SIMS to perform cellular activities, employing a hypoosmotic solution may cause the cells to burst; therefore, it is essential to wash the cells with an iso-osmotic solution, such as ammonium formate. Consequently, the reduction in ionic strength could not have been caused by salt residues from the biological components. However, to investigate the ion suppression mechanism of the seven unidentified compounds, a large number of chemical compounds and further research are required.

In conclusion, understanding the ionisation mechanism and efficiency of ToF-SIMS, especially in biological matrices, remains a considerable challenge. While the presence of tissue homogenates may impact the ionisation efficacy of drugs, further research is needed to uncover the specific mechanisms and factors contributing to ion suppression. By examining various chemical compounds and refining experimental methods, researchers can develop a more comprehensive understanding of the factors influencing ion intensity in SIMS analysis. This knowledge can consequently improve

the accuracy and applicability of ToF-SIMS in various scientific and industrial applications.

3.3.3 Analytical capabilities of OrbiSIMS at the cellular level

3.3.3.1 Detection of 15 anti-cancer agents in MCF-7 cells

OrbiSIMS, with its larger cluster primary ion beam and Orbitrap analyser, can identify a greater number of endogenous metabolites and possesses a higher mass resolution. This technique can effectively detect small molecules functioning as anti-cancer drugs, in addition to identifying endogenous metabolites. However, it remains unclear whether OrbiSIMS can perform this task at the single-cell level, because tissues are composed of specialised cells with distinct functions. Consequently, OrbiSIMS analysis was conducted on the breast cancer cell line MCF-7, with a single dose (10 μ M) of 15 anti-cancer compounds, to evaluate its efficacy in detecting anti-cancer drugs at the cellular level.

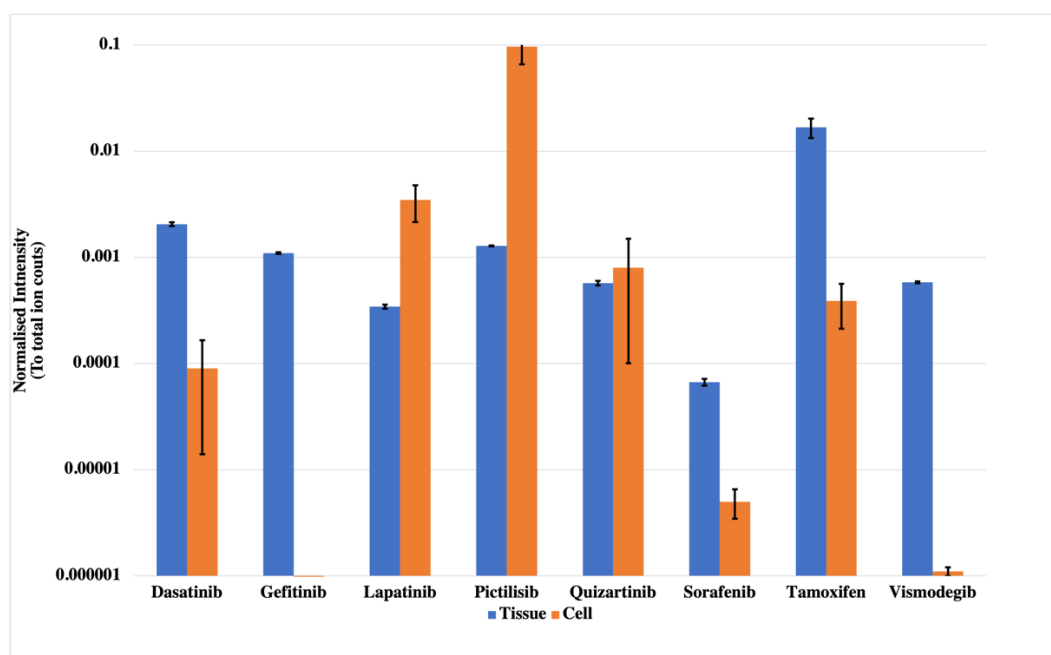


Figure 3. 11 Ion intensity of active compounds identified by OrbiSIMS in tissue and cell. The intensity of $[M+H]^+$ ion was counted and normalised to the total ion counts. The bar chart reflects normalized ion intensity (to total ion counts) values obtained from three separate experiments (mean $\pm$ s.d). Blots are representative of the data sets (N=3).

The intensity observed in the cell work, as shown in Figure 3.11, differs from that found in the tissue homogenate. While analysing the tissue homogenate, eight distinct

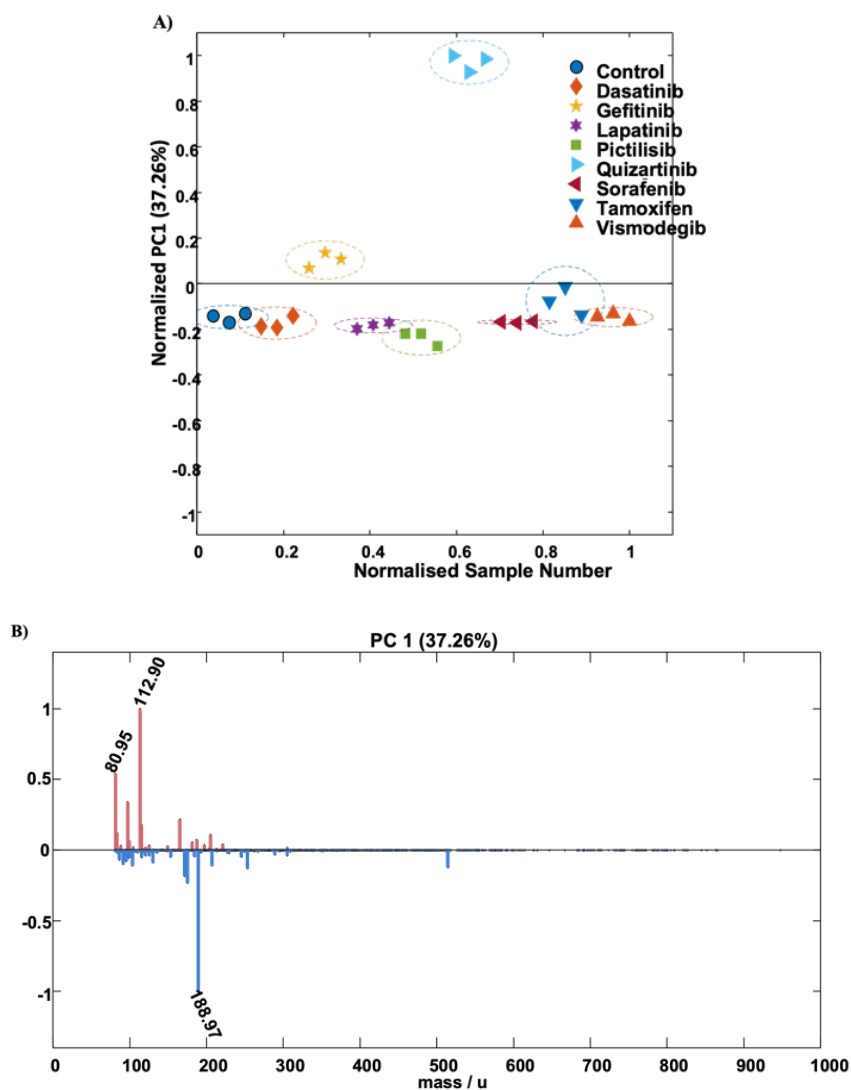
drug molecule ions were identified from the tissue biological matrix. Similar to the homogenate analysis conducted earlier, these eight drug molecules were identified in the cells. However, only six of these ions were clearly identifiable in the cellular matrix. Although gefitinib and vismodegib were challenging to discern in Figure 3.11, they were still identified based on their spectra. In contrast, tamoxifen displayed the highest potency in the doped tissue homogenate, whereas vismodegib had the highest intensity. Notably, most drug ion intensities decreased compared to that of the tissue biological matrix, with only pictilisib and lapatinib showing higher intensities. Therefore, despite the differences in drug molecule intensity between the cell and homogenate experiments, these eight compounds could still be identified using OrbiSIMS analysis.

Multiple reasons could contribute to the intensity differences between the homogenate and cell experiments. When the homogenate is spiked with a drug, no biological activities occur, as the BCR-185R biological matrix is deactivated before the addition of each anti-cancer agent. This suggested that drug molecules will remain after the homogenate has been treated with the drug. In contrast, living MCF-7 cells undergo treatment with individual drugs for 2 h, potentially exhibiting diverse reactions to various substances based on reaction mechanisms. For example, vismodegib is a PI3K inhibitor that prevents the synthesis of phosphatidylinositol (3,4,5)-trisphosphate by interacting with the ATP-binding pocket of PI3K, whereas tamoxifen acts as an oestrogen antagonist, blocking the effects of oestrogen on subsequent pathways (208-209). The results in Figure 3.11 could be attributed to drug molecules being taken into or out of the cell because of varied cellular responses.

3.3.3.2 Chemical differentiation of anti-cancer agents using multivariate data analysis (MVA)

In this study, a diverse panel of anti-cancer agents with varying mechanisms was employed to test the potential of OrbiSIMS for detecting capabilities within the biological matrix. The previous section demonstrated that OrbiSIMS can be used to detect eight anti-cancer agents within the biological matrix. However, this represents only one aspect of OrbiSIMS analysis, as it is unable to observe the endogenous response to drug treatment because each spectrum contains at least 600 individual

peaks, rendering the visual comparison of the differences difficult. Consequently, principal component analysis (PCA) was conducted to highlight any changes in ion intensity between sample types, referred to as untargeted analysis (162-164).



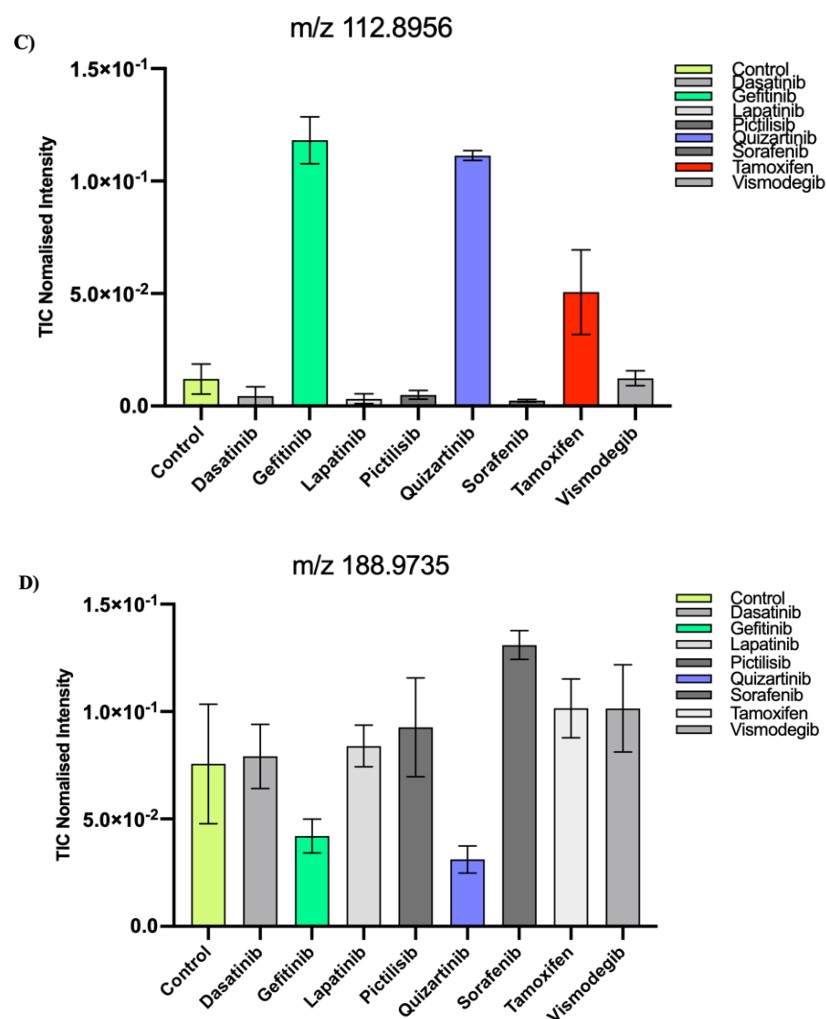


Figure 3. 12 Untargeted endogenous metabolite changes between identified drug treatment. A) Multivariate analysis of OrbiSIMS depth profile data under positive mode from the extracted normalized (to total ion counts) intensities based on treatment of eight identified drugs (10 μ M) in MCF-7 cells. B) Loading plots of OrbiSIMS depth profile data from the extracted normalized (to total ion counts) intensities based on treatment of eight identified drugs. C) Statistical analysis of metabolite m/z 112.8956 in MCF-7 cell after eight identified drugs for single treatment. D) Statistical analysis of metabolite m/z 188.9735 in MCF-7 cell after eight identified drugs for single treatment. Loading plots and Score plots are shown for each group and n=3 per treatment group.

PCA generates score plots that display the greatest variance between samples, whereas scatter loading plots highlight the variables that contribute to this variance. The data for the recognised group sample revealed notable patterns, as illustrated by

the score plot in Figure 3.12A. This figure presents the formation of two distinct groups: quizartinib as one group and the other seven drugs as another. This finding suggests that the endogenous metabolite modifications in these groups underwent changes. The corresponding loading plot revealed the ions associated with each sample, with variables located far from the plot origin identified as the most contributors to the variation. The first principal component suggested that the quizartinib treatment group should be considered distinct from the other groups. The loading plot indicated that the difference might be attributable to variations in the m/z 112.8956 ion, which could not be recognised. Figure 3.12B displays the results of the statistical analysis conducted on this ion for each treatment group, showing a discernible variation in the peak intensities. In addition to the quizartinib treatment, the ion at m/z 188.9735 could be attributed to the other seven treatment groups separated from the quizartinib group. Statistical analysis confirmed this in Figure 3.12C, with considerable changes in the intensity of the presence of this ion in each of the seven treatment groups, including the control group.

3.3.3.2.1 Elucidating endogenous molecules using secondary ion mass spectrometry with mass filtered prediction (SIMS-MFP)

To assign structural properties to the numerous detected ions and to delineate the intricate chemical composition of the cell, the recently established SIMS-MFP method (210) was employed to investigate the significant variables identified from PCA analysis (Figure 3.12 C; D). This approach hinges on the precise mass determination of an elemental formula and anticipates the molecular formula of the species within a 3D OrbiSIMS dataset and categorises them based on their double-bond equivalencies. This, consequently, facilitates the targeted investigation of various compound classes according to the presence of specific functional groups. Owing to the extensive array of species present, our study concentrated on the data acquired in the positive ion mode.

Table 3 Ions elucidated by SIMS-MFP

Chemical formula	DBE	Assignment	Structure
K ₂ Cl ⁺	0.5		

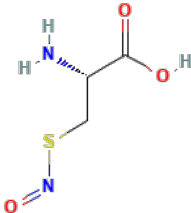
(m/z		
112.8956)		
$C_3H_6N_2O_3SK^+$		
(m/z	2	
118.9735)		
		S-Nitroso cysteine ($C_3H_6N_2O_3S$)
		

Table 3 illustrates that through SIMS-MFP and cross-referencing with prior literature, the chemical formula of m/z 112.8956 can potentially be assigned to K_2Cl^+ that has been reported by previous research as well (211). This finding suggested that the administration of quizartinib, gefitinib, and tamoxifen may potentially influence endogenous potassium activity, as the m/z 112.8956 ion emerged as an important variable in the treatment groups involving these three drugs. Several studies have reported that quizartinib may act as a potassium channel blocker, subsequently leading to impaired potassium activity (212-213). Moreover, patients treated with gefitinib demonstrated an approximate 40% probability of developing hypokalaemia syndrome, while other medications yielded a considerably lower likelihood of 1–5% (214-215). Additionally, tamoxifen administration may inhibit myocardial K^+ currents through an unidentified mechanism (216).

The probable chemical structures of the other two ions are presented in the Table 3. The 188.9735 ion is likely S-nitroso cysteine ($C_3H_6N_2O_3SK^+$). For the 174.9723 ion, two potential formulae have been proposed: $C_4H_4N_2O_2PS^+$ and $C_6H_4N_2SNa^+$. These molecules play a crucial role in the development of cancer, and the capacity to detect and identify these ions at the cellular level will be advantageous for cancer researchers.

3.3.3.3 *In vitro* localisation of tamoxifen in breast cancer cells

The state-of-the-art OrbiSIMS instrument, which combines ToF and Orbitrap analysers, possesses the ability to visualise the distribution of anti-cancer agents within cells, in addition to its powerful profile analysis capabilities (133). The imaging capabilities of this method outperformed those of the commonly used chromatographic methods, thus rendering easy separation of sample sections related to biological sections and the glass substrate possible and demonstrating the differences in the spatial distribution of many chemical components within the biological matrix (133). Although previous studies have demonstrated imaging capabilities by revealing the distribution of amiodarone within macrophages (133), there is insufficient research on the imaging of anti-cancer agents under therapeutic conditions. Consequently, cells were treated with tamoxifen before freeze-drying and studied to simultaneously enhance the mapping capacity of label-free drugs in MCF-7 cells. This approach aimed to facilitate a more accurate interpretation of the results. Using both LMIG and ToF analysers enabled the creation of a 2D ion image, whereas a chemical ion image was used to map a region up to $250 \times 250 \mu\text{m}$ in size to determine the location of distinctive ions.

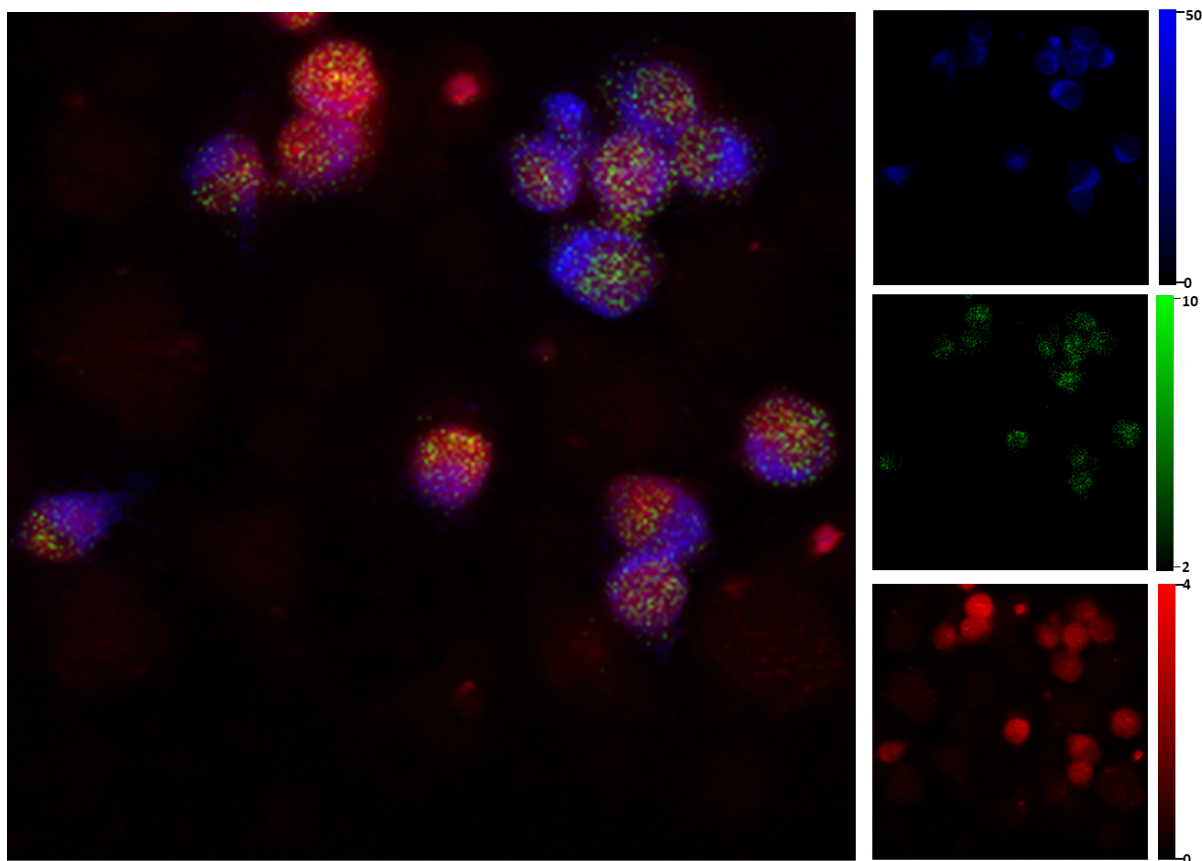


Figure 3. 13 The RGB overlayed ion images of tamoxifen incubated with MCF-7 cells. Nuclear was marked as red, phosphocholine fragment was marked as blue and drug molecules were marked as tamoxifen (green)

As illustrated in Figure 3.13, tamoxifen (green) coincided with MCF-7 cells (red and blue). Notably, the distribution of drug ions could be used to investigate drug delivery efficacy at the cellular level during novel drug development. Although a majority of the drugs were found near the nucleus, trace levels of residual ions (Figure 3.13C) were observed near the membrane. This suggested that most of the targeting sites of tamoxifen, akin to those of an oestradiol antagonist, are situated in the nucleus, with only a few in close proximity to the membrane (209).

Overall, the results of this section demonstrate that the targeted compound can be visualised at a resolution suitable for cellular level of biological matrices. To enhance the efficacy of this work, the recently demonstrated ability of OrbiSIMS to visualise biofilms under cryogenic conditions could be expanded (217). Consequently, endogenous metabolites would maintain their natural state under cryogenic conditions, facilitating the evaluation of biological responses to drug treatment. A more comprehensive understanding of drug transport and delivery efficiency at the cellular level would considerably improve the impact of future drug development efforts.

3.3.4 Frozen hydrated sample preparation vs freeze dried sample preparation

Finally, this study investigated the impact of cryogenic environments on the ionic strength of medications where tamoxifen, lapatinib and sorafenib has been selected as example. Cryogenically preserved samples offer a material dispersion that more closely mirrors natural conditions compared to freeze-dried samples (218-220). Freeze-drying, a prevalent sample preparation technique, entails rapid freezing followed by vacuuming to eliminate residual water. However, this method can lead to cell disintegration, and molecular rearrangements within cells and tissues may transpire (221-224). As a result, this section delves into the effects of sample preparation on ion's intensity. The findings could provide insights into the most suitable sample preparation method for subsequent phases of this project.

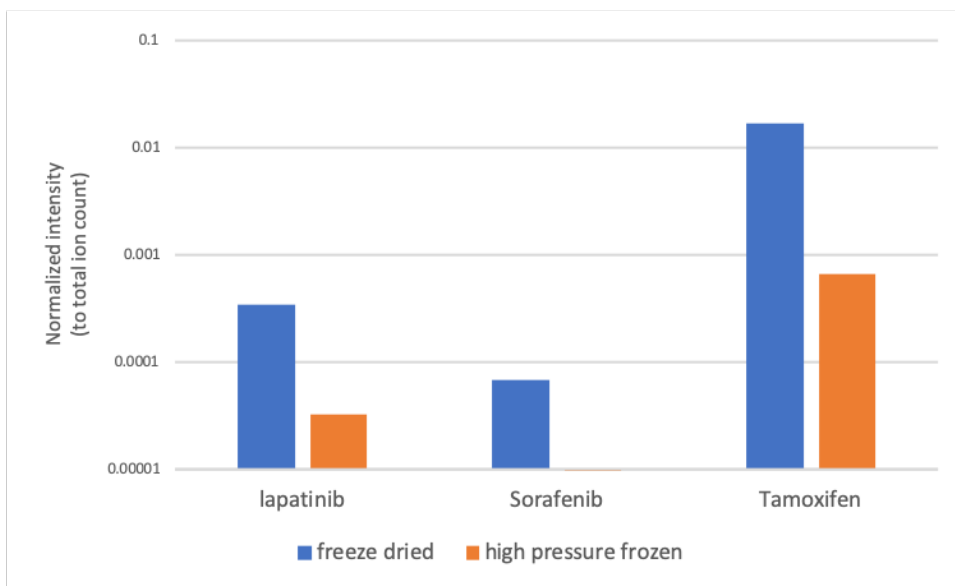


Figure 3. 14 Comparison of active compounds intensity between the freeze dried and high-pressure frozen sample in the biological matrix BCR-185R by OrbiSIMS analysis. The ion intensity was normalised to total ion counts.

To ensure comparability with previous experiments, each drug was individually administered into the tissue homogenate to achieve a final concentration of 167 $\mu\text{g/mL}$, and total ion counts were used to standardise ion intensity. Figure 3.14 demonstrates that although the three chemicals could be detected in the cryogenic state, their relative intensities were markedly suppressed. For example, the relative strength of tamoxifen ions was considerably lower in cryogenic conditions than in freeze-dried samples. Cryo-SIMS analysis identified only drug molecules and a significant number of solvent molecules. The presence of a solvent may be responsible for the reduced ionic concentration.

Initially, pure chemicals were prepared and dissolved in DMSO. An appropriate amount of the chemical was subsequently added to the tissue homogenate solution to achieve the desired drug concentration. Unlike freeze-drying, which allows the solvent to evaporate, frozen-hydrate sample analysis retains the solvent throughout the process. Given that SIMS is a sensitive technique, the presence of a solvent can result in matrix effects and inaccurate measurement of drug ionic strength. The treatment and preparation method used in this study could cause the reference tissue

homogenate BCR-185R to lose its original biological properties, such as drug carrier and biological activities.

In experiments involving living cells, the DMSO concentration is typically maintained below 0.1% to avoid interference with cellular activities or induction of cell death. When the DMSO concentration is $<0.1\%$, its interference with SIMS analysis may be negligible (225). Further investigation is required to examine the potential interference of the DMSO signal with cellular functions and to explore this issue in greater depth.

3.4 Conclusion

In the present chapter, we examined the initial application of SIMS, specifically the investigation of analysing biomolecules and pharmaceutical compounds within biological matrices using ToF-SIMS and OrbiSIMS. The objective was to assess the relationship between the signal intensity and the chemical and physical properties of the drug molecules. Furthermore, the efficacy of OrbiSIMS for obtaining information about biomolecules was evaluated.

This chapter commenced with an assessment of the effectiveness of OrbiSIMS in gathering biomolecular data, followed by an examination of the correlation between compound intensity and their physical and chemical properties. Crucial information encompasses the analysis of chemical ionisation in both biological matrices and neat samples. Therefore, the ability to precisely predict the responsiveness of a molecule to ionisation using SIMS could be valuable. Consequently, although a clear correlation was not identified between feasibly tested matrices and compound signals in biological matrices, it can be concluded that neat compound testing is the preferred approach for evaluating compound ionisation. This is because neat compound testing did not impede any of the studied compounds from advancing to cell assays. Conversely, if the compounds were tested solely in various biological matrices such as liver cells or tissues, they would have been excluded. Moreover, this technique does not require tissues, cells, or liver-derived products, enabling faster completion and reduced cost.

No association was found between a physicochemical attribute and SIMS ionisation. This lack of correlation was observed for the logP value and when comparing logD against compound intensity. This finding contradicts those of existing research, as logD is essentially logP adjusted for cellular pH neutrality.

Owing to the specificity and complexity of SIMS analysis data, we employed multivariate analysis to interpret the SIMS data matrix, as other methods cannot represent the data effectively. PCA, a widely used multivariate technique, reduces the dimensions of the data matrix and estimates variance to elucidate patterns within the data matrix. By applying PCA, several unassigned ion changes were successfully identified in the cell response data matrix. Consequently, PCA was employed in the subsequent chapters of this study.

The application of Principal Component Analysis (PCA) has proven instrumental in discerning the variations between individual treatment data obtained via OrbiSIMS analysis. To further elucidate the peaks identified from PCA loading plots, an additional layer of analysis—SIMS-MFP—was implemented. This approach successfully identified endogenous peaks, notably those associated with K_2Cl^+ and S-nitroso cysteine. Given their pivotal role in cancer development, the ability to detect and identify these ions at the cellular level may present considerable benefits for researchers in the field of oncology.

In addition to conducting biomolecular profiling, the unique imaging capabilities of instrumentation have enabled us to successfully map the distribution of targeted drug molecules at the subcellular level. Utilizing the robust imaging capabilities of OrbiSIMS facilitates a more comprehensive understanding of drug transport and delivery efficiency at the cellular level, which could significantly enhance the impact of future drug development endeavours.

Finally, we investigated the alteration of the ionic strength of a drug under cryogenic conditions. The distribution of the drug in a hydrated frozen sample is as close as possible to that in the native condition, compared to a freeze-dried sample. Although endogenous metabolite concentrations may increase under cryo-conditions relative to freeze-dried samples, the drug concentration experiences variable levels of

suppression, potentially due to the presence of solvent. Therefore, freeze-drying was the preferred method for sample preparation in the following chapters, given that the primary focus of this research was on the pharmaceutical compounds themselves.

Chapter 4. *In vitro* investigation of the effects of anti-cancer agents on endogenous metabolites by OrbiSIMS

4.1 Introduction

The investigation of small molecules found within biological systems, i.e., endogenous metabolites, as well as their interactions and responses to external and internal factors is a burgeoning field that employs sophisticated analytical techniques, such as mass spectrometry and nuclear magnetic resonance spectroscopy (73-77). These techniques are used to identify and quantify these molecules and their associated pathways with the goal of enhancing our understanding of the fundamental biochemical processes that regulate the physiology and pathology of living organisms (78-80).

Small molecules can act as biochemical signatures to monitor the physiological effects of drugs by measuring their concentration and identity. Increased energy metabolism is reflected by high levels of phosphate compounds and elevated enzyme activity (226-230). Analysing metabolites in cells through a cell-based approach is a powerful tool used to investigate cancer diagnosis and treatment (231-233). This allows for a thorough examination of the metabolites present in cells, thereby revealing insights into the underlying biochemical mechanisms involved in cancer progression and identifying possible therapeutic targets (234-240)

Recent studies have demonstrated that perturbations in endogenous metabolites are linked to numerous diseases, including cancer (241-244). Therefore, analysing the impact of anti-cancer agents on endogenous metabolites has the potential to provide valuable insights into their mechanisms of action and potential therapeutic applications.

4.2 Chapter aims

The purpose of this chapter was to investigate the potential application of the methods developed in Chapter 3 for identifying the effects of selected anti-cancer agents on the small molecule response of cells. We selected two drugs for this study: tamoxifen and lapatinib. Tamoxifen was chosen due to its high intensity observed within the tissue homogenate in both the ToF-SIMS and OrbiSIMS systems. In contrast, lapatinib was

only detected using OrbiSIMS and not with the ToF-SIMS system. In this chapter, we examined the response of MCF-7 cells to various concentrations and exposure times of the selected anti-cancer agents, as these two factors are crucial to the treatment conditions.

OrbiSIMS was used to investigate the cellular response to anti-cancer agent treatment. In Chapter 3, this technique was shown to be capable of identifying intact endogenous metabolites and assessing changes in metabolite levels resulting from anti-cancer agent treatment.

To fully explore the effects of the selected anti-cancer agents, the targeted data analysis approaches were employed. The targeted approach specifically investigated the energy metabolism response to the selected anti-cancer agents, as cancer cells exhibit distinct energy metabolism patterns compared to normal cells. The data generated from this study will contribute to our understanding of the impact of selected anti-cancer agents on cellular metabolism, while providing valuable insights into the metabolism of these drugs and their therapeutic efficacy. The results of this investigation may facilitate the development of novel strategies for cancer treatment by elucidating the molecular mechanisms underlying the metabolic effects of anti-cancer drugs.

4.3 Results and discussion

Owing to the advanced capabilities of large cluster ion beam (GCIB) technology including high mass resolution and improved production of intact molecules, there is considerable interest in investigating the minimum detectable concentration and time range. Such enquiry would enable the monitoring of biological responses and endogenous metabolite changes resulting from variable concentrations of compounds of interest. Notably, the preparation method for OrbiSIMS analysis is simpler and faster than conventional analytical methods such as MALDI or LC/MS, as previously reported (245).

4.3.1 Determining half maximal inhibitory concentration values (IC_{50}) of selected anti-cancer agents

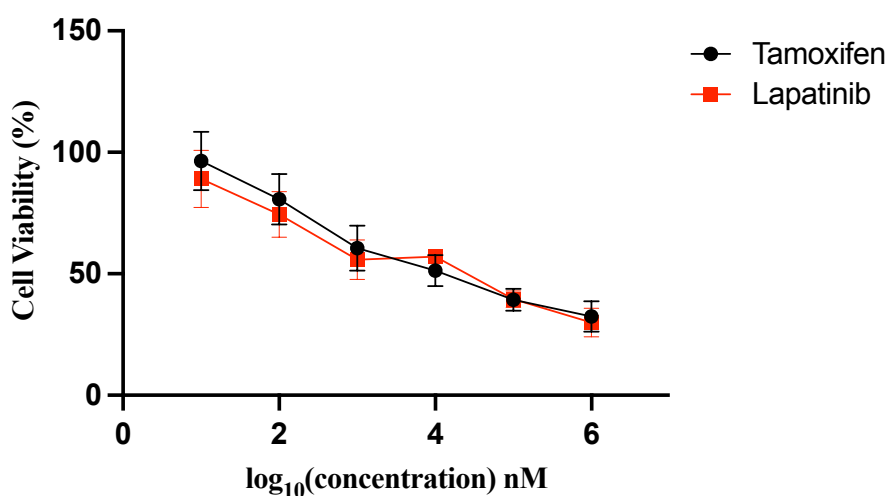


Figure 4. 1 Cytotoxicity of tamoxifen and lapatinib in MCF-7 breast cancer cell line. Cells were treated with increasing concentrations of tamoxifen (black) and lapatinib (red). The cell viability is determined by the MTT assay. The point reflects the cell viability values obtained from three separate experiments (mean $\pm$ s.d). Blots are representative of the data sets (N=3).

To assess cytotoxic effects and calculate the effective dosage of tamoxifen and lapatinib on MCF-7 cells, cells were individually subjected to various concentrations of tamoxifen and lapatinib for 48 h. As anticipated (Figure 4.1), MCF-7 cells exhibited a response to the antiproliferative action of tamoxifen, with an estimated IC_{50} of 10 μ M (indicated by the black trend line). Moreover, cell viability of the MCF-7 cell line remained affected by progressively increasing concentrations of lapatinib, as evidenced by the ability to predict the IC_{50} that was calculated at approximately 15 μ M (represented by the red trend line). This result indicated that MCF-7 cells exhibited a high degree of response to tamoxifen and lapatinib treatment. Tamoxifen and lapatinib treatment demonstrated a significant antiproliferative effect on MCF-7 cells with IC_{50} values of 10 μ M for tamoxifen and 15 μ M for lapatinib.

4.3.2 Evaluating OrbiSIMS in drug analysis under varied conditions

As a sophisticated and potent analytical instrument, OrbiSIMS can examine both drug molecules and endogenous metabolites owing to its enhanced sensitivity and mass resolution (133;183-184;210). Nevertheless, whether this technique can effectively evaluate drug levels and endogenous responses under varying treatment conditions, timeframes, and concentrations remains unclear, as these factors are fundamental determinants in drug treatment. Consequently, the initial investigation sought to determine whether OrbiSIMS is capable of monitoring drug molecules under these circumstances.

4.3.2.1 Dose-Dependent Drug Response

4.3.2.1.1 Tamoxifen dose response curve

Tamoxifen was first selected owing to its pronounced intensity in tissue homogenate analyses, as demonstrated in both ToF-SIMS and OrbiSIMS systems. Figure 4.2 illustrates the ion intensity changes of tamoxifen as the concentration was gradually altered. The highest concentration tested in this study was 10 μM , which corresponds to the IC_{50} of tamoxifen. The concentration progressively decreased to a minimum of 0.005 μM , representing a 2000-fold dilution relative to the IC_{50} value. As shown in Figure 4.2, the protonated tamoxifen ion exhibited a gradual increase in intensity, peaking at 10 μM . The increase followed an approximately linear relationship with incubation concentration and revealed considerable variation in uptake behaviour at the single-cell level.

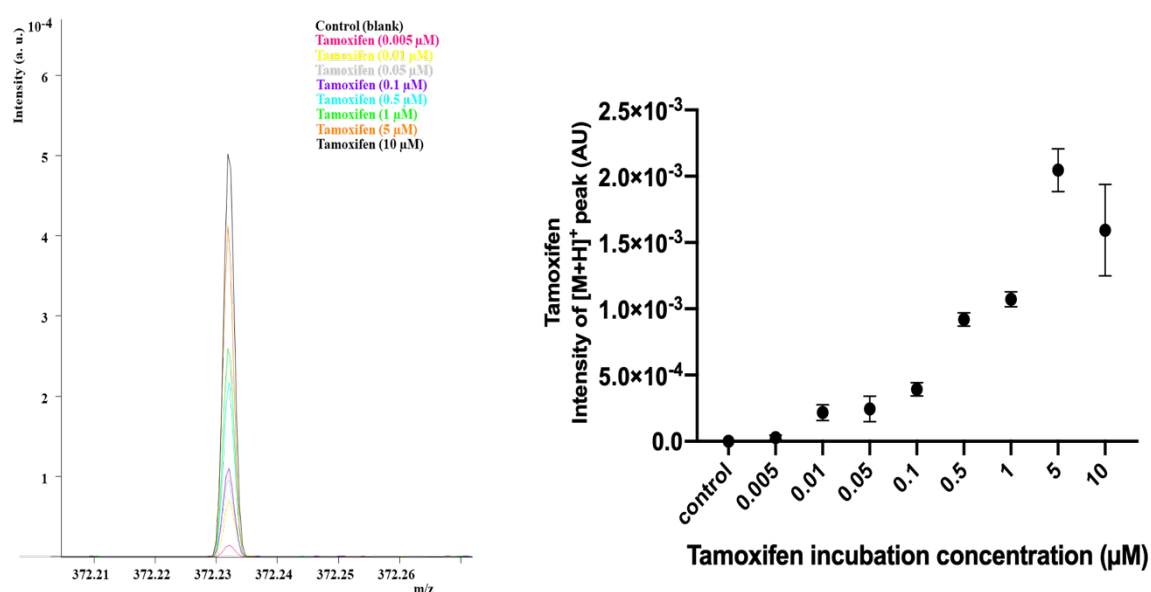


Figure 4. 2 OrbiSIMS data highlight the differences in example spectra showing peak intensities for a single data set and exported peak intensities for 9 treatment groups, control; 0.005 μM ; 0.01 μM ; 0.05 μM ; 0.1 μM ; 0.5 μM ; 1 μM ; 5 μM and 10 μM (a) spectra showing peak intensities of protonated tamoxifen molecule ion $[\text{M}+\text{H}]^+$, (b) exported average peak intensities for each group.

Furthermore, the improved mass-resolved power resulted in a sharper shape and narrower peak width in the spectrum of protonated tamoxifen ions, thus enabling the accurate chemical characterisation of tamoxifen. Notably, even at a concentration of 0.005 μM , which is 2000 times diluted relative to the IC_{50} value, the peak of tamoxifen was detected with excellent mass resolution and a narrow peak width. Additionally, the total ion count observed at 0.005 μM was notably higher, which is critical for the detection of native compounds that may be present at lower concentrations.

In summary, the data presented in Figure 4.2 demonstrate the concentration-dependent changes in tamoxifen ion intensity and highlight the enhanced resolution and sensitivity offered by the analytical method. These findings have considerable implications for the detection and characterisation of compounds at low concentrations and contribute to our understanding of the pharmacokinetics and pharmacodynamics of tamoxifen.

Notably, the ion intensity of tamoxifen exhibited a marginal decrease at 10 μM relative to that at 5 μM , which may be attributed to the matrix effect previously discussed in Chapter 3. SIMS analysis is an exceptionally sensitive technique, and the presence of other compounds can potentially interfere with the detection and output of tamoxifen. The matrix effect, which arises from the influence of the sample matrix on the ionisation efficiency, is a well-documented phenomenon in mass spectrometry (147;149;166;205).

The observed decrease in ion intensity highlights the importance of careful consideration and control of the experimental conditions in SIMS analysis. By controlling for the matrix effect and other potential sources of interference, more

accurate and reliable measurements of the ion intensity of tamoxifen can be obtained. These findings have considerable implications for the interpretation and application of SIMS in pharmacological and clinical research.

4.3.2.1.2 Lapatinib dose response curve

Based on the results of the previous experiments outlined in Chapter 3, lapatinib was selected as a candidate compound for further investigation alongside tamoxifen. Notably, tamoxifen exhibited the highest ion intensity in both the ToF and OrbiSIMS systems, whereas lapatinib was detected solely in the OrbiSIMS system. Lapatinib, a type of inhibitor, inhibits human epidermal growth factor receptor type 2 (HER2/ERBB2) and serves as a tyrosine kinase inhibitor of epidermal growth factors (HER1/EGFR/ERBB1) (246).

Upon binding to the intracellular domain of phosphorylation, lapatinib prevents receptor phosphorylation upon ligand binding. As depicted in Figure 4.3, the intensity of the lapatinib molecule ions varied with different treatment concentrations. These changes in ion intensity offer insights into the pharmacological activity of lapatinib, which may be harnessed for therapeutic purposes. These findings highlight the importance of using both ToF and OrbiSIMS systems in tandem for comprehensive analyses of drug compounds.

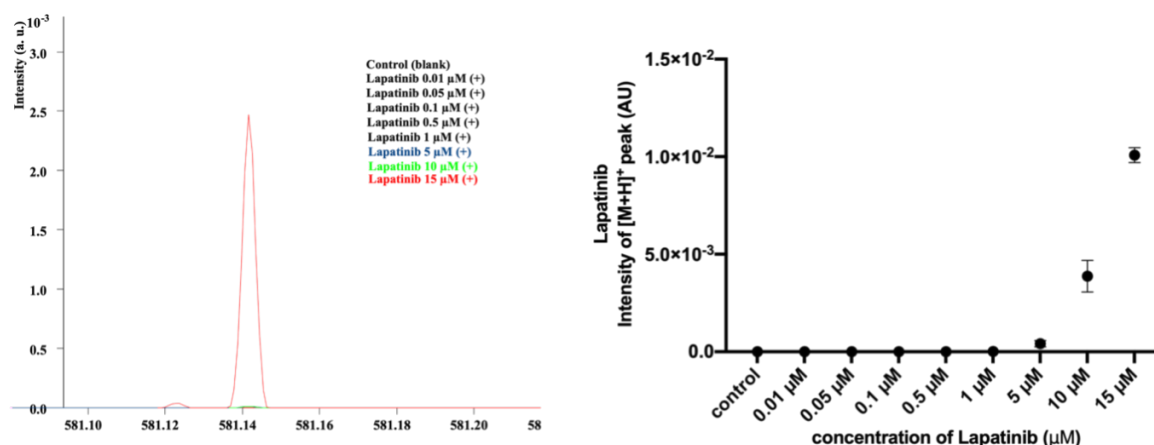


Figure 4. 3 OrbiSIMS data highlight the differences in example spectra showing peak intensities for a single data set and exported peak intensities for 9 treatment groups, control; 0.01 μM; 0.05 μM; 0.1 μM; 0.5 μM; 1 μM; 5 μM; 10μM and 15 μM (a) spectra showing peak intensities of protonated tamoxifen molecule ion [M]⁺, (b) exported average peak intensities for each group.

The concentration of lapatinib gradually decreased from the highest concentration, which was set at an IC_{50} of 15 μM in this study, to a minimum concentration of 0.01 μM . Notably, unlike tamoxifen, which could be easily identified at minimum dosage, no protonated lapatinib ions were identified at lower dosages ranging from 0.01–5 μM . However, at a concentration of 5 μM , the intensity of lapatinib began to increase and peaked at the IC_{50} concentration of 15 μM . These data suggest that tamoxifen displays higher permeability into cells than does lapatinib, possibly owing to several reasons, including the higher logP value of tamoxifen compared to that of lapatinib (6.35 and 4.64, respectively). This difference in logP values could aid the diffusion of tamoxifen across the lipophilic cell membrane and facilitate intracellular targeting.

4.3.2.1.3 Unravelling the impact of drug molecules on energy metabolism of cancer MCF-7 cells

After investigating the detection range of anti-cancer agents, the endogenous responses of cancer cells were examined, aiming to fully elucidate the potential of OrbiSIMS in biomedical research. We focused on analysing the impact of anti-cancer treatment on energy metabolism, as cancer cells exhibit distinct energy metabolism patterns in comparison to normal cells.

4.3.2.1.3.1 Impact of tamoxifen on glutaminolysis and tricarboxylic acid (TCA) cycle metabolism in cancer cells

OrbiSIMS can identify endogenous metabolites and explore their energy metabolism in response to changes in treatment conditions, as illustrated in Figure 4.4.

Deprotonated glutamine ions were identified in all treatment groups, and while the observations did not exhibit a linear trend, they revealed that the tamoxifen treatment dose affected the expression of glutamine, compared to the control group.

associated with the inhibition of cellular glutamine use, resulting in oxidative stress and induction of apoptosis (251-253). Advanced OrbiSIMS analysis confirmed that tamoxifen treatment leads to an inhibitory effect on glutamine intensity.

Furthermore, we examined the impact of tamoxifen treatment on TCA cycle intermediates in cancer cells using OrbiSIMS analysis. Our results revealed substantial inhibition of TCA cycle intermediates throughout tamoxifen treatment. Figure 4.5 displays noticeable alterations in the concentrations of key TCA cycle intermediates, including citrate, succinate, fumarate, and malate. A considerable decline in the levels of these intermediates was observed upon exposure to tamoxifen compared with that in the control group. This finding indicated that tamoxifen treatment interferes with the regular functioning of the TCA cycle in cancer cells, subsequently causing a reduction in energy production.

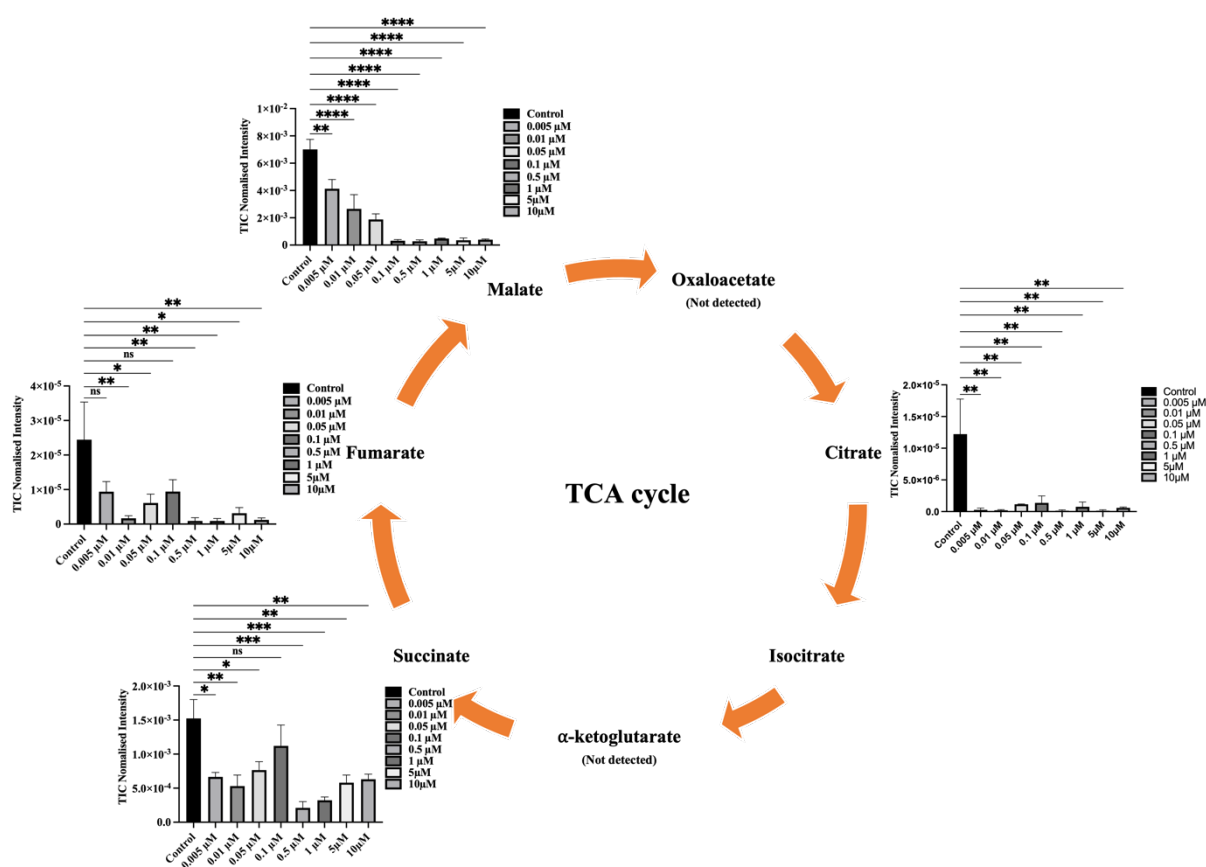


Figure 4. 5 TCA cycle intermediates changes for MCF-7 incubated with tamoxifen by different dose. Dose response curve for cells from 9 treatment groups, control; 0.005 μM; 0.01 μM; 0.05 μM; 0.1 μM; 0.5 μM; 1 μM; 5 μM; 10 μM. All data acquired by GCIB Ar3000+. The bar chart reflects normalized ion intensity (to total ion counts) values obtained from three separate experiments (mean ± s.d). Blots are representative

of the data sets (N=3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant

The inhibition of TCA cycle intermediates can be attributed to the known effects of tamoxifen on glutamine utilisation (251-253). As glutamine is a crucial nutrient for cell growth and proliferation, its inhibition by tamoxifen may lead to a reduction in the availability of key substrates required for the TCA cycle, such as citrate. Consequently, this could result in diminished energy metabolism, oxidative stress, and induction of apoptosis in cancer cells.

The detected disruption of the TCA cycle aligns with prior reports suggesting that tamoxifen can trigger metabolic changes in cancer cells, thereby contributing to its anti-cancer properties (253-255). Our results further substantiate the capability of OrbiSIMS as an effective tool for investigating metabolic alterations induced by anti-cancer treatments, offering valuable insights into their mechanisms of action and potential pathways for refining therapeutic approaches.

4.3.2.1.3.2 Revealing the impact of lapatinib on glutamine and TCA cycle metabolism

In contrast to tamoxifen, which is an SERM, lapatinib is a type of tyrosine kinase inhibitor that targets both the epidermal growth factor receptor (EGFR)/HER2 and is typically used in the treatment of HER2-positive breast cancer (246). The effects of lapatinib on cellular energy metabolism primarily focused on its ability to inhibit glucose consumption, potentially leading to glucose deprivation. However, it is unclear whether lapatinib affects glutaminolysis.

Figure 4.6 illustrates that lapatinib treatment can induce effects similar to those observed with tamoxifen treatment in MCF-7 cells. While no linear trend was observed, the intensity of deprotonated glutamine decreased compared to that in the control MCF-7 group, suggesting that lapatinib treatment can affect glutamine uptake and metabolism.

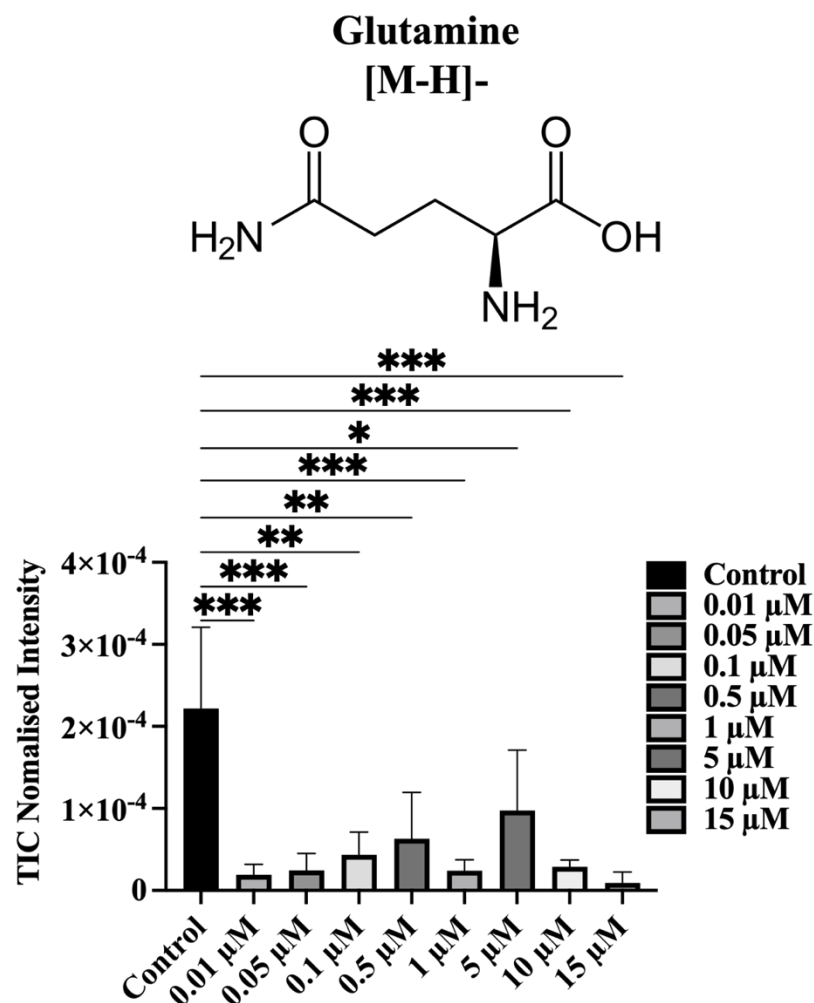


Figure 4. 6 Glutamine changes for MCF-7 incubated with lapatinib by different dose. Dose response curve for cells from 9 treatment groups, control; 0.005 μ M; 0.01 μ M; 0.05 μ M; 0.1 μ M; 0.5 μ M; 1 μ M; 5 μ M; 10 μ M. All data acquired by GCIB Ar3000+. The bar chart reflects normalized ion intensity (to total ion counts) values obtained from three separate experiments (mean $\pm$ s.d). Blots are representative of the data sets (N=3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant

Previous studies have explored the role of glutamine metabolism in lapatinib resistance in breast cancer cells, highlighting the potential impact of lapatinib-induced stress on glutamine metabolism (256-257). While the specific metabolic pathways affected by lapatinib were not examined in this study (258), OrbiSIMS analysis provides a broad view of how lapatinib treatment can impact cancer cells at the cellular level.

In addition, we used OrbiSIMS analysis to investigate the impact of lapatinib treatment on TCA cycle intermediates in cancer cells (Figure 4.7). Similar to tamoxifen treatment, lapatinib treatment induced changes in the concentrations of key intermediates, including citrate, succinate, fumarate, and malate, compared to the control group. This suggests that lapatinib treatment may interfere with the normal functioning of the TCA cycle, potentially affecting energy production.

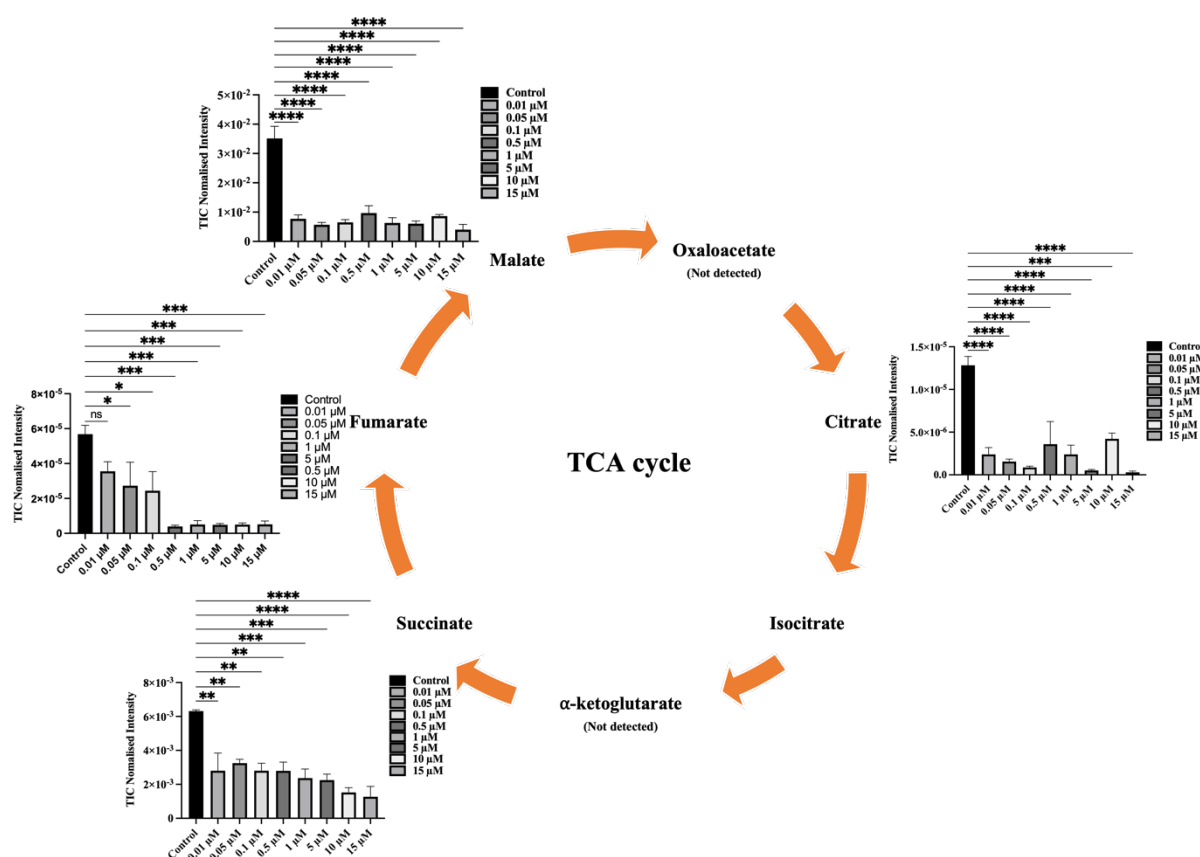


Figure 4. 7 TCA cycle intermediates changes for MCF-7 incubated with lapatinib by different dose. Dose response curve for cells from 9 treatment groups, control; 0.005 μM; 0.01 μM; 0.05 μM; 0.1 μM; 0.5 μM; 1 μM; 5 μM; 10 μM. All data acquired by GCIB Ar3000+. The bar chart reflects normalized ion intensity (to total ion counts) values obtained from three separate experiments (mean ± s.d). Blots are representative of the data sets (N=3). *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, not significant

Lapatinib is a dual tyrosine kinase inhibitor that targets the EGFR/HER2 pathways, which regulate cellular metabolism. Inhibition of these pathways by lapatinib may lead to the observed changes in TCA cycle intermediates. These changes in cancer cell metabolism may reflect an adaptation to the reduction in nutrient availability due

to the inhibition of growth factor signalling by lapatinib. The alterations in the TCA cycle observed in our study are consistent with findings of prior research (258-260) indicating that lapatinib induces metabolic changes in cancer cells, contributing to its anti-cancer effects.

4.3.2.2 Time-dependent drug response

4.3.2.2.1 Time-dependent response curve of tamoxifen

In addition to drug concentration, treatment duration is another crucial variable in drug therapy. Consequently, the time-dependent response of MCF-7 cells to tamoxifen treatment was also explored using OrbiSIMS depth profile analysis.

The minimum treatment duration was set at 10 s, while the maximum was set at 2 h, and the concentration was set at an IC₅₀ of 10 μ M. Consistent with the time-dependent response, an increase in incubation time led to a corresponding increase in the intensity of protonated tamoxifen ions, as shown in Figure 4.8.

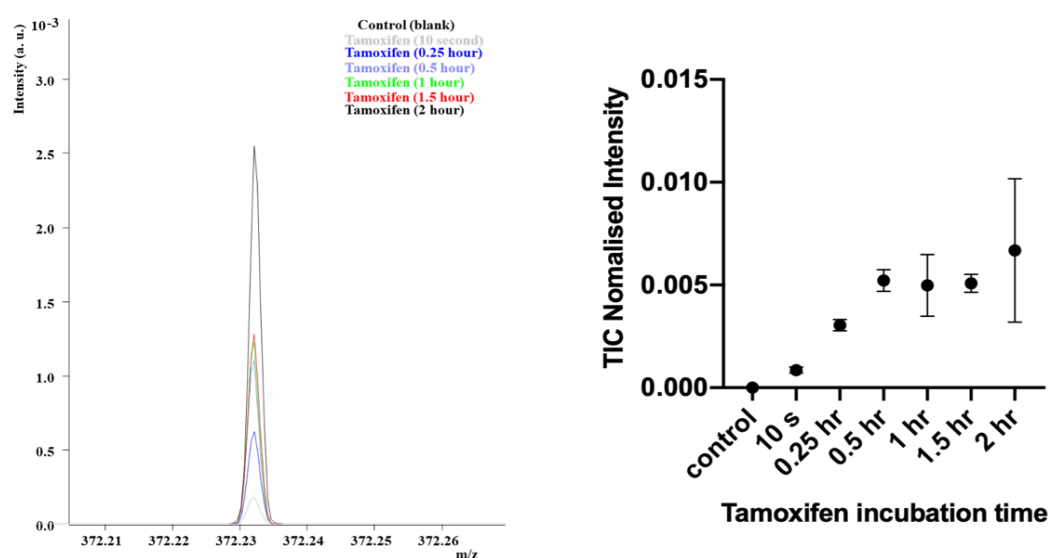


Figure 4. 8 OrbiSIMS data highlighting the differences in example spectra showing peak intensities for a single data set and exported peak intensities for 7 treatment groups, control; 10 seconds; 0.25 hour; 0.5 hour; 1 hour; 1.5 hour and 1-hour (a) spectra showing peak intensities of protonated tamoxifen molecule ion $[M+H]^+$, (b) exported average peak intensities for each group.

Notably, the intensity of tamoxifen ions gradually increased from 10 s to 0.5 h of incubation and remained constant thereafter until 2 h (Figure 4.8). The mass spectrum

of tamoxifen ions displayed a sharp peak with a narrow width. Notably, even when MCF-7 cells were treated with tamoxifen for only 10 s, the tamoxifen molecule exhibited an intense peak with excellent mass accuracy, which facilitated the identification of protonated tamoxifen ions. This observation suggested that OrbiSIMS is capable of detecting small-molecule ions at low levels and has potential for further analysis.

4.3.2.2.2 Time-dependent response curve of lapatinib

As lapatinib exhibited the highest intensity at the therapeutic dosage (IC_{50}), further investigation was conducted using this concentration to examine the time-dependent effects of lapatinib on MCF-7 cells using OrbiSIMS depth profile analysis. The intensity of protonated lapatinib ions progressively increased with increasing incubation time and reached a maximum at 2 h. Notably, quick incubation of MCF-7 cells (10 s) with lapatinib at the IC_{50} concentration (15 μ M) resulted in a lower ion intensity (Figure 4.9).

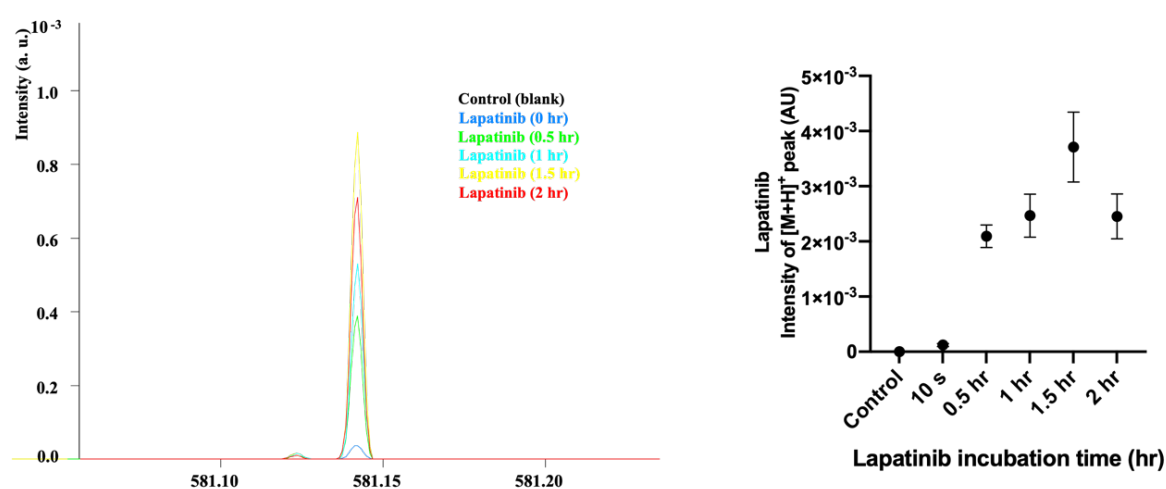


Figure 4. 9 OrbiSIMS data highlighting the differences in example spectra showing peak intensities for a single data set and exported peak intensities for 7 treatment groups, control; 10 seconds; 0.5 hour; 1 hour; 1.5 hour and 2-hour (a) spectra showing peak intensities of protonated tamoxifen molecule ion $[M+H]^+$, (b) exported average peak intensities for each group.

In contrast to tamoxifen, which produced molecular ions at a considerable level following brief incubation, lapatinib exhibited a lower ion intensity. There are several possible explanations for this observation: First, the logP value of tamoxifen is higher than that of lapatinib (6.35 and 4.64, respectively), suggesting that tamoxifen is more readily able to cross the cell membrane to reach its target site at the cellular level. Additionally, tamoxifen and lapatinib have different mechanisms of action; while tamoxifen primarily acts as an antagonist of ERs (209), lapatinib is primarily targeted as a tyrosine kinase inhibitor on the HER2 receptor (246). This difference in the mechanism may also contribute to the observed difference in ion intensity. Furthermore, previous research by Fabian in 1981 reported that tamoxifen exhibited a high binding affinity to MCF-7 cells, while lapatinib did not. This may explain why, under the same conditions of a brief 10-s incubation time, the intensity of tamoxifen molecule ions was higher than that of lapatinib (261).

4.3.2.2.3 Exploring effects of drug incubation time on glutamine uptake and cancer metabolism

As with the energy metabolism response to varying treatment concentrations discussed in the previous section, it remains unclear whether OrbiSIMS can monitor the endogenous response to different treatment durations. Therefore, this study aimed to investigate the impact of different incubation times of tamoxifen and lapatinib on the endogenous response.

4.3.2.2.3.1 Tamoxifen's Time-Dependent Impact on Glutamine Intensity and Metabolism

As illustrated in Figure 4.10, the trend observed in the study was similar to that of the time-response curve, where the control group exhibited the highest intensity levels for glutamine. The intensity of glutamine decreased by approximately half compared to the control group upon rapid treatment with tamoxifen. Upon increasing the incubation time, glutamine intensity drastically decreased at the 15-min treatment time point, gradually decreasing thereafter, and reaching its lowest point when MCF-7 was treated with tamoxifen for 1.5 h. Notably, when the treatment time was increased to 2 h, the intensity levels of glutamine marginally increased compared to that at the 1.5-h time point.

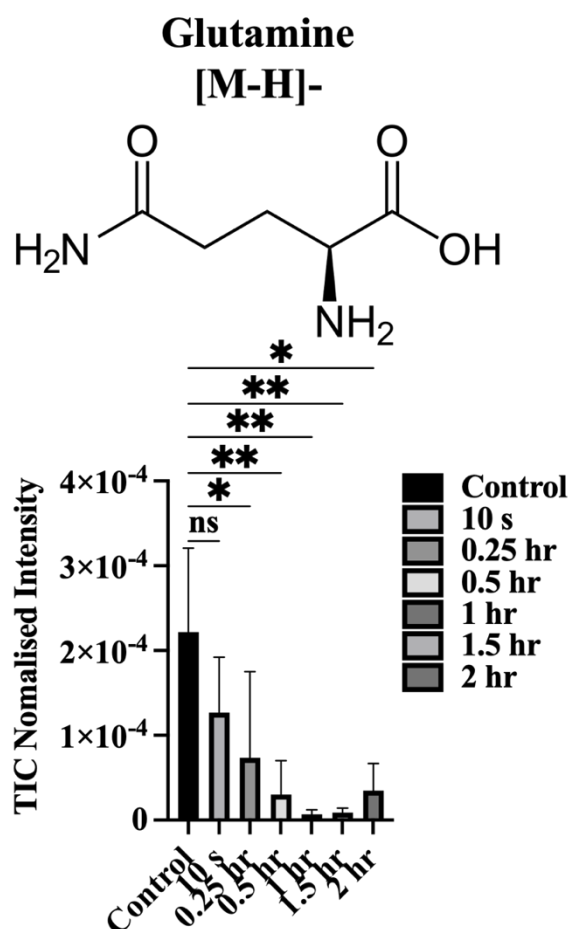


Figure 4. 10 Glutamine changes for MCF-7 incubated with tamoxifen by different times. Time-dependent response curve for cells from 6 treatment groups, control; quick (10 s); 0.25 hr; 0.5 hr; 1 hr; 1.5 hr and 2 hr. All data acquired by GCIB Ar3000+.The bar chart reflects normalized ion intensity (to total ion counts) values obtained from three separate experiments (mean ± s.d). Blots are representative of the data sets (N=3). *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, not significant

In addition to the observed effects on glutamine intensity, the intermediates of the TCA cycle were affected by varying durations of tamoxifen treatment. As depicted in Figure 4.11, TCA cycle intermediates displayed a time-response pattern similar to that of glutamine, with the control group showing the highest levels of these intermediates.

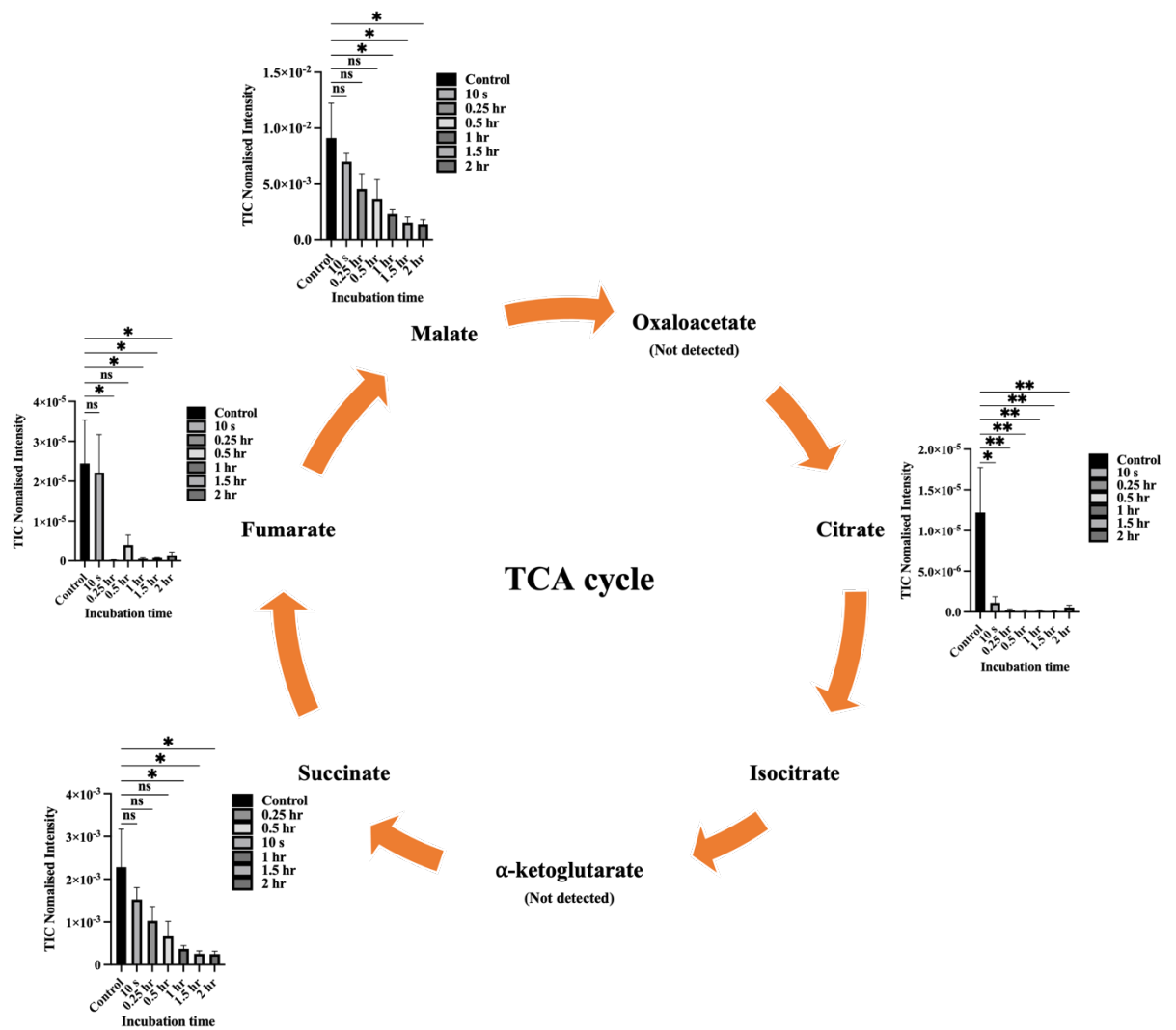


Figure 4. 11 TCA cycle intermediates changes for MCF-7 incubated with tamoxifen by different time. Time response curve for cells from 6 treatment groups, control; quick (10 s); 0.25 hr; 0.5 hr; 1 hr; 1.5 hr and 2 hr. All data acquired by GCIB Ar3000+. The bar chart reflects normalized ion intensity (to total ion counts) values obtained from three separate experiments (mean $\pm$ s.d). Blots are representative of the data sets (N=3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant

In cancer cells, amino acids are selectively channelled into metabolic pathways that are often enhanced and altered, typically in conjunction with the aerobic glycolysis characteristic of tumours (262-263). Due to the limited vascularisation of solid tumours, cancer cells must have efficient means of collecting plasma amino acids to compete with host tissues (264). Consequently, amino acid transporters in cancer cells are selected for and expressed at higher levels than those in the parent tissue, with features that confer advantages to cell development and survival (265-266). As the

figures shown (Figure 4.10-4.11), it suggested that tamoxifen inhibits glutamine uptake and its TCA cycle intermediates. In previous studies, it has indicated that the downregulation of glutathione may be dependent on glutamine availability, given that tamoxifen inhibits glutathione synthesis in MDA-MB-231 cells, which are triple-negative breast cancer cells (262). Therefore, tamoxifen treatment could potentially inhibit glutamine metabolism to inhibit cancer activity.

4.3.2.2.3.2 Time-dependent impact of lapatinib on glutamine intensity and cancer cell metabolism

In contrast to tamoxifen, the response curves for glutamine gradually changed with increasing incubation time of lapatinib. As shown in Figure 4.12, the intensity of deprotonated glutamine ions gradually decreased, with the control group exhibiting the highest intensity. Upon rapid incubation of MCF-7 cells with lapatinib, the intensity of deprotonated glutamine ions decreased to half of that of the control, indicating a stress response to lapatinib treatment.

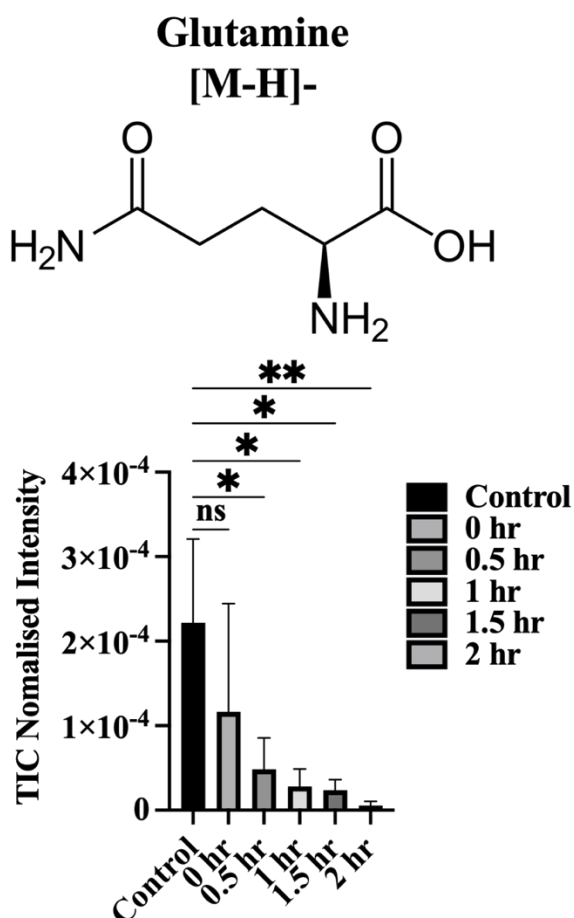


Figure 4. 12 Glutamine changes for MCF-7 incubated with lapatinib by different time.

Time response curve for cells from 6 treatment groups, control; quick (10 s); 0.5 hr; 1 hr; 1.5 hr and 2 hr. All data acquired by GCIB Ar3000+. The bar chart reflects normalized ion intensity (to total ion counts) values obtained from three separate experiments (mean $\pm$ s.d). Blots are representative of the data sets (N=3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant

Similar to the observed effects on glutamine, the intermediates of the TCA cycle also exhibited considerable alterations in response to varying incubation times of lapatinib. As illustrated in Figure 4.13, the intensities of key TCA cycle intermediates, such as citrate, succinate, and malate displayed a time-dependent modulation upon lapatinib treatment. Compared with the control group, which maintained relatively stable levels of these intermediates, the experimental groups showed a gradual reduction in their concentrations with increasing incubation time of lapatinib.

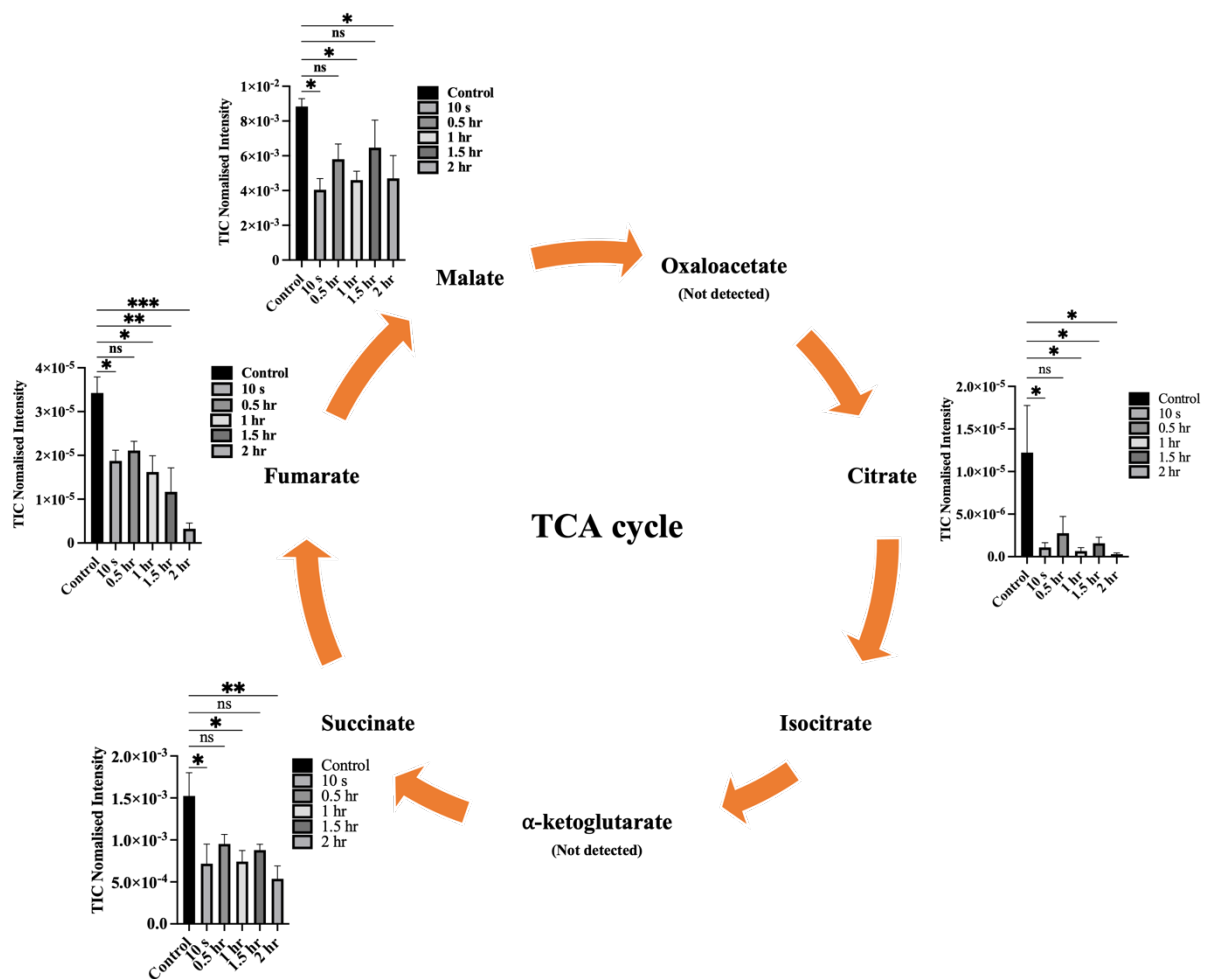


Figure 4. 13 TCA cycle intermediates changes for MCF-7 incubated with tamoxifen by different time. Time response curve for cells from 6 treatment groups, control; quick

(10 s); 0.5 hr; 1 hr; 1.5 hr and 2 hr. All data acquired by GCIB Ar3000+. The bar chart reflects normalized ion intensity (to total ion counts) values obtained from three separate experiments (mean $\pm$ s.d). Blots are representative of the data sets (N=3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant

This trend is particularly noteworthy during the initial phase of lapatinib treatment, when the most pronounced decrease in TCA cycle intermediates is observed. This implies a potential stress response in MCF-7 cells as they attempt to cope with the metabolic perturbations induced by lapatinib. As the treatment duration was prolonged, the cells appeared to partially adapt to drug-induced metabolic changes, resulting in a less pronounced decrease in TCA cycle intermediates. However, even with extended incubation times, the levels of these intermediates remained significantly lower than those observed in the control group.

In summary, our data suggest that both lapatinib and tamoxifen treatment affects glutamine metabolism and has a substantial impact on the TCA cycle, with time-dependent alterations observed in the concentrations of key intermediates. This highlights the complex nature of cellular responses to lapatinib and the importance of considering the effects of the time-course when studying the impact of the drug on cellular metabolism.

If stress persists during anti-cancer treatment, cancer cells may not be able to adapt and reach homeostasis through various signalling pathways, such as the activation of the integral stress response (ISR) (267-269). This can lead to the activation of transcription factor 4 (ATF4) and, ultimately, apoptosis (270). The intensity of deprotonated glutamine ions decreased by half following a 30-min incubation with lapatinib, gradually decreasing thereafter, and barely identifiable at the 2-h incubation condition. These results suggest that with increasing incubation time, both drug treatment (tamoxifen and lapatinib) has the potential to inhibit glutamine uptake, leading to glutaminolysis dysfunction, which is the primary energy process in tumours, alongside glycolysis.

As stated earlier, cancer cells exhibit considerable changes in their energy metabolism. Glutamine, one of the most abundant amino acids in human blood, plays

a crucial role in cancer growth, bioenergetics, and antioxidant production via glutaminolysis (271). In glutamine-dependent cancer cells, glutaminolysis regulates the conversion of glutamine into metabolites that enter the TCA cycle to produce ATP, primarily derived from glutamine. Glutaminase is a crucial enzyme in this process (272).

Targeting glutaminolysis can be an effective approach to disrupt cancer metabolism and tumour progression. Although previous studies have not provided clear evidence of the effect of lapatinib and tamoxifen on glutamine and its metabolism, OrbiSIMS analysis in this study revealed that lapatinib and tamoxifen treatment inhibits the uptake of glutamine in a time- and dose-dependent manner. As uptake was affected, downstream processes, such as glutaminolysis, were also affected. Further studies are required to investigate the mechanism underlying the inhibitory effect of both drugs (lapatinib and tamoxifen) on cancer metabolism. Nevertheless, the results suggest the potential use of OrbiSIMS in pre-clinical studies of anti-cancer agents by providing a comprehensive analysis of different types of biomolecules and their downstream metabolic pathways, such as glutamine and metabolism explored in this study.

4.3.2.3 Uncovering Metabolic Responses in the Absence of Drug Molecule Identification

The advanced instrumentation previously discussed in the literature review, namely OrbiSIMS, is capable of profiling local information from a complex chemical environment. As demonstrated in the previous section, OrbiSIMS can monitor changes in endogenous metabolites in response to anti-cancer agents. Moreover, it is important to investigate whether endogenous metabolites react to undetectable concentrations of drugs, as this can help the pharmaceutical industry fully understand the mechanisms of compounds of interest. The outcome of the dose-response of lapatinib is an excellent example, as no lapatinib molecules were identified until the concentration reached 5 μ M.

Following this line of inquiry, it becomes crucial to assess the impact of these drug concentrations on cellular biochemical pathways, especially those associated with our compound of interest - lapatinib. The lapatinib targeted effects on EGFR/HER2

signalling could indirectly impact phosphocholine levels. For example, EGFR/HER2 signalling pathways have been shown to regulate phospholipase C (PLC) enzymes, which are involved in phosphocholine metabolism (246; 256). By inhibiting these signalling pathways, lapatinib may indirectly affect phosphocholine levels and metabolism in cancer cells (258-260).

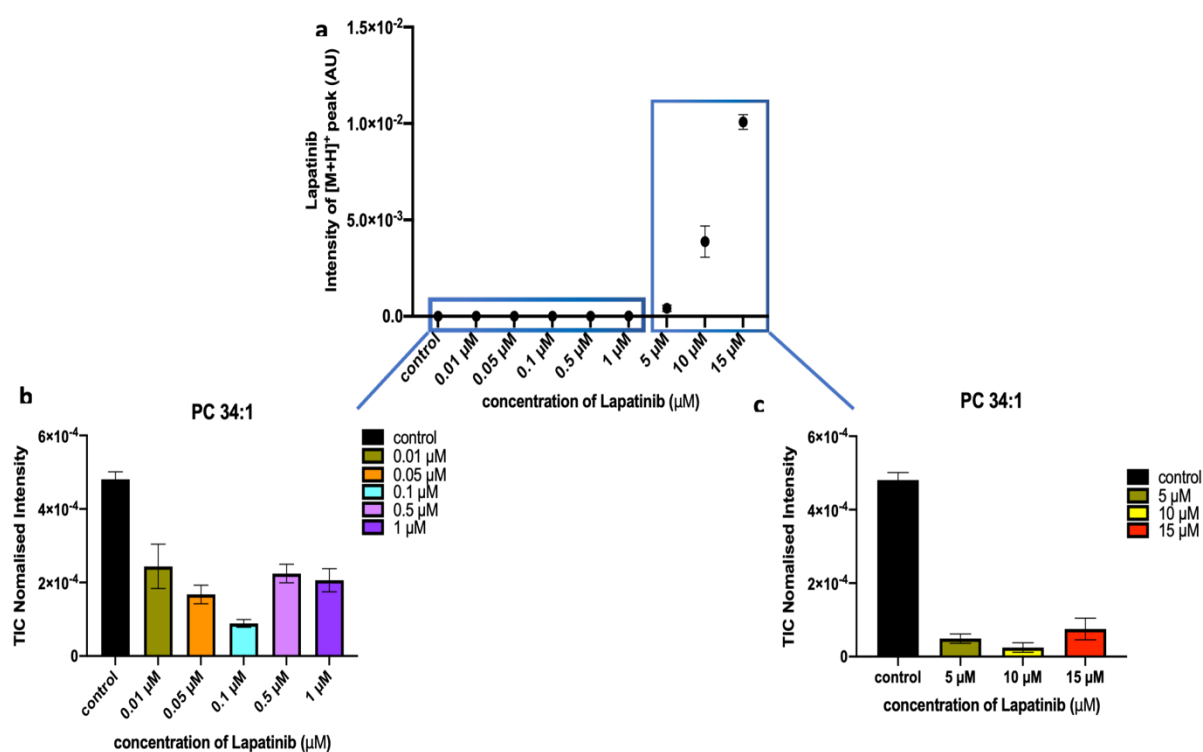


Figure 4. 14 Intact lipid response against different dose of lapatinib. Dose response curve for cell's PC (34:1) from 9 treatment groups, control; 0.01 μ M; 0.05 μ M; 0.1 μ M; 0.5 μ M; 1 μ M; 5 μ M; 10 μ M and 15 μ M (a) protonated molecule ion of lapatinib $[M+H]^+$, (b) PC (34:1) response to un-identified lapatinib range, (c) PC (34:1) response to identified lapatinib range All data acquired by GCIB Ar3000+. The bar chart reflects normalized ion intensity (to total ion counts) values obtained from three separate experiments (mean $\pm$ s.d).

This study investigated the metabolic changes in the intact lipid at m/z 758.570, which was putatively deprotonated as phosphocholine (PC) (34:1) $C_{42}H_{81}NO_8P^-$, in MCF-7 cells from nine lapatinib-treated groups (Figure 4.14). These groups included a control (0 μ M), five groups without the identification of lapatinib molecules (0.01, 0.05, 0.1, 0.5, and 1 μ M), and three groups where the drug ions were identified (5, 10,

and 15 μM). As expected, the average intensity of intact lipids decreased under lapatinib-induced stress, with the control group showing the highest intensity of PC 34:1. Upon lapatinib intervention, the intensity decreased to half of its original value and reached its minimum at a lapatinib concentration of 0.1 μM . When the lapatinib concentration increased to 0.5 μM , the intensity of PC 34:1 increased to a level similar to that observed at a lapatinib concentration of 0.01 μM , and this trend persisted at a flat level up to a lapatinib concentration of 1 μM . When the concentration of lapatinib reached 5 μM , protonated ions were observed, and the intensity of PC 34:1 began to decrease until it reached its minimum at a lapatinib concentration of 10 μM . Notably, PC 34:1 marginally increased at the therapeutic dosage of lapatinib (IC_{50}).

Such details can only be revealed through single-cell analysis. Phosphatidylcholine species, including PC (34:1), can be affected by various factors such as cellular signalling, metabolic changes, and enzyme activity. However, it is important to note that the relationship between lapatinib treatment and the levels of specific phosphatidylcholine species like PC (34:1) is not well-established and may require further research to elucidate the precise effects of the drug on phosphatidylcholine metabolism.

In conclusion, OrbiSIMS, an advanced instrumentation, enables the profiling of local information in complex chemical environments. This study demonstrated its effectiveness in monitoring changes in endogenous metabolites in response to anti-cancer agents. The investigation focused on lapatinib's dose-response and its indirect impact on phosphocholine levels. The intensity of PC (34:1) decreased with lapatinib-induced stress, reaching its minimum at 0.1 μM . At 0.5 μM , the intensity increased, while protonated ions appeared at 5 μM . Further research is needed to establish the precise effects of lapatinib on phosphatidylcholine metabolism and its implications in cancer treatment. OrbiSIMS offers valuable insights for future oncology advancements.

4.3.3 Application of OrbiSIMS for the analysis of known biomarkers

Advanced instrumentation in OrbiSIMS enables the profiling of biomolecules from targeted cells, including endogenous lipids. Lipids play a crucial role in oncogenesis, and their impact on immune activation and disease development is profound (273-274). Among the lipid classes, phospholipids are the most widely recognised in mammalian cells. Their enzymatic products are involved in multiple physiological processes and malignant transformations. Alterations in tissue lipid composition have been observed in several diseases, including cancer (273-274). Notably, abnormal lipid metabolism is a feature of cancer, where extra lipids are required to support the abnormal proliferation and activities of cancer cells (274-277). Thus, identification of known lipid biomarkers using OrbiSIMS is of great significance in cancer research and treatment.

To comprehensively analyse the distribution, functions, and implications of lipid variations in disease progression, it is imperative to employ sophisticated analytical techniques. This necessity arises from the current paucity of methodologies capable of concurrently examining lipid content, trafficking, and spatial distribution (118; 126-128).

MSI combines the selectivity of mass spectrometry with precise spatial data, enabling the development of images that reflect ion intensities at specific tissue locations. This information is particularly useful when analytes, such as lipids, are compartmentalised in tissues rather than dispersed uniformly. OrbiSIMS is a type of MSI that has been used to identify biomarkers in cancer research, providing the ability to view thousands of analytes concurrently with the label-free nature of the approach, which is a noteworthy advantage over conventional imaging and analytical techniques used in clinical diagnosis (133).

Based on the literature review, eight potential biomarkers were successfully identified using OrbiSIMS depth profile analysis in MCF-7 cells, which have been previously presented in pre-clinical and clinical studies. The corresponding labels, formulae, and m/z values for each biomarker are listed in Table 4. These biomarkers belong to two different lipid families, namely phosphatidylinositol (PI) and phosphatidylcholine (PC), with PI (34:1), PI (38:4), PI (40:6), PI (40:5), PC (32:1), PC (32:0), PC (34:0),

and PC (34:1) identified with the respective molecular formula and m/z values (278–282).

Table 4 Potential biomarkers retrieved from literature.

Potential Biomarkers		
Label	Formula	m/z
PI (34:1)	C ₄₃ H ₈₁ O ₁₃ P	836.5351
PI (38:4)	C ₄₇ H ₈₃ O ₁₃ P	886.5513
PI (40:6)	C ₄₉ H ₈₃ O ₁₃ P	910.5501
PI (40:5)	C ₄₉ H ₈₅ O ₁₃ P	912.5643
PC (32:1)	C ₄₀ H ₇₈ NO ₈ P	731.5543
PC (32:0)	C ₄₀ H ₈₀ NO ₈ P	733.5716
PC (34:0)	C ₄₂ H ₈₄ NO ₈ P	761.5801
PC (34:1)	C ₄₂ H ₈₂ NO ₈ P	759.5741

As these biomarkers are associated with the PC and PI families, they are involved in the composition of cell membranes. The lipid bilayer of cell membranes, composed of two layers of phospholipids, contains PC and sphingomyelin (SM) on the outer side and phosphatidylserine (PS), phosphatidylethanolamine (PE), and PI on the inner side (283). These biomarkers play a crucial role as second messengers in various biochemical processes, such as metabolic, signal transduction, and apoptosis pathways.

Evidence demonstrating the effect of tamoxifen and lapatinib on these biomarkers, particularly when using mass spectrometry imaging techniques, is lacking. Therefore, we employed depth profiling analysis to investigate the effects of tamoxifen and lapatinib on these eight potential biomarkers (284). The results of this study may provide valuable insights into the mechanism of action of these drugs and their potential use as therapeutic agents for the treatment of breast cancer.

4.3.3.1 Differential Response of PC and PI Biomarkers to Tamoxifen Treatment: A Dose and Time-Dependent Analysis

Figure 4.15 illustrates a dose-response curve for eight potential biomarkers in response to tamoxifen treatment. Notably, the intensities of PC and PI exhibited

distinct trends. Particularly, the PC family demonstrated the highest intensity in the control group, with the intensity decreasing gradually as tamoxifen concentration increased to 0.1 μM . However, at the 0.5 μM concentration of tamoxifen, the intensity of these four biomarkers abruptly decreased approximately eight-fold in comparison to the intensity observed at the 0.1 μM concentration of tamoxifen.

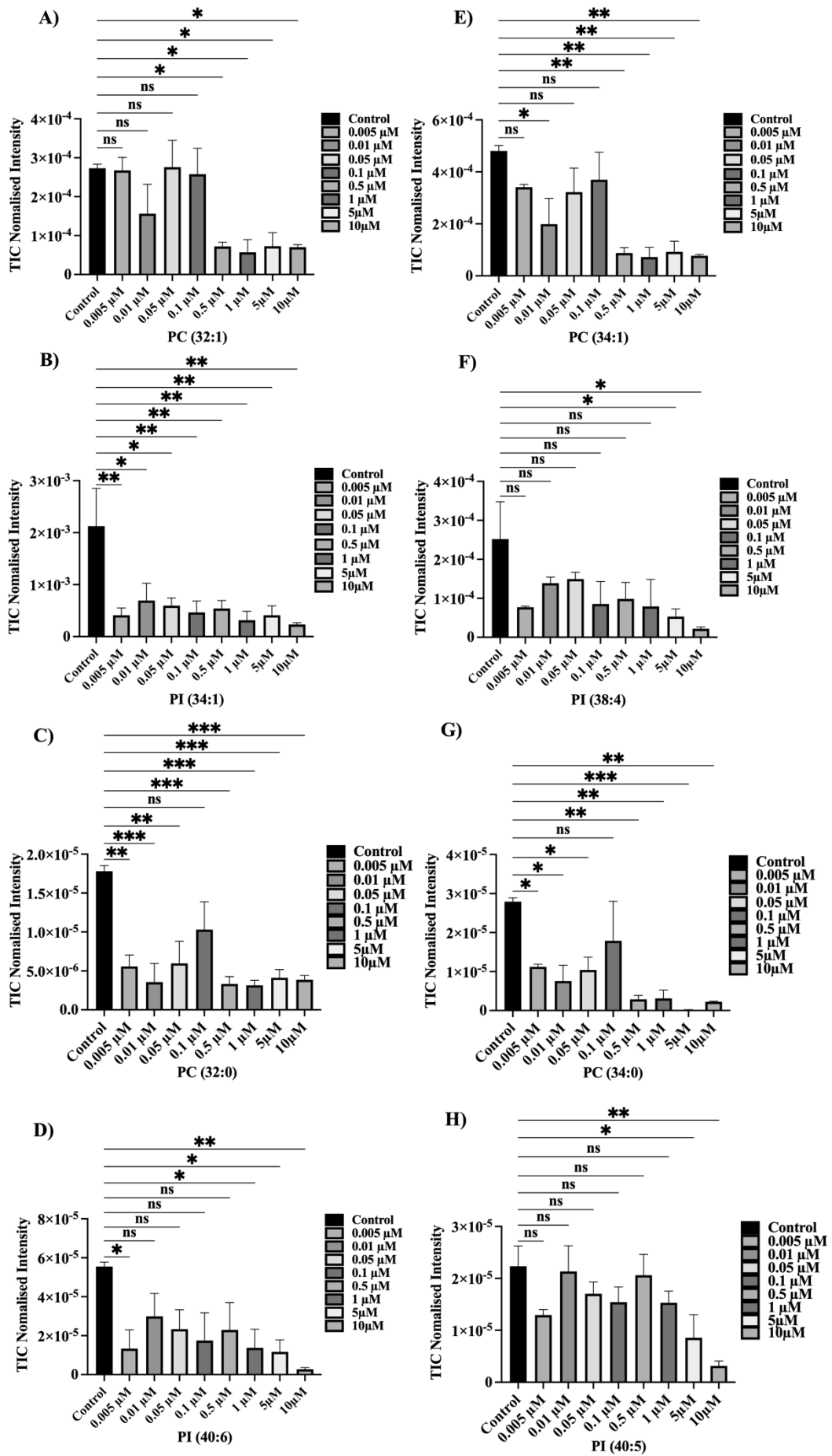


Figure 4. 15 Metabolite changes for MCF-7 incubated with tamoxifen by different dosage. Dose response nine treatment groups, control; 0.005 μ M; 0.01 μ M; 0.5 μ M; 1 μ M; 5 μ M; 10 μ M. All data acquired by GCIB Ar3000+. The bar chart reflects normalized ion intensity (to total ion counts) values obtained from three separate experiments (mean $\pm$ s.d). Blots are representative of the data sets (N=3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant

The intensities of PC family biomarkers moderately changed from 0.5 μ M to the highest concentration of 10 μ M. At the therapeutic dosage of tamoxifen (10 μ M), the intensity of the PC family was the lowest among all treatment dosages, except for PC 32:0, which exhibited a gradual increase in intensity from 0.5 μ M to 10 μ M. Although no significant trend was observed, these results suggest that tamoxifen treatment may inhibit potential biomarkers belonging to the endogenous PC family.

In contrast to the three PI biomarkers, the intensity changes pattern of PI (38:4) was marginally different. It (38:4) remained relatively flat from the control group to 0.05 μ M, and began to decrease from 0.1 μ M, exhibiting the same response pattern as that of other PC and PI ions to the therapeutic dosage of tamoxifen, which was the lowest in the set dose range.

In addition to the dose-response curve to tamoxifen for the eight biomarkers, the effect of incubation time on ion intensity was investigated (Figure 4.16). As observed in the previous analysis, the ion intensity trend differed between the PC and PI families. Within the PC family, the ion intensity trend was consistent across biomarkers. Notably, the control group did not exhibit the highest intensity; rather, upon rapid incubation with tamoxifen, the intensity rapidly increased and peaked.

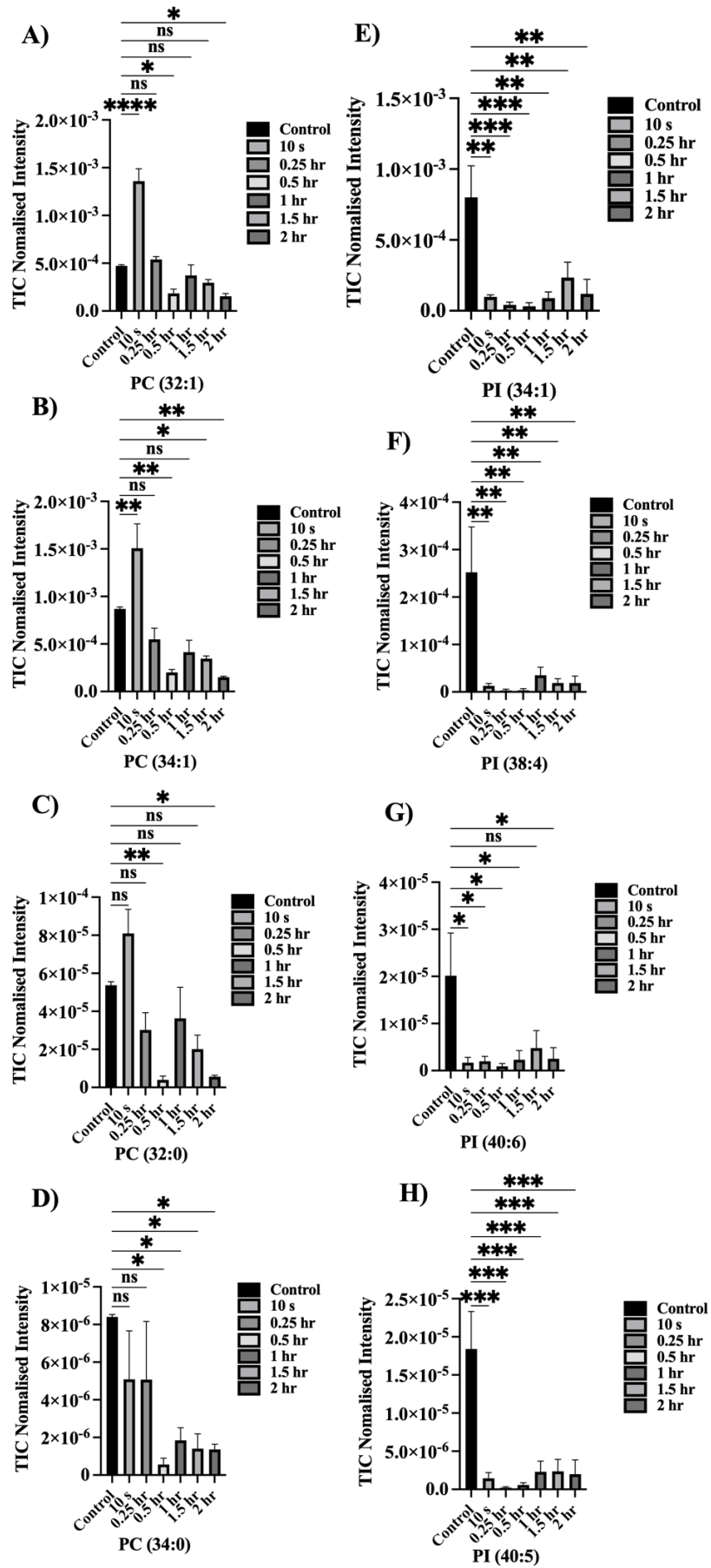


Figure 4. 16 Metabolite changes for MCF-7 incubated with tamoxifen by different time. Time response seven treatment groups, control; 10 s; 0.25 hr; 0.5 hr; 1 hr; 1.5 hr; 2 hr. All data acquired by GCIB Ar3000+. The bar chart reflects normalized ion intensity (to total ion counts) values obtained from three separate experiments (mean $\pm$ s.d). Blots are representative of the data sets (N=3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant

Subsequently, the intensity of the biomarkers gradually decreased with increasing incubation time, reaching a minimum at 0.5 h of tamoxifen treatment. When the treatment time was increased to 1 h, the intensity of PC (32:1) and PC (34:1) increased two-fold compared to the 0.5-h treatment, before gradually decreasing between 1 and 2 h, and ultimately stabilising at 2 h, where the intensity was lower than that of the control group. Notably, although PC (34:0) was not clearly visible in Figure 4.16, its trend was similar to that of the other three PC biomarkers. In particular, PC (34:0) exhibited an initial increase upon treatment with tamoxifen for 10 s and 15 min (0.25 h), followed by a decrease to a very low intensity at 30 min (0.5 h), an increase at 1 h, and a subsequent decrease with increasing incubation time, ultimately stabilising at 2 h of treatment.

4.3.3.2 Lapatinib-induced Alterations in PC and PI biomarker intensities: a comprehensive dose- and time-dependent study

Lapatinib, as an alternative anti-cancer agent, exhibits a different mechanism to tamoxifen (246). Therefore, the present study aimed to investigate the time- and dose-dependent responses of eight potential biomarkers to lapatinib treatment using the OrbiSIMS depth profile technique. In the time-response curve, the untreated group displayed the highest intensity of the four PC family biomarkers.

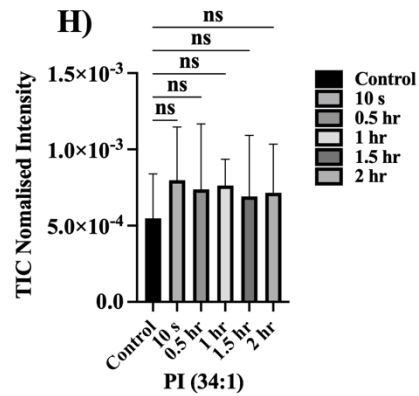
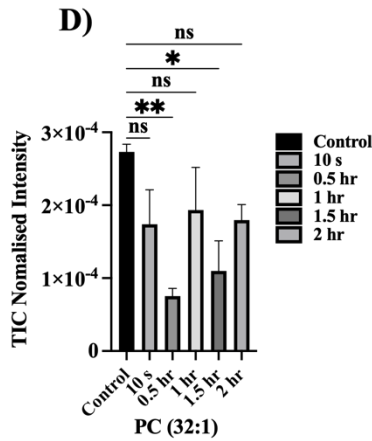
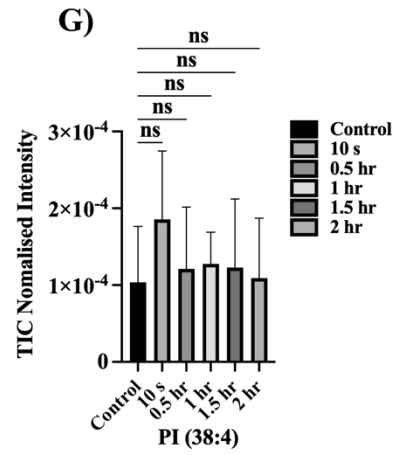
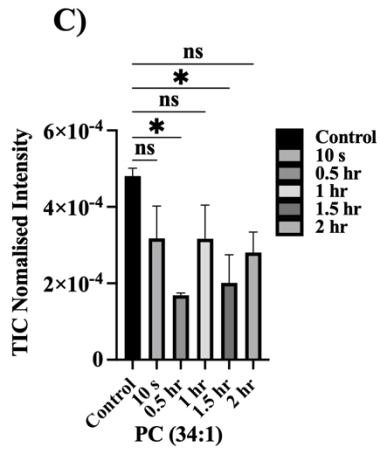
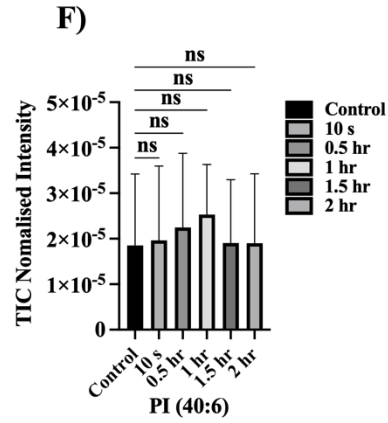
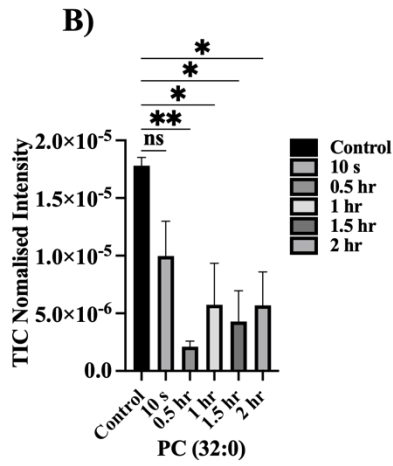
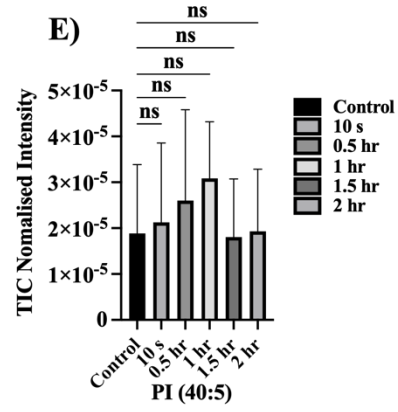
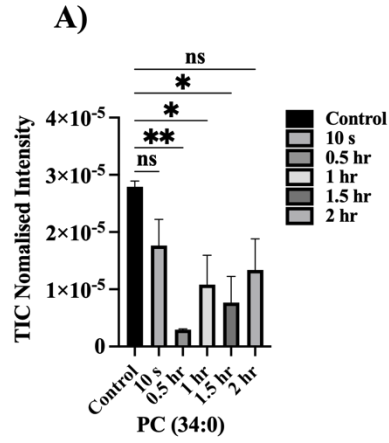


Figure 4. 17 Metabolite changes for MCF-7 incubated with lapatinib by different time. Time response six treatment groups, control; 10 s; 0.5 hr; 1 hr; 1.5 hr; 2 hr. All data acquired by GCIB Ar3000+. The bar chart reflects normalized ion intensity (to total ion counts) values obtained from three separate experiments (mean $\pm$ s.d). Blots are representative of the data sets (N=3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant

Upon incubation with lapatinib, the four biomarkers demonstrated distinct levels of reduction (Figure 4.17). However, at 0.5 h of incubation, the intensity decreased and reached its lowest level across all biomarkers. At the 1-h incubation point, the intensity of the four PC biomarker ions increased with the incubation time, and at 1.5 h, the intensity marginally decreased; however, it remained higher than that at the 0.5-h incubation point. At the 2-h incubation point, the ion response increased to a similar level observed at the 1-h time point for all four PC lipids. Nevertheless, the intensity remained lower than that in the control group, suggesting that lapatinib inhibits endogenous PC biomarkers in a time-dependent manner. OrbiSIMS depth profile analysis provides valuable insights for identifying these inhibitory effects.

In addition to the extracellular biomarker PC, the intracellular membrane lipid biomarker PI was examined for its response to lapatinib treatment. As shown in Figure 4.17, the intensity trends of PI (34:1) and PI (38:4) at different incubation times are similar. The control group did not exhibit the highest intensity; however, it was the highest when MCF-7 cells were quickly washed with 15 μ M lapatinib (within 10 s), which is the therapeutic IC₅₀ dose in this study. However, there was no significant change in intensity from the 10-s time point to the 2-h incubation time for these two PI biomarkers. In contrast, PI (40:6) and PI (40:5) showed different responses to lapatinib treatment. At the first time point (control and 10 s), these two PI biomarkers exhibited the same pattern as PI (34:1) and PI (38:4), with an abrupt increase

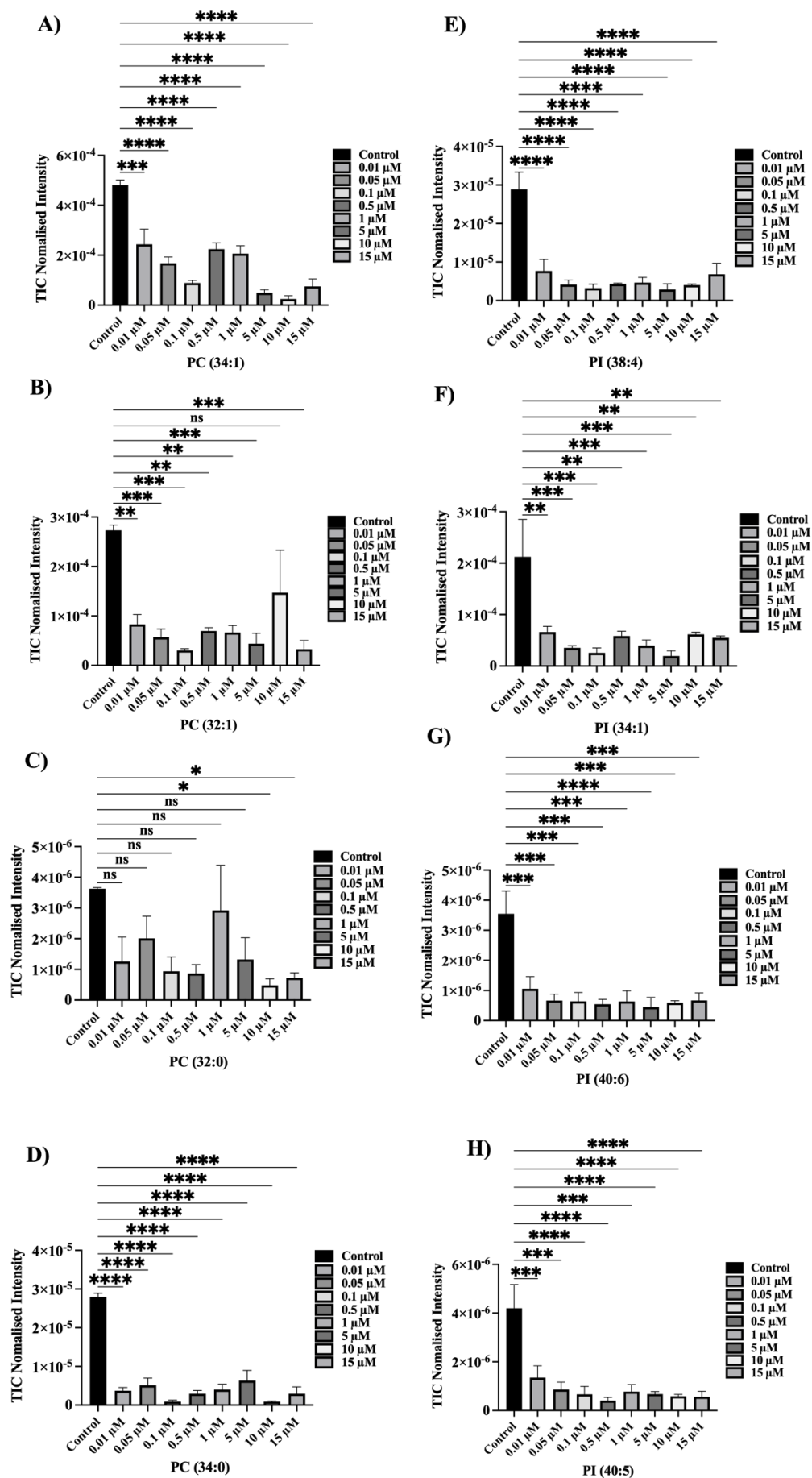


Figure 4. 18 Metabolite changes for MCF-7 incubated with lapatinib by different dosage. Time response nine treatment groups, control; 0.01 μM ; 0.05 μM ; 1 μM ; 5 μM ; 10 μM ; 15 μM . All data acquired by GCIB Ar3000+. The bar chart reflects normalized ion intensity (to total ion counts) values obtained from three separate experiments (mean $\pm$ s.d). Blots are representative of the data sets (N=3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant

in intensity under the 10-s incubation condition. However, when the incubation time was increased to 0.5 h, various levels of intensity decrease were observed for PI (40:6) and PI (40:5). Thereafter, the intensity marginally increased at 1 h of incubation and gradually decreased to the lowest level.

Apart from different times, the response of these biomarkers to different concentrations of lapatinib has also been explored (Figure 4.18). Among the PI family biomarkers, three species (PI (40:5), PI (40:6), and PI (38:4)) exhibited a consistent decreasing trend in response to lapatinib treatment, with a gradual decline in intensity that was evident at low concentrations (0.01 μM). The highest intensity was observed in the control group, which rapidly decreased in response to lapatinib, despite its low concentration. Subsequently, the intensities of these three biomarkers gradually decreased in a concentration-dependent manner. In contrast, PI (34:1) displayed a unique response profile. Its intensity was the highest in the control group and decreased to one-third of the intensity at a lapatinib concentration of 0.01 μM , followed by further decline at 0.05 μM and 0.1 μM . At 0.5 μM , the intensity of PI (34:1) returned to levels similar to those observed at 0.01 μM , and subsequently decreased at concentrations ranging from 0.5–5 μM . Notably, the intensity of PI (34:1) increased to levels similar to those observed at a lapatinib concentration of 0.01 μM when the concentration was increased to 10 μM and the IC_{50} was 15 μM .

Based on these results, it was evident that tamoxifen and lapatinib treatments exhibit varying degrees of inhibition of PC and PI biomarkers. Previous research has demonstrated that tamoxifen has the potential to induce apoptosis in MCF-7 cells by activating phospholipase C, and this is primarily achieved through the hydrolysis of PC (285–286). With its straightforward pre-treatment, OrbiSIMS expedites

conclusion-drawing. The results of the OrbiSIMS depth profile presented here indirectly support this finding, indicating that tamoxifen can induce apoptosis. Furthermore, the phospholipase C (PLC) pathway can be modulated via the PI3K/Akt signalling pathway, which is a major cancer survival pathway (287). Both growth factor receptor tyrosine kinases and G protein-coupled receptors activate PI3K, leading to the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) to generate phosphatidylinositol 3,4,5-trisphosphate (PIP3). However, PI lipids can also be phosphorylated to generate PIP2 and PIP3 (288). Therefore, these results indicate that the inhibition of PI by tamoxifen and lapatinib may result in the suppression of the PI3K pathway. Thus, OrbiSIMS could be applied to assess the impact of drug molecules on the endogenous metabolite response. Such details can only be revealed at the cellular level with advanced OrbiSIMS instrumentation.

4.4 Conclusion

The primary objective of this chapter was to evaluate the potential of OrbiSIMS for monitoring endogenous metabolite responded to drug treatment and examining its effectiveness as a semi-quantitative approach.

In this study, we investigated the effect of selected anti-cancer agents on the energy metabolism of cells using the methods developed in the previous chapter. Tamoxifen and lapatinib were selected as anti-cancer agents because of their high intensity in the biological matrix in both ToF-SIMS and OrbiSIMS analyses, and the exclusive detection of lapatinib in OrbiSIMS profile analysis. Using OrbiSIMS, we also identified the drug molecules at very low concentrations.

To explore the factors contributing to data variability and the application of OrbiSIMS as a quantitative method for examining the selected effect of anti-cancer agents on the metabolism of breast cancer cells, we conducted experiments under a range of different conditions. The initial experiments involved different treatment conditions at specific times and concentrations. Our results demonstrated the successful application of OrbiSIMS depth profile analysis in detecting changes in endogenous metabolites in human breast cancer. Anti-cancer treatment was found to alter several lipid species, including the membrane lipids PC and PI.

To determine the detection limit of OrbiSIMS, we evaluated a range of doses of anti-cancer agents. The instrument's high sensitivity allowed for the detection of a maximum 2000-fold dilution of an anti-cancer drug. Furthermore, our findings indicated an intrinsic decrease in glutamine and its TCA cycle intermediates related to the change in anti-cancer agents, which was also reported only in another type of breast cancer cells. This is a novel observation, as no other studies have evaluated the effect of anti-cancer drugs on MCF-7 cell metabolism. The observed inhibition of glycolysis and glutaminolysis may have potential applications in future studies.

Our analysis identified eight different biomarkers, same as from reported research, in addition to the endogenous metabolite responded to anti-cancer treatment. These eight biomarkers belong to two different lipid families, PC and PI, because lipids play an active role in the proliferation of cancer cells. The present study demonstrated that the identified biomarkers manifested a reduced response to tamoxifen and lapatinib, indicative of the inhibitory impact of these therapeutic interventions on PC and PI lipid biomarkers. These results emphasise the practicality of OrbiSIMS for monitoring biological activity and identifying potential biomarkers, warranting additional exploration in subsequent research endeavours.

Chapter 5. Investigating metabolic alterations in tamoxifen-resistant breast cancer cells

5.1 Introduction

Breast cancer poses a significant risk to women's health and is a leading cause of mortality worldwide, with >80% of cases diagnosed as oestrogen receptor (ER) positive (289). In ER-positive breast cancer, endogenous oestrogen molecules bind to the ER, modulating gene activity related to cell proliferation and survival through oestrogen response elements and growth factor signalling pathways (290). This modulation leads to the proliferation and survival of ER-positive cancer cells.

Endocrine therapies, such as tamoxifen, have been developed to target the oestrogen dependence of ER-positive malignancies. As a first-line endocrine therapy, tamoxifen is frequently prescribed to female patients, particularly premenopausal women, reducing breast cancer recurrence by 50% and the mortality rate by 31% (291). As a selective ER modulator, tamoxifen competes with oestrogen for ER binding, modulating downstream signalling pathways and inhibiting the oestrogenic action responsible for tumour growth and proliferation (292).

Despite high ER expression levels, many ER-positive patients with cancer exhibit innate resistance to hormonal treatment. Among those initially responsive to tamoxifen, approximately 40% develop *de novo* or acquired resistance. This acquired resistance involves complex processes such as tamoxifen target modification and activation of alternative signalling pathways (293-304).

Metabolism is pivotal in the development of resistance when multiple factors are involved, such as c-Src kinase (Src). Src augments glycolysis and glucose flux by activating hexokinase (HK) through the pentose phosphate pathway and modulating intracellular glucose levels (305). Moreover, Src participates in a variety of biomolecular processes, including cell proliferation, differentiation, and migration, and is implicated in numerous signalling pathways (306-307). Prior studies have demonstrated that extended tamoxifen treatment amplifies Src activity, resulting in increased breast cancer cell invasion, while attenuating Src activity exerts a favourable influence on the development of tamoxifen resistance (308).

Src plays a pivotal role in cell metabolism and the development of drug resistance, making it a promising therapeutic target. Dasatinib, a potent and bioavailable tyrosine kinase inhibitor, has been extensively studied for its role as an Src inhibitor (309). In recent clinical studies, combining dasatinib with endocrine therapy has shown promise. Specifically, breast cancer patients receiving dasatinib in combination with letrozole exhibited favourable tolerability and minimal toxicity (310). In parallel, in vitro studies have demonstrated that adding dasatinib to tamoxifen-resistant cell models can restore drug sensitivity (311). Despite these findings, there's a gap in understanding the underlying mechanisms by which dasatinib and endocrine therapy combination impacts breast cancer cells. This knowledge is especially crucial when considering its effect on cellular metabolisms, particularly energy metabolism.

5.2 Chapter aims

The primary objective of this investigation was to determine the applicability of the workflow established in the preceding two chapters for examining metabolic alterations in the tamoxifen-resistant breast cancer cells, thus identifying potential novel therapeutic approaches. This study aimed to investigate whether the novel application of OrbiSIMS can yield pertinent information to differentiate between common breast cancer cells and tamoxifen-resistant breast cancer cells. The metabolites of interest are predominantly endogenous, with a majority being small molecules that fall within the detection range of OrbiSIMS. This technique will also be used to explore the potential benefits of combination therapy involving tamoxifen and dasatinib. Furthermore, the subcellular distribution of the drugs was visualised using the unique imaging capabilities of OrbiSIMS, enabling the analysis of their differences and permeation compared to tamoxifen treatment alone. This research endeavour aimed to serve as an exemplar for addressing and understanding the genuine biomedical challenges inherent in cancer research.

5.3 Results and discussion

5.3.1 Effects of dasatinib and tamoxifen on tamoxifen-resistant breast cancer cells

In the previous section (Chapter 4), OrbiSIMS was employed to examine the effects of tamoxifen and lapatinib in MCF-7 cells under varying exposure durations and

concentrations. The results elucidated the biological responses of MCF-7 cells, reflecting their reactions to drug treatments. These findings demonstrated the ability of the instrument to monitor metabolite responses during drug exposure. Subsequent studies were conducted to investigate the combined effects of tamoxifen and dasatinib in tamoxifen-resistant MCF-7/TamR-1 cells.

5.3.1.1 Cytotoxic effects of dasatinib and tamoxifen on MCF-7/TamR-1 cells

To assess the combined effects of dasatinib and tamoxifen on MCF-7/TamR-1 cells, cells were subjected to various concentrations of dasatinib in conjunction with a fixed concentration of tamoxifen for 48 h. Initially, a cytotoxicity assay was performed to investigate the antitumour efficacy of the combination treatment on MCF-7/TamR-1 cells. Cell death was more pronounced when both agents were administered together, as opposed to their individual use. MCF-7/TamR-1 cells responded to the antiproliferative action of dasatinib alone, with an estimated IC_{50} of 20 μ M (indicated by the light blue trend line of Figure 5.1).

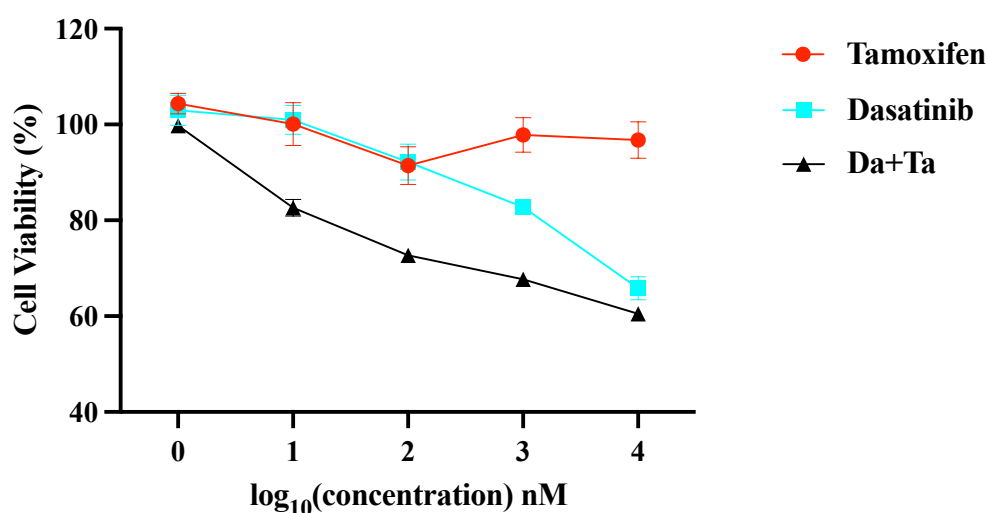


Figure 5. 1 Combination treatment effect on MCF-7 and MCF-7/TamR-1 cell. Cytotoxicity effects of Dasatinib alone or in combination with tamoxifen in tamoxifen resistance breast cancer cell line. Cells were treated with increasing concentrations of dasatinib (blue) and tamoxifen (red), or fixed concentration of dasatinib combined with increasing concentrations of tamoxifen (Black). The cell viability is determined by MTT assay.

As anticipated in Figure 5.1, the viability of the MCF-7/TamR-1 cells remained unaffected by progressively increasing concentrations of tamoxifen, as evidenced by the inability to predict the IC_{50} (represented by the red trend line). This result indicates that MCF-7/TamR-1 cells exhibited a high degree of resistance to tamoxifen.

However, the addition of dasatinib restored the sensitivity of MCF-7/TamR-1 cells to tamoxifen, as illustrated by the black trendline. Consequently, the combination of dasatinib and tamoxifen demonstrated a markedly enhanced antiproliferative effect on tamoxifen-resistant cells compared to the separate administration of tamoxifen and dasatinib.

5.3.1.2 The role of dasatinib in re-sensitizing MCF-7/TamR-1 breast cancer cells

The results of the cytotoxicity assay demonstrated that MCF-7/TamR-1 cells were resistant to tamoxifen treatment, as cell viability did not exhibit significant changes. Moreover, the proliferation of MCF-7/TamR-1 cells was affected by dasatinib alone, enabling the prediction of its IC_{50} value for resistant cancer in this study. However, whether the potency of tamoxifen potency is reduced in the resistant cell line remains unknown.

Two primary factors are implicated in the development of drug resistance in cancer: increased drug efflux and endogenous mechanisms. Cell membrane proteins, which are responsible for regulating the distribution, absorption, and excretion of various substances, protect cells from cytotoxicity caused by excessive intracellular drug concentrations, potentially interfering with therapeutic efficacy. Overexpression of these proteins has been directly associated with the emergence of drug resistance (312-315). Conversely, resistance may be attributed to enhanced xenobiotic metabolism, improved DNA repair capabilities, or growth and genetic factors, either individually or in combination. Although a sufficient quantity of drug molecules may enter the cell, endogenous factors can hinder their ability to effectively target the desired site (312-315). Hence, discerning the underlying factors contributing to tamoxifen resistance in MCF-7 cancer cells is crucial for distinguishing between these scenarios and informing future research.

As demonstrated in the previous chapter, OrbiSIMS is capable of monitoring varying concentrations of intracellular drug molecules. Consequently, a depth profile analysis was employed to further investigate this phenomenon. OrbiSIMS was performed following the cytotoxicity assessment of tamoxifen and dasatinib in MCF-7 and MCF-7/TamR-1 cells to investigate whether tamoxifen molecules are located in resistant cell lines. This is because resistance conditions typically increase efflux activity and decrease drug accumulation.

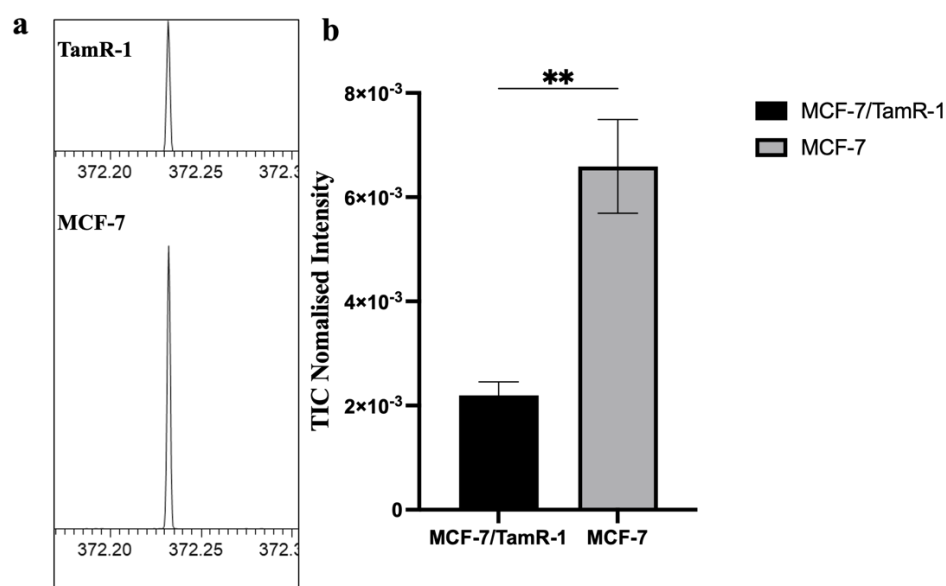


Figure 5.2 a). OrbiSIMS spectrum of tamoxifen molecule in MCF-7 and TamR-1 cells. Protonated tamoxifen molecule $[M+H]^+$ in TamR-1 (up) and MCF-7 (below). b). Protonated tamoxifen molecule $[M+H]^+$ intensity comparison between TamR-1 and MCF-7. Blots are representative of the data sets (N=3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant

Depth profile analysis revealed that although protonated tamoxifen molecules $[M+H]^+$ could be identified in both MCF-7 and MCF-7/TamR-1 cell lines (Figure 5.2A), the intensity was markedly reduced in resistant cell lines compared to that in MCF-7 cells. In the spectra, the intensity area of tamoxifen molecules $[M+H]^+$ substantially increased in MCF-7 compared to that in MCF-7/TamR-1, indicating that only a small amount of tamoxifen remained within the resistant cancer cell lines, and the efflux of tamoxifen was enhanced compared to that of MCF-7 cells. Statistical comparison of

tamoxifen intensity supported this observation. In resistant cell lines, tamoxifen intensity was approximately three times lower than that in MCF-7 cells (Figure 5.2B).

Consequently, the development of tamoxifen resistance is potentially due to the enhanced efflux of tamoxifen molecules, as there is a lower intracellular tamoxifen ion concentration than that in tamoxifen-sensitive MCF-7 cells. Notably, most studies have suggested that the development of tamoxifen resistance is attributable to genetic mutations. The primary objective of cytotoxic drugs is to damage cell components that are vital for cell survival. Owing to frequent gene mutations in tumour cells, they can undergo alterations in response to target molecules, rendering them resistant to specific medications. The product of the mutant gene retains its function; however, it can no longer bind to the drug owing to the changes in its stereochemical structure. The gradual depletion of ERs in altered cells leads to complete unresponsiveness in tamoxifen-resistant breast cancer. It is likely that oestrogen is no longer required for the survival and proliferation of tumour cells (313-318).

Contrary to previous findings, the OrbiSIMS experiment revealed that enhanced tamoxifen efflux could potentially be responsible for resistance. At the beginning of this chapter, the cytotoxicity assay demonstrated that the dasatinib interaction exhibited a markedly anti-proliferative effect on tamoxifen-resistant cells compared to treatment with tamoxifen and dasatinib separately. These results suggest that, as an Src kinase inhibitor, dasatinib could potentially re-sensitise MCF-7/TamR-1 cells to tamoxifen. Therefore, the intracellular intensity of tamoxifen has been investigated in conjunction with dasatinib, as cytotoxicity assays have demonstrated that this combination therapy results in the restoration of tamoxifen sensitivity.

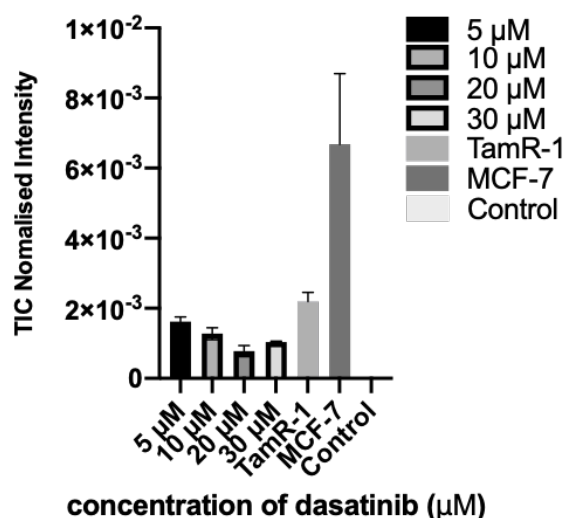


Figure 5. 3 Protonated tamoxifen $[M+H]^+$ intensities within seven increasing concentrations of Dasatinib, control; MCF-7; TamR-1; 5 μ M; 10 μ M; 20 μ M; 30 μ M. The bar chart reflects normalized ion intensity (to total ion counts) values obtained from three separate experiments (mean $\pm$ s.d).

As shown in the Figure 5.3, increasing the dasatinib concentration did not result in increased levels of tamoxifen within cells, as evidenced by the protonated tamoxifen molecule ions $[M+H]^+$. As expected, there were no tamoxifen ions in the control group. Similar to the results presented in Figure 5.2, MCF-7 cells exhibited the highest tamoxifen ion intensity. In contrast, tamoxifen intensity decreased upon the addition of dasatinib. When dasatinib was introduced at the lowest concentration (5 μ M), tamoxifen exhibited the highest intensity. As the dosage increased, the tamoxifen ion intensity gradually decreased, reaching its lowest point when the dosage of dasatinib was at the IC_{50} value (20 μ M).

The observed decrease in tamoxifen intensity suggests that tamoxifen ions did not accumulate in MCF-7/TamR-1 cells. This outcome indicated that other mechanisms may be involved during re-sensitisation to tamoxifen when dasatinib is added to MCF-7/TamR-1 cells in conjunction with tamoxifen.

5.3.2 Differentiating between MCF-7 and MCF-7/TamR-1 cells by PCA analysis

Following the assessment of the effect of the combination treatment on MCF-7 and MCF-7/TamR-1 cells, principal component analysis (PCA) was used to examine

potential endogenous metabolite alterations between MCF-7 and the resistant cell line, MCF-7/TamR-1. As continuous tamoxifen treatment can lead to the development of resistance in breast cancer therapy, PCA may offer indirect insights into changes in endogenous metabolites in response to persistent stress from tamoxifen treatment. The resulting outcomes could contribute to a better understanding of the pathology of developed resistance.

Here, a data matrix was constructed using the significant peak areas as variables associated with the full spectra obtained from MCF-7 and MCF-7/TamR-1 cells. Figure 5.4 displays the PCA scores and loadings of the positive ion spectra for the MCF-7 and MCF-7/TamR-1 samples. A biplot representation was used to present the scores and loadings of the first two principal components from the OrbiSIMS data matrix. Biplots offer a convenient method for simultaneously presenting scores and loadings derived from PCA. The peaks with significant loadings on PC1 are labelled in the figure. These loadings signify the weights of the variables contributing to the differentiation of cells, as explained by the score plot (319-326).

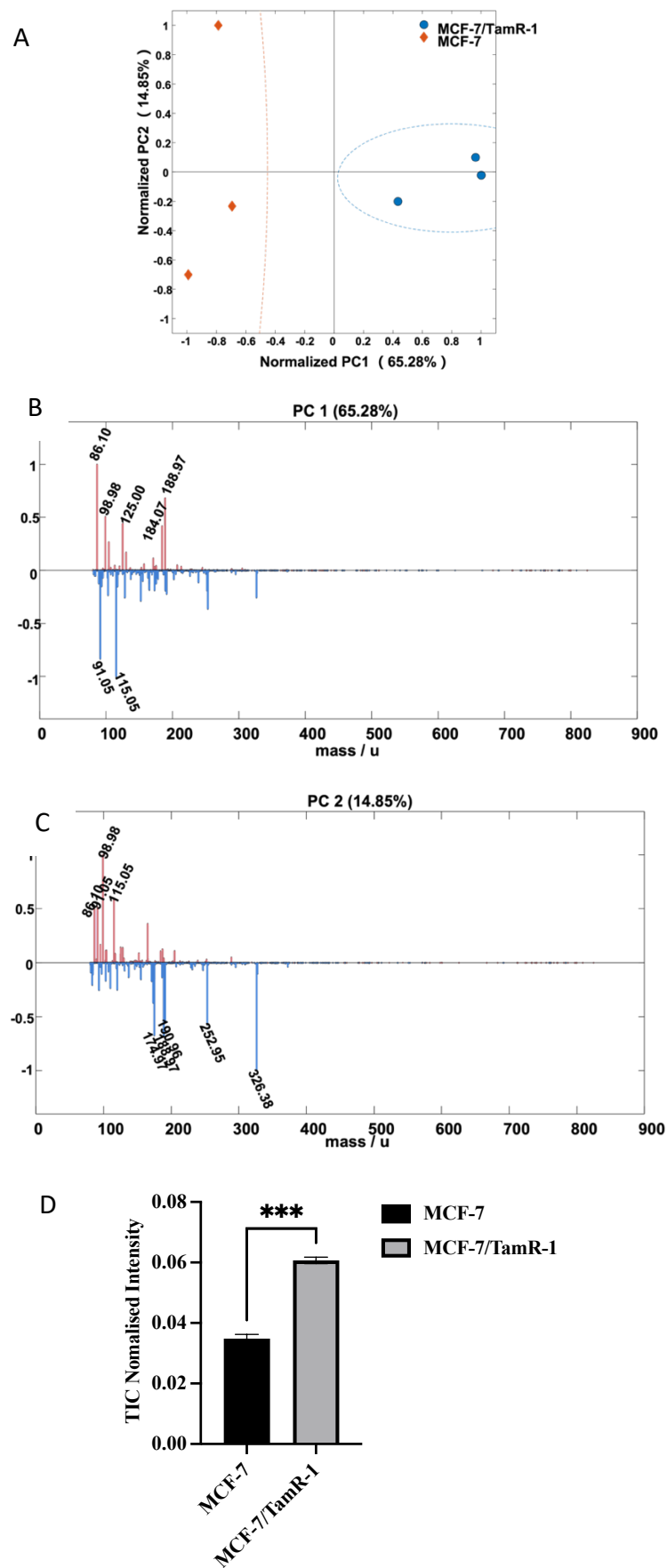


Figure 5. 4 Untargeted endogenous metabolite changes between MCF-7 and MCF-

7/TamR-1 cells. A) Multivariate analysis of OrbiSIMS depth profile data from the extracted normalized (to total ion counts) intensities based on treatment of tamoxifen (10 μ M) in MCF-7 and MCF-7/TamR-1 under positive mode. B) Loading plots of PC1. C) Loading plots of PC2. D) Statistical analysis of $C_9H_7^+$ between MCF-7 and MCF-7/TamR-1 after tamoxifen treatment Loading plots and Score plots are shown for each group and n=3 per treatment group.). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant

The first two PCs accounted for 80.13% of the total variance in the data matrix. PC1 was responsible for 65.28% of the variance in OrbiSIMS data, while PC2 accounted for the remaining 14.85%. As depicted in the figure, the two sample groups are separated in the score plot. The loadings characterise the weight of the variables in the differentiation of the two cell types, as explained by the score plot. The ions responsible for the differences between the samples were identified from the loading plots of PC1. PC1 primarily distinguished between MCF-7 and MCF-7/TamR-1 cells, both treated with 10 μ M tamoxifen.

In the loading plots Figure 5.4B, the substantial variance that allows for the separation of tamoxifen-sensitive and tamoxifen-resistant cancer cells under pathological conditions could be attributed to the leucine fragment peaks at m/z 86.10 (positive PC1 loadings). These peaks, associated with leucine ($C_5H_{12}N^+$), are attributed to the resistant cancer cell line MCF-7/TamR-1 (positive PC1 scores), with minor contributions from phospholipid ions at m/z 188.97 and 98.98, as illustrated in the figure. Notably, negative scores representing MCF-7 cells are closely related along PC1 with increasing peaks at m/z 115.0542 (negative PC1 loadings), which are associated with $C_9H_7^+$ and are assigned to MCF-7 cells. Additionally, a non-negligible contribution was observed in the ion peaks at m/z 91.05. A negative correlation was observed between MCF-7 and MCF-7/TamR-1 cells from PC1 loading, owing to leucine, as confirmed by the statistical comparison shown in the Figure 5.4D

PCA analysis of the OrbiSIMS results revealed that leucine was responsible for the difference between the common breast cancer cell line MCF-7 and tamoxifen-resistant MCF-7/TamR-1 cells. Metabolic reprogramming enables cancer cells to

continue growing under stressful conditions, such as nutrient deficiency and pharmacological treatment (327-328). Cancer cells exhibit dysregulated amino acid absorption and abnormal cell surface expression of amino acid transporters, and their involvement in ER+ breast cancer is beginning to be understood (327-328). Previous studies have demonstrated that a decrease in leucine levels suppresses tumour cell proliferation, whereas an increase enhances it. Furthermore, research suggests that low-leucine diets may be beneficial for patients with ER+ breast cancer (329-330).

Leucine, an essential amino acid obtained from food, is among the 20 building blocks of proteins (328). Decreasing leucine levels suppressed the division of ER+ breast cancer cells, whereas a tenfold increase in leucine levels enhanced cell division. Additionally, scientists discovered that cells resistant to tamoxifen could grow even when leucine levels were low. Resistance to tamoxifen was associated with higher levels of the surface protein SLC7A5, which is responsible for transporting leucine into cells. When SLC7A5 is overexpressed in cells, leucine is absorbed more efficiently, enhancing the resistance of breast cancer cells to tamoxifen. Inhibiting SLC7A5 using chemical inhibitors results in the shrinkage of ER+ tumours in mice (328-330).

Apart from the reported membrane protein SLC9A5, recent research suggests that the SCRIB/LLGL polarity complex regulates membrane trafficking of the SLC7A5/SLC3A2 amino acid transporter complex in cancer cells. SLC7A5/SLC3A2 plays a vital role in multiple cancers, indicating that the SCRIB/LLGL2 complex may have an important yet unknown role in promoting multiple malignancies (329-330). This finding also revealed that cancer cells can repurpose polarity protein complexes involved in normal cell polarity to enhance cell growth and treatment resistance to cancer.

5.3.3 Differences of metabolic profile between MCF-7 and MCF-7/TamR-1 cells

In addition to the findings from the untargeted PCA, which indicated that leucine was associated with the development of tamoxifen resistance in MCF-7 cells and could potentially contribute to the acquisition of tamoxifen resistance. A subsequent

OrbiSIMS depth profile analysis was conducted to investigate the differences in energy metabolism between MCF-7 and MCF-7/TamR-1 cells.

Compared to healthy cells, cancer cells exhibit a heightened demand for glucose. Even in the presence of oxygen, they derive energy through both aerobic oxidation and lactate fermentation, rather than relying solely on aerobic oxidation. Consequently, breast cancer cells may exploit the increased glucose demand and augmented glutaminolysis (252, 259, 327). Thus, targeted analysis of endogenous metabolites was performed to investigate whether specific ion alterations could reflect metabolic differences, particularly focusing on key molecules generated through metabolic processes between MCF-7 and MCF-7/TamR-1 cells.

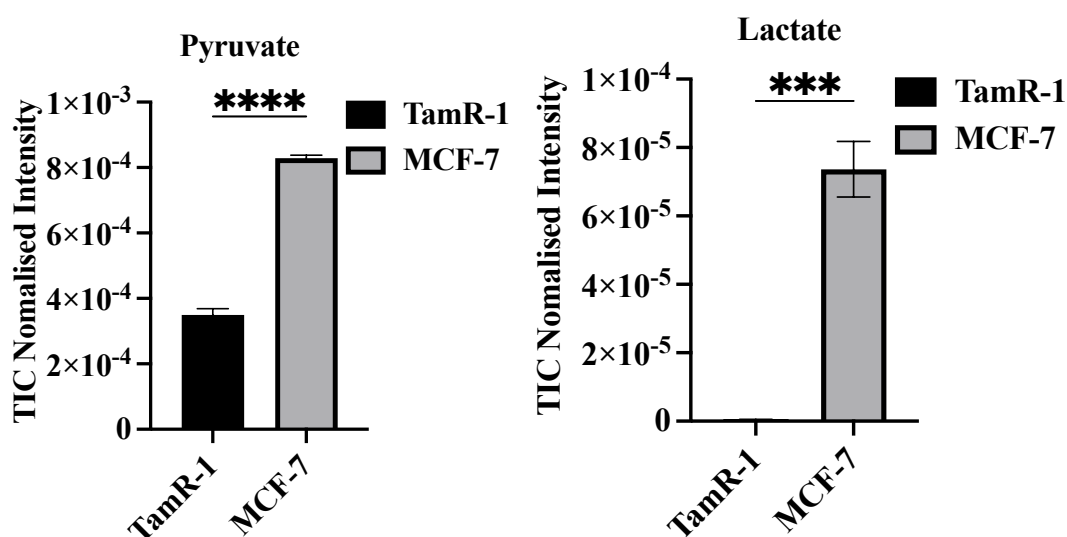


Figure 5.5 Deprotonated pyruvate molecule $[M-H]^-$ intensity and lactate molecule $[M-H]^-$ comparison between TamR-1 and MCF-7 cells. The bar chart is shown for each group and $n=3$ per treatment group.). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant

The findings revealed that the intracellular concentrations of pyruvate and lactate in the investigated cell line were significantly ($p < 0.0001$ for pyruvate and $p < 0.001$ for lactate) lower than those in the classical breast cancer cell line MCF-7. Given that glucose is metabolised into pyruvate and lactate during glycolysis, the low levels of these downstream metabolites observed in the OrbiSIMS depth profile suggest that the cell line may also exhibit reduced glucose levels (Figure 5.5). As the main source of energy, glucose is responsible for the production of these downstream molecules in

cellular metabolism. Therefore, the glucose peak (Figure 5.6) and other intermediates of TCA (Figure 5.7) were also examined. In comparison to the control group, which maintained relatively stable levels of these intermediates, the MCF-7/TamR-1 groups showed a markedly lower intensity.

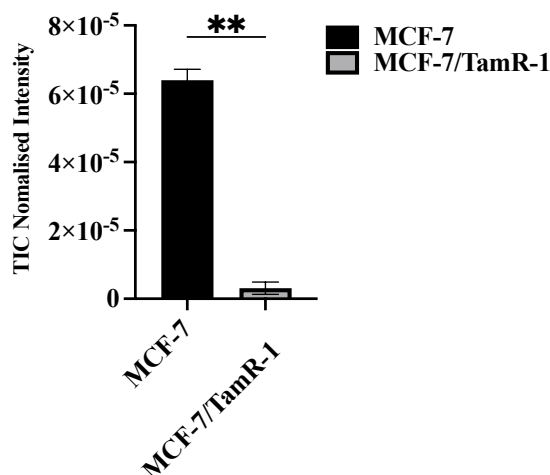


Figure 5. 6 Deprotonated glucose molecules $[M-H]^-$ intensity comparison between TamR-1 and MCF-7. The bar chart is shown for each group and n=3 per treatment group.). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant

The metabolic reprogramming observed in cancer cells with developed resistance supported the typical physiological functions associated with malignancy.

Consequently, metabolism in resistant cancer cells is expected to be augmented to support the higher demand for metabolism. As tumour cells exhibit high glycogen consumption, glucose concentrations within the tumour microenvironment are reduced, necessitating supplementary energy sources to maintain the normal physiological activity of these cancer cells (331).

In cancer-associated fibroblasts, overproduction of pyruvate and lactate occurs because of glycolysis, which consequently enhances mitochondrial activity, rendering neighbouring cancer cells resistant to various clinically employed medications (332), such as tamoxifen. In the present study, however, MCF-7/TamR-1 cells were cultured in isolation, thus not receiving any additional glucose from the microenvironment. This could potentially account for the observed lower intensities of pyruvate and lactate in the resistant cell line MCF-7/TamR-1 compared to MCF-7 cells.

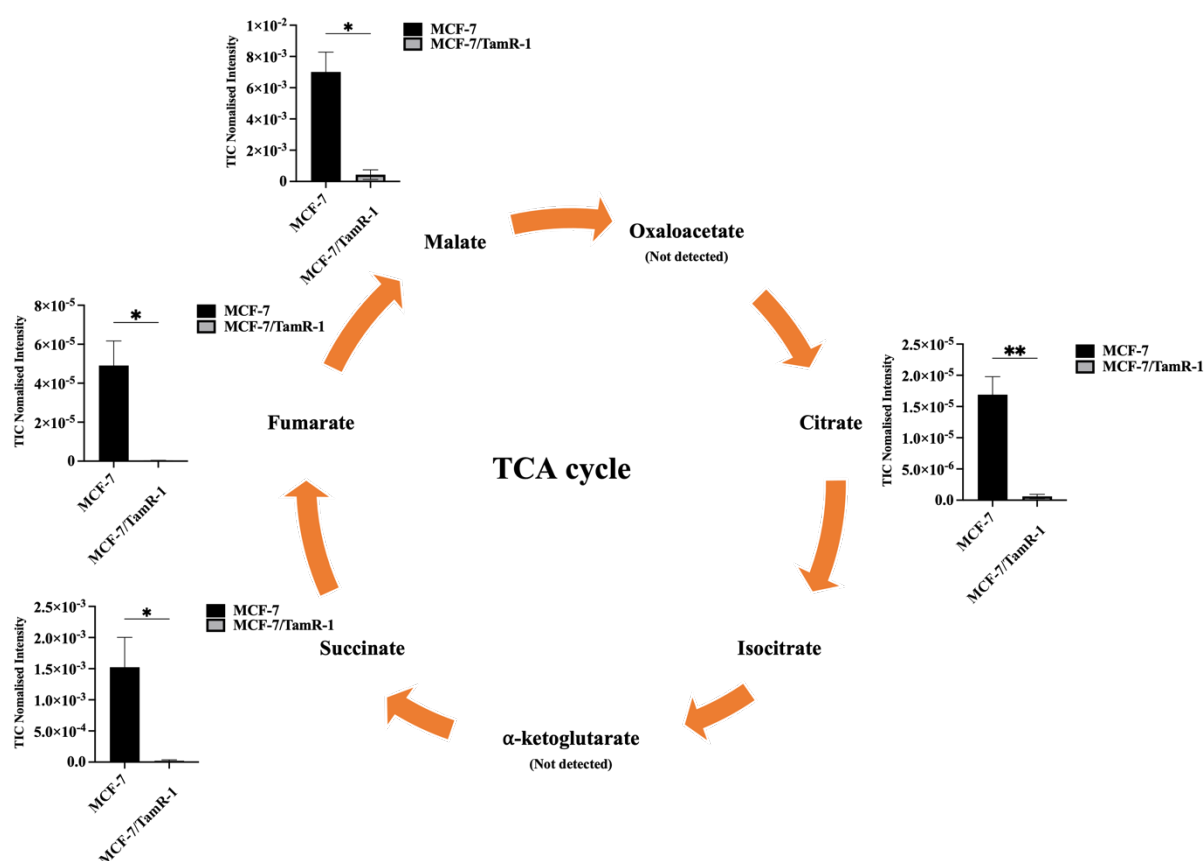


Figure 5. 7 Alterations of TCA cycle intermediates between MCF-7 and MCF-7/TamR-1 cells. All data are acquired by GCIB Ar3000+. The bar chart reflects normalized ion intensity (to total ion counts) values obtained from three separate experiments (mean $\pm$ s.d). Blots are representative of the data sets (N=3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant

Furthermore, the cytotoxicity assay results from the preceding section (Figure 5.1) demonstrated that the introduction of dasatinib in tamoxifen-resistant cells can restore the sensitivity of MCF-7/TamR-1 cells to tamoxifen. However, the intracellular intensity of tamoxifen did not increase in conjunction with dasatinib, suggesting that the enhanced cytotoxicity may be independent of tamoxifen, indicating the presence of an additional mechanism at play.

5.3.4 Effects of dasatinib on energy metabolism of MCF-7/TamR-1 cells

Following the comparison of MCF-7 and MCF-7/TamR-1 cells, the metabolic effects of the combination of dasatinib and tamoxifen were investigated to evaluate the potential impact of dasatinib on MCF-7/TamR-1 cellular metabolism. As a second-generation tyrosine kinase inhibitor, dasatinib targets BCR, Src, c-Kit receptors, and

other tyrosine kinases, such as ephrin receptors. Previous studies have demonstrated that dasatinib can induce apoptosis in MCF-7 cells and reduce reactive oxygen species production in these cancer cells (333-335). Owing to its antioxidant properties, dasatinib may represent a promising therapeutic strategy for cancer treatment.

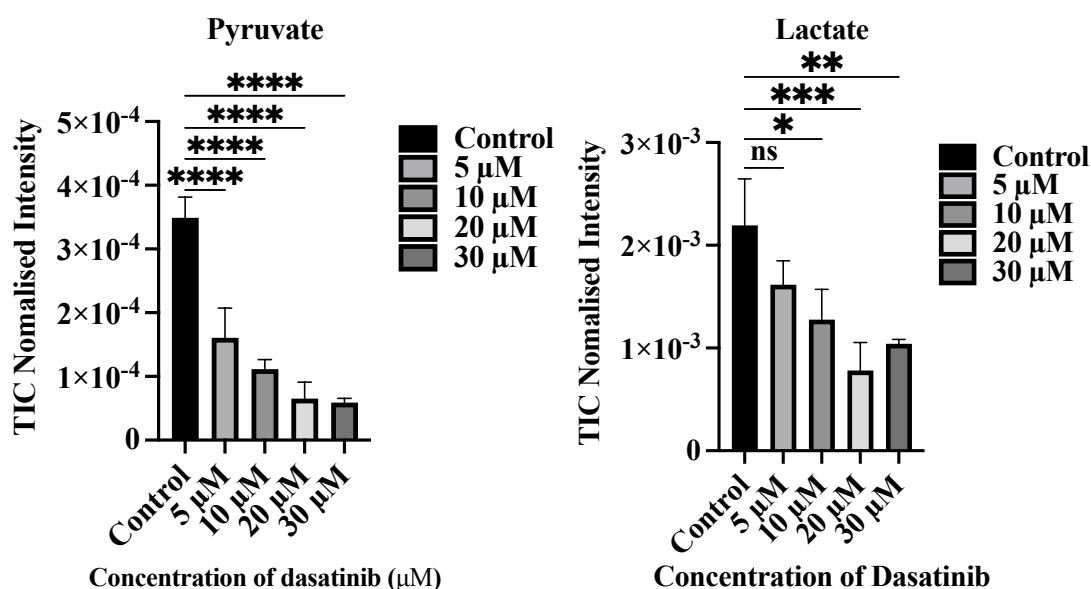


Figure 5. 8 Deprotonated pyruvate $[M-H]^-$ and lactate molecule $[M-H]^-$ intensity within increasing concentrations of dasatinib. Cells were treated by increased concentrations of dasatinib (5 μ M, 10 μ M, 20 μ M, 30 μ M) with a fixed concentration of tamoxifen (10 μ M). The bar chart reflects normalized ion intensity (to total ion counts) values obtained from three separate experiments (mean $\pm$ s.d). Blots are representative of the data sets (N=3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant

As depicted in the Figure 5.8, prior to the addition of dasatinib, MCF-7/TamR-1 cells were treated with a constant tamoxifen dose of 10 μ M, which did not have any observable impact on the cells. When dasatinib was introduced at the lowest concentration of 5 μ M, lactate intensity reached its highest level. However, as the concentration of dasatinib increased, the intracellular lactate intensity progressively declined, reaching minimum levels when dasatinib was present at a concentration of 20 μ M (Figure 5.8). Notably, when the dasatinib concentration was further elevated to a maximum of 30 μ M, there was a marginal increase in lactate intensity compared to that observed at 20 μ M.

Analogous to lactate, the ion intensity of pyruvate, another important product of cellular metabolism, was examined, and the pattern observed was similar to that of lactate. The highest intensity of pyruvate was observed at the lowest concentration of dasatinib (5 μ M). However, unlike lactate, which exhibited the lowest intensity at a dasatinib concentration of 20 μ M, pyruvate intensity displayed a slightly different trend. As the concentration of dasatinib increased, the intracellular pyruvate intensity consistently decreased, reaching minimal levels when the concentration of dasatinib reached a maximum of 30 μ M.

Lactate and pyruvate, the final products of the TCA cycle in energy metabolism, prompted an investigation into the response of other TCA cycle intermediates (335-336). Similar to the changes observed in lactate and pyruvate, TCA cycle intermediates exhibited substantial alterations in response to varying dosages of dasatinib. As depicted in the Figure 5.9, dasatinib treatment influenced the concentrations of crucial TCA cycle intermediates such as citrate, isocitrate, and succinate in a dose-dependent manner. In contrast, the control group maintained relatively stable levels of these intermediates, whereas the experimental groups experienced a gradual decline in concentrations with increasing dosages of dasatinib.

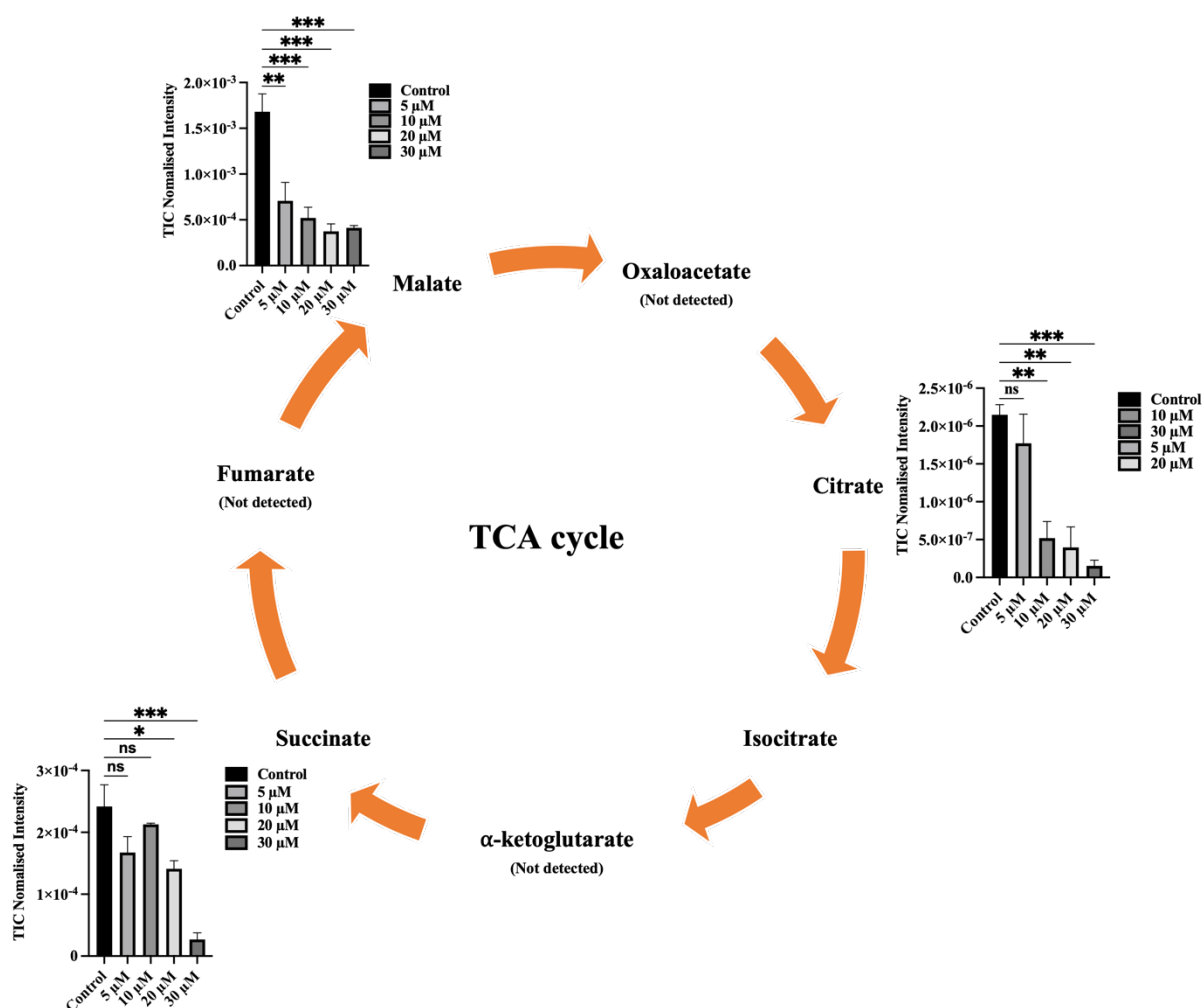


Figure 5. 9 Alterations of TCA cycle intermediates with increasing concentrations of dasatinib. All data are acquired by GCIB Ar3000+. Cells were treated by increased concentrations of dasatinib (5 μ M, 10 μ M, 20 μ M, 30 μ M) with fixed concentration of tamoxifen (10 μ M). The bar chart reflects normalized ion intensity (to total ion counts) values obtained from three separate experiments (mean $\pm$ s.d). Blots are representative of the data sets (N=3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant

The most pronounced decrease in TCA cycle intermediates was observed during the initial phase of dasatinib treatment (Figure 5.9). This phenomenon suggests a potential stress response in MCF-7/TamR-1 cells, as they attempt to adapt to metabolic perturbations induced by dasatinib. Over time, the cells appeared to partially adjust to these drug-induced metabolic changes, resulting in a less drastic decline in TCA cycle intermediate levels as the treatment continued. However, even

with extended dasatinib exposure, the concentrations of these intermediates remained substantially lower than those observed in the control group.

As a tyrosine kinase inhibitor, dasatinib has been used as a first-line therapy in the treatment of chronic myelogenous leukaemia (337). Therefore, dasatinib may play a substantial role in breast cancer progression. Triple-negative breast cancer, typically characterised as basal-type breast cancer, is affected by dasatinib. Moreover, according to Finn et al., basal-type and epithelial-to-mesenchymal transition (post-EMT) breast cancer cell lines exhibit greater responsiveness to the growth-limiting capabilities of dasatinib than do luminal-type breast cancer cell lines (338-339). Owing to the limited sensitivity of luminal breast cancer cell lines to dasatinib, the present study aimed to investigate the effects of dasatinib on multidrug resistance in the MCF-7 cell line, which represents a subtype of luminal breast cancer cell lines.

In this investigation, MCF-7/TamR-1 cells were found to be tamoxifen-resistant (Figure 5.1). Subsequent studies have demonstrated that reversal doses of dasatinib markedly enhance the cytotoxicity of tamoxifen in MCF-7/TamR-1 cells. Moreover, dasatinib notably decreased the accumulation of tamoxifen in resistant MCF-7/TamR-1 cells, which may have facilitated its efflux.

According to Yu (2019), dasatinib induced a shift in glucose metabolism from aerobic glycolysis to gluconeogenesis in patients with primary chronic lymphocytic leukaemia (340). Consequently, rather than directly inhibiting tamoxifen efflux, it can be hypothesised that dasatinib reverses tamoxifen resistance by suppressing metabolic pathways. This observation suggests an indirect inhibitory effect of dasatinib on cellular metabolism.

5.3.5 Co-localization of dasatinib and tamoxifen molecular ions visualised by OrbiSIMS imaging

In addition to integrating SIMS profiles with PCA to elucidate untargeted biological responses to pharmaceutical agents, OrbiSIMS offers a promising approach for mapping the intracellular distribution of anti-cancer compounds. This potential arises from the synergistic combination of ToF and Orbitrap analytical techniques.

Consequently, the speed and spatial resolution of ToF analysis are complemented by the superior mass-resolving capabilities of Orbitrap, resulting in optimal fusion of these two analytical methods.

MCF-7/TamR-1 cells were subjected to dasatinib treatment prior to freeze-drying and analysis to establish a method for simultaneous label-free drug mapping within cells. Using the LMIG and ToF analyser, a two-dimensional ion image was generated. To determine the spatial distribution of characteristic ions, a chemical ion image was obtained by mapping an area up to $250 \times 250 \mu\text{m}$. Analysis of the imaging results revealed a correlation between dasatinib (depicted in green) and MCF-7/TamR-1 cells (represented in red and blue in Figure 5.10). Notably, this approach has the potential to facilitate the evaluation of drug delivery efficiency at the cellular level following the introduction of novel therapeutic compounds (Figure 5.10).

As depicted in the Figure 5.10, ions associated with abundant chemicals in specific cellular structures were used for identification. The secondary ion fragment $\text{C}_5\text{H}_5\text{O}^+$ (m/z 81 Da), unique to K_2PO_4^+ within cells, was selected as the nuclear marker (indicated in red). K_2PO_4^+ , a major constituent of DNA and RNA structures, is found in relatively high concentrations within the nucleus (133). The phosphatidylcholine fragment from lipids (m/z 184 Da), represented by the blue colour, was employed as a cellular marker. The green colour illustrates the distribution of the intact ion of the compound, corresponding to the dasatinib signal.

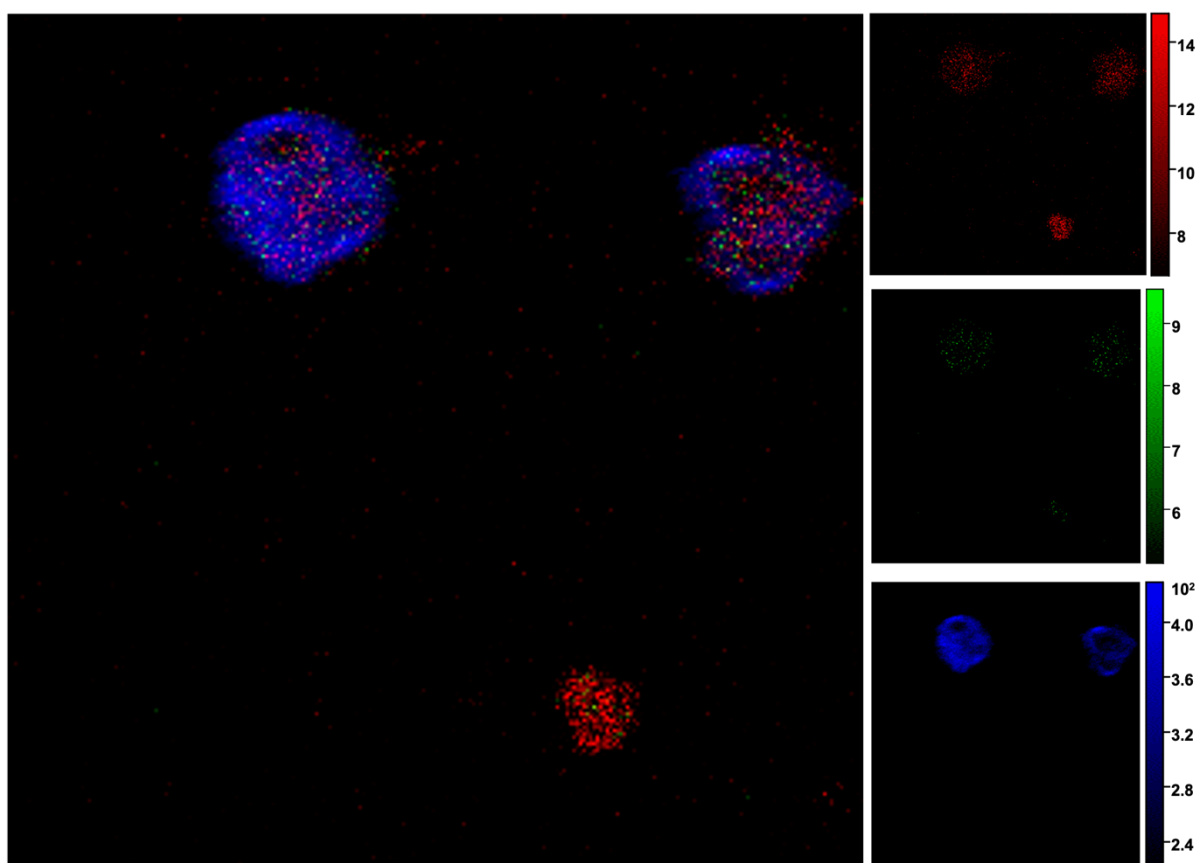


Figure 5. 10 The RGB overlaid ion images of tamoxifen incubated with MCF-7/TamR-1 cells. Nuclear was marked as red, phosphocholine fragment was marked as blue and drug molecule dasatinib was marked as green.

In the Figure 5.10, the signal intensity is denoted by the brightness of the colour; a brighter colour indicates a higher concentration of the selected ion per pixel. In this experiment, the pixel size was $0.5 \mu\text{m}^2$. Based on these results, a majority of the dasatinib signal (green) were accumulated within the larger MCF-7/TamR-1 cells, predominantly in the cytoplasm, as the primary target of dasatinib appears to be localised in the inner region of the membrane. It was also evident that the K_2PO_4^+ signal was concentrated in the nucleus, although it was not exclusive to this area. Although the nucleus contains an amount of K_2PO_4^+ , this molecule is also present in RNA and proteins, both of which are found in the cytoplasm. Consequently, the K_2PO_4^+ signal in the cytoplasm was diffuse, whereas the signal in the nucleus was more distinct.

Although the addition of dasatinib might help to overcome resistance, the depth profile revealed a decrease in intracellular tamoxifen intensity. Consequently, after mapping dasatinib independently in the resistant cancer cell lines, the localization of tamoxifen was investigated to determine whether intracellular tamoxifen consumption was diminished.

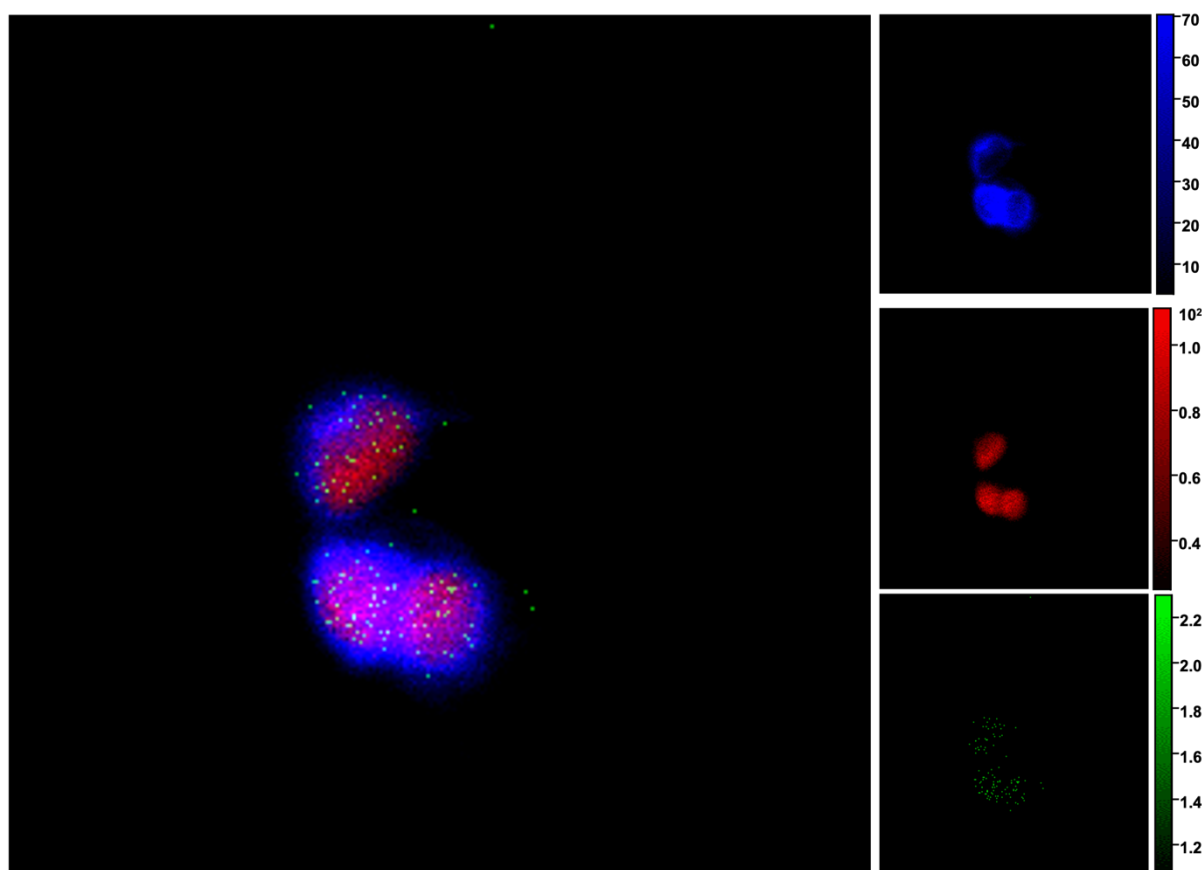


Figure 5. 11 The RGB overlaid ion images of tamoxifen incubated with MCF-7/TamR-1 cells. Nuclear was marked as red, phosphocholine fragment was marked as blue and drug molecule tamoxifen was marked as green.

Analogous to the outcomes observed from isolated dasatinib treatment, the data revealed that MCF-7/TamR-1 cells exhibited an increased accumulation of the compound (represented by green), predominantly localised in the cytoplasm. In this image analysis, the ribose fragment ($C_5H_5O^+$) served as a nuclear marker. It is evident that the ribose signal exhibits a higher concentration within the nucleus (341-342). As depicted in Figure 5.11, upon the introduction of dasatinib, the protonated tamoxifen

[M+H]⁺ molecules were detected. However, the intracellular delivery of tamoxifen to tamoxifen-resistant cancer cells does not undergo substantial alterations, as evidenced by depth profile analysis.

The imaging results demonstrated that the co-culture of dasatinib and tamoxifen did not enhance the accumulation of tamoxifen, which is consistent with the depth profile findings. This dual outcome suggests the existence of a mechanism potentially independent of tamoxifen. Previous research supports the interaction between the ER and Src kinase, which promotes nuclear export by phosphorylating ER at tyrosine 537. Inhibition of Src prevents this action, leading to nuclear sequestration of the ER (343-347). This observation may aid in elucidating the growth-promoting effect of dasatinib in wild-type MCF-7 cells in the absence of oestradiol (E2), enabling the residual nuclear receptor to continue binding oestrogen response elements and promoting cell survival. This increase in transcription was also observed in the MCF7-TAMR cell line; however, it appeared insufficient to counteract the upstream inhibitory effects on ERK1/2 and AKT, resulting in an overall decrease in proliferation. This phenomenon can be partially explained by the cellular context. Src and ER have been reported to interact with PELP-1 and phosphatidylinositol-3-kinase (PI3K) at the cell membrane, activating the ERK1/2 and AKT pathways, which subsequently leads to a marked reduction in proliferation (348-349).

5.3.6 Alterations in biomarker level expression following dasatinib treatment

Advanced OrbiSIMS instrumentation provides three-dimensional information on targeted compounds, visualising and evaluating whether an analyte is able to achieve its target. Owing to the significance of biomarkers, changes in the eight lipids identified in the previous section were also investigated during combination treatment with tamoxifen and dasatinib. As illustrated in the Table 4 presented in the previous chapter (Chapter 4.5.6), these eight biomarkers primarily belonged to lipid families. Lipids, proteins, and nucleic acids are essential components of biological membranes and cellular building blocks (350-351). Lipids serve as crucial signalling molecules in various cellular processes. Regulation of lipid metabolism, including lipid absorption, synthesis, and hydrolysis, is vital for maintaining cellular homeostasis. Cancer cells exploit lipid metabolism to support their rapid proliferation, survival, migration,

invasion, and metastasis within tumour microenvironments, where nutrient supply constantly fluctuates throughout tumour evolution (352).

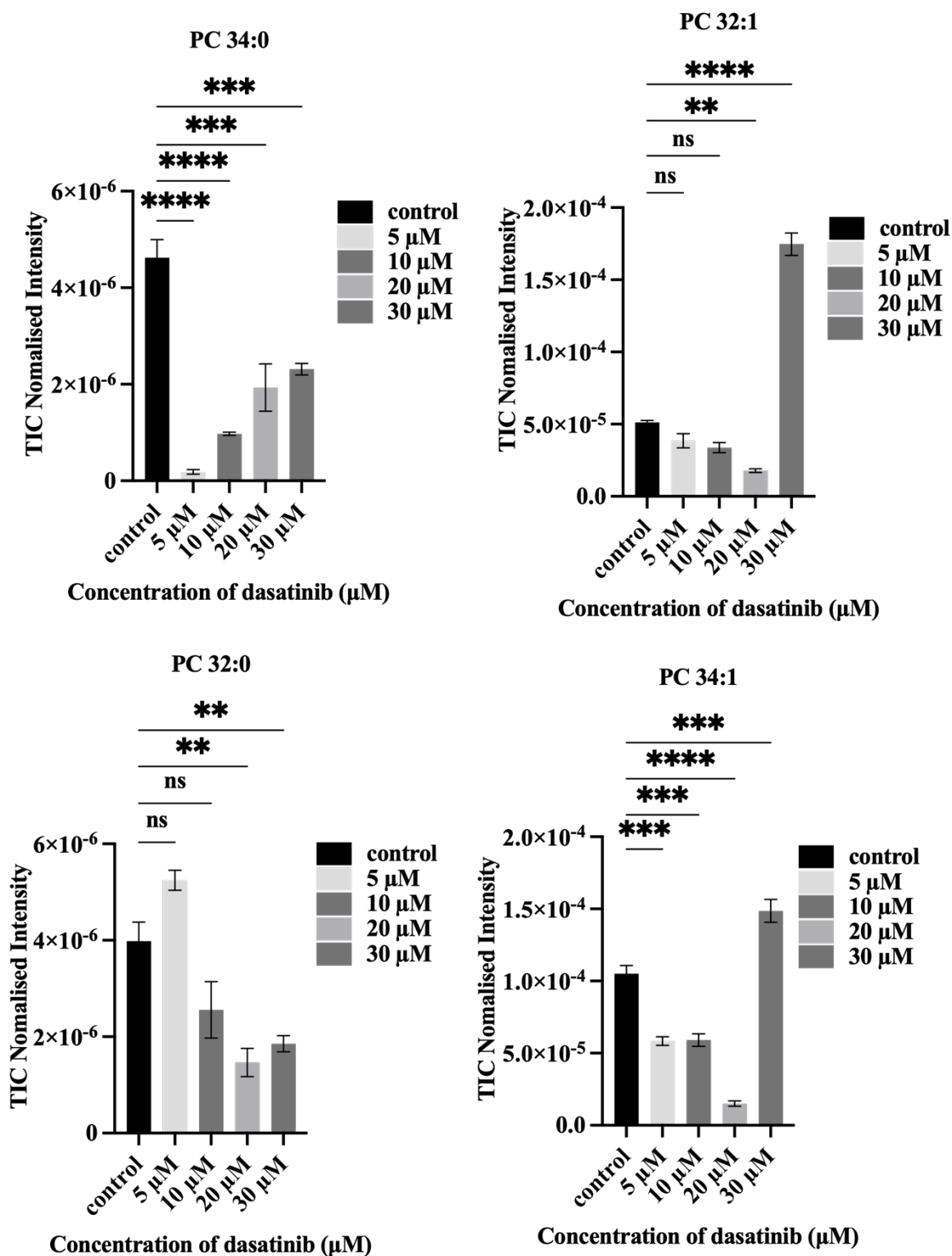


Figure 5. 12 Statistical comparisons of biomarkers (PC34:0, PC32:1, PC32:0, PC34:1) analysed in MCF-7/TamR1 cells for control versus different doses of dasatinib plus 10 μM of tamoxifen. Error bar shows standard error of the mean (n=3 for each group). The

ion intensity was normalised to total ion counts. PC, phosphatidylcholine.

In Figure 5.12, changes in the four phosphatidylcholine (PC) biomarkers are presented, each exhibiting different levels of alteration. PC (32:1) and PC (34:1) displayed a similar trend: intensities gradually decreased between the control and the 20 μ M dasatinib treatment; however, when the dasatinib concentration was increased to 30 μ M, the cellular intensity of PC (32:1) and PC (34:1) sharply increased. In contrast, PC (34:0) exhibited a pattern with an enhancement that, although not higher than that of the control group, was noteworthy. No significant pattern was observed for PC (32:0). The considerable impact of dasatinib on these biomarkers was evident, despite the lack of patterns for all four biomarkers.

In addition, the changes in phosphatidylinositol (PI) biomarkers did not present a substantial pattern (Figure 5.13). However, when dasatinib was introduced, PI intensity increased compared to that in the control group. Notably, PI intensities reached their lowest level when concentration of dasatinib was at the IC₅₀ therapeutic dose of 20 μ M.

As discussed in the previous chapter, the suppression of PC and PI levels resulting from anti-cancer treatments may induce apoptosis by regulating the PLC and PI3K pathways (287-288; 348-349). However, this mechanism may differ in tamoxifen-resistant breast cancer. The reduction in PC and PI levels can potentially disrupt their molecular and biomolecular interactions, as they form lipid rafts in cells, which serve as functional platforms for protein molecule interactions in tamoxifen-resistant cells.

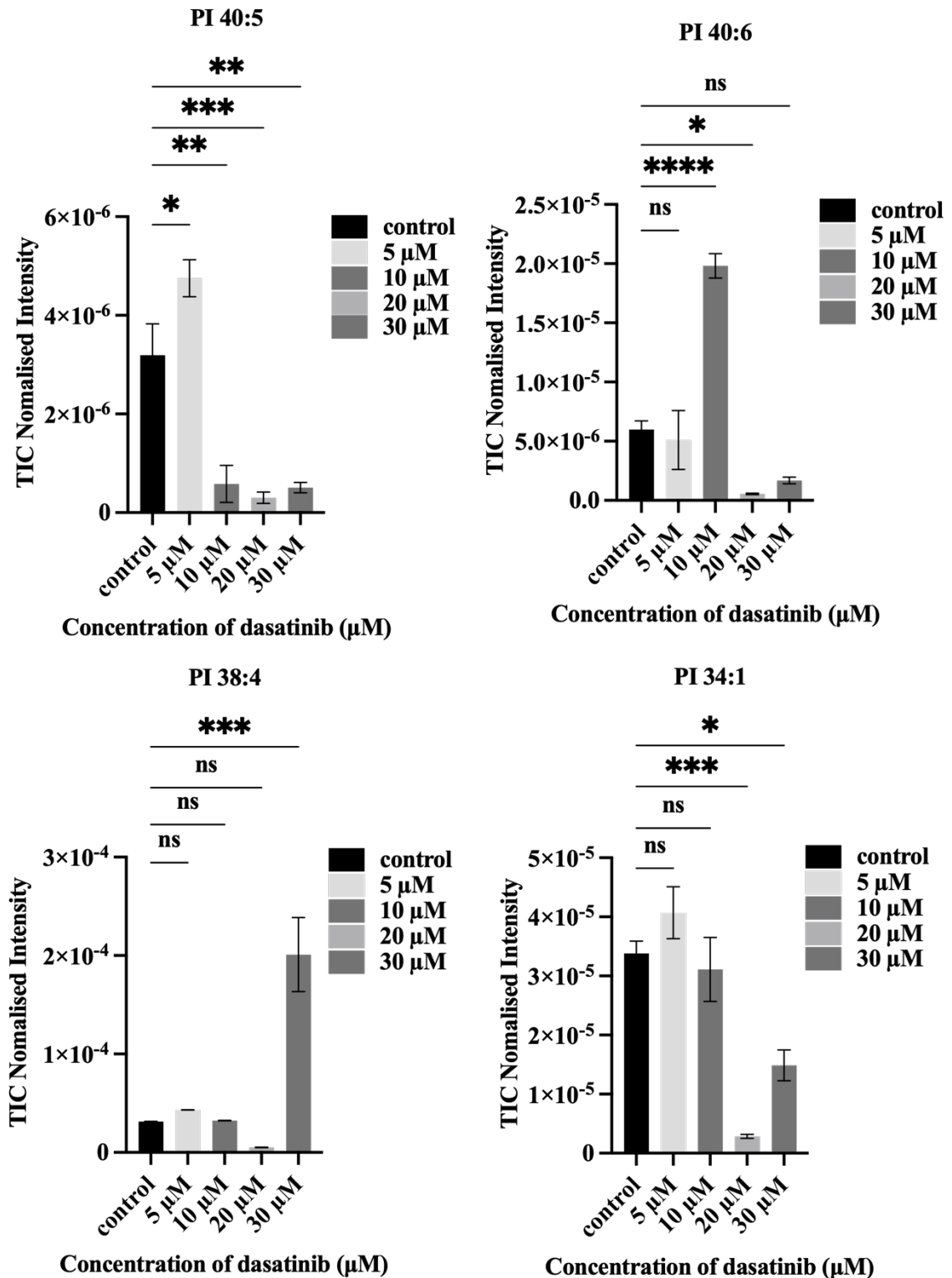


Figure 5. 13 Statistical comparisons of biomarkers (PI 40:5; PI 40:6; PI 38:4; PI 34:1) analysed in MCF-7/TamR1 cells for control versus different doses of dasatinib plus 10 μM of tamoxifen under negative mode. Error bar shows standard error of the mean (n=3 for each group). The ion intensity was normalised to total ion counts. PI, phosphatidylinositol

The disruption of lipid rafts may result in the malfunction of membrane proteins, including the FAT/CD36 protein, which belongs to the type B scavenger receptor family and is responsible for fatty acid uptake (353-354). High levels of FAT/CD36 expression can attenuate the inhibitory effect of tamoxifen in MCF-7 cells, and it is overexpressed in MCF-7/TamR-1 cells (354). Hence, FAT/CD36 potentially plays a key role in the development of tamoxifen-resistant breast cancer. Previous studies have suggested that dasatinib can reduce FAT/CD36 expression in chronic myeloid leukaemia cells, potentially inhibiting it in MCF-7/TamR-1 cells and blocking the Src/PI3K signalling pathway (355-357). This, consequently, increases apoptosis in tamoxifen-resistant cells. Although more biomolecular research is needed to validate these findings, they provide novel insights into how dasatinib interacts with tamoxifen-resistant breast cancer.

5.4 Conclusion

Endocrine resistance in breast cancer is caused by various processes, some of which remain unknown. Targeting key resistance pathways is essential for improving the survival of patients with breast cancer. This chapter presents a successful analysis of metabolic profiles derived from tamoxifen-resistant breast cancer cells using the OrbiSIMS workflow, that was developed in the previous chapter, to explore potential changes in metabolism using combination treatment with tamoxifen and dasatinib. Additional bio-evaluation methods, such as the cytotoxicity assay, were used to differentiate the response of tamoxifen-resistant MCF-7/TamR-1 cells to the anti-cancer agents - tamoxifen or dasatinib along, or the combination treatment of both.

The method developed in Chapter 3 for MCF-7 Cells was successfully applied in this Chapter for comparing metabolic profiles in tamoxifen-sensitive MCF-7 cells. Leucine appears to be a key driver of tamoxifen-resistant cell growth, which is consistent with previous reports suggesting that high leucine levels may play a role in the development of tamoxifen resistance. Overall, Tamoxifen-resistant MCF-7/TamR-1 cells did not possess higher intracellular tamoxifen intensity, and the addition of dasatinib did not enhance intracellular tamoxifen intensity. This indicates that the interaction of dasatinib with other potential mechanisms may be mainly responsible for tamoxifen resistance. The evidence that a combination of tamoxifen with an Src

inhibitor resulted in complete growth arrest and cell death in tamoxifen-resistant cell line supports the idea that tamoxifen treatment should precede dasatinib therapy to prevent ER-independent growth.

This study produced unprecedented 3D chemical profiles, highlighting the distribution of anti-cancer agents in targeted cancer cell line. The successful use of OrbiSIMS to analyse cell samples is thought to be the first of its kind, as it had previously only been accomplished for highly sensitive compounds in cancer cell models.

Furthermore, the method developed in Chapter 3 was used to monitor depth profiling and imaging experiments, suggesting that dasatinib can act on the metabolism of MCF-7/TamR-1 cells independently of tamoxifen.

Chapter 6. Conclusion and future perspectives

6.1 Chapter Summary

The thesis described herein provides a pioneering in situ approach for profiling endogenous metabolites present in cancer cells. This was achieved through the utilization of an Ar3000+ cluster primary ion beam, which enabled the identification of distinctive endogenous responses in an in vitro cellular model. In addition, this research linked these observed responses with the administration of assorted anti-cancer drugs, employing 3D OrbiSIMS technology for an in-depth examination. These conducted studies hold significant importance for monitoring cellular drug concentrations under varying treatment conditions, while also probing the intrinsic responses to pharmaceutical compounds. A comprehensive understanding of these endogenous responses can prove instrumental in evaluating the potency of drugs that specifically target malignant cells, hence mitigating detrimental off-target effects on normal tissues. The capability to extract biomolecular information from the intrinsic responses of cancer cells to anti-cancer therapeutics will bolster advancements in cancer research. It is anticipated that the unique chemical specificity and imaging capacities of OrbiSIMS will furnish novel perspectives within this field.

During this doctoral research, techniques were established for the direct examination of metabolic patterns in human monocyte breast cancer cells at the cellular level. These methods also studied the cells' reactions to specific anti-cancer agent treatments in vitro using 3D OrbiSIMS, which are elaborated upon in Chapter 4 and Chapter 5. Notably, the OrbiSIMS technique enables the detection of unique metabolites in individual macrophages. This method is efficient in identifying lipids, amino acids, and smaller molecules, bypassing the need for liquid extraction or damaging sample preparations, as seen in LC-MS/MS methods. This method could theoretically visualize metabolite distribution, though this potential wasn't fully tapped into due to the faint signals from most metabolites. A striking benefit of OrbiSIMS is that it needs considerably fewer cells than conventional mass spectrometry methods. The cells also remain undisturbed on their original base. Additionally, OrbiSIMS can analyse cells directly on the surface without chemical fixation or the need for antibody-based markers.

The superior mass resolving power of OrbiSIMS enables the precise assignment of metabolic signatures. When comparing its spectra to that of traditional ToF-SIMS, common peaks between the two SIMS systems were identified. Notably, OrbiSIMS displays sharp and significant ion peaks. Additionally, when examining the endogenous metabolite and anti-cancer agents, our findings differed from existing studies; while many suggest a correlation between specific physicochemical properties (like logP and logD) and SIMS ionisation, we found no such link. Given the intricate nature of SIMS data, we employed Principal Component Analysis (PCA) to discern ion changes and differentiate treatment data using OrbiSIMS. The supplemental SIMS-MFP analysis further identified crucial peaks relevant to oncology. OrbiSIMS also showcased its proficiency in mapping drug molecule distribution at a subcellular level. Our investigation into sample preparation using OrbiSIMS led to the optimized identification of anti-cancer agents, as detailed in Chapter 3.

After that the capacity of OrbiSIMS to oversee responses of endogenous metabolites to pharmacological interventions and its semi-quantitative potential was assessed (Chapter 4). Leveraging methodologies delineated in prior chapters, the effects of anti-cancer agents, notably Tamoxifen and lapatinib, on cellular energetic metabolism were scrutinized. In a bid to validate the quantitative precision of OrbiSIMS, diverse experimental paradigms were employed, shedding light on its adeptness in detecting perturbations in metabolites inherent to human breast carcinoma, particularly post anti-cancer therapeutic regimens. When delineating the detection threshold of OrbiSIMS, the instrument exhibited the capacity to discern drug dilutions up to a factor of 2000. Our empirical endeavours led to the novel discovery of the impact of oncological pharmaceuticals on MCF-7 cellular metabolic pathways. Concurrently, our analysis converged with prior research in identifying eight salient biomarkers associated with the lipid classes PC and PI, integral to neoplastic cell proliferation dynamics.

In the end, the OrbiSIMS methodology, was further employed to assess the metabolic profiles of tamoxifen-resistant breast cancer cells (Chapter 5). Specifically, the study examined the metabolic implications of a combined treatment regimen of tamoxifen and dasatinib. Notably, MCF-7/TamR-1 cells resistant to tamoxifen did not exhibit an

augmented intracellular tamoxifen presence upon dasatinib inclusion, leading to the inference that dasatinib might engage with alternative pathways to potentiate tamoxifen resistance. This investigation underscored leucine's critical role in the proliferation of tamoxifen-resistant cells and pioneered the use of OrbiSIMS for detailed 3D chemical profiling in cell sample analysis.

6.2 Future perspectives

In summary, this research offers essential insights into the efficacy of anticancer drugs, creating a robust foundation for further exploration of cancer using OrbiSIMS. In addition, this technique has shown exceptional capacity in collecting detailed information from ex vivo samples.

Evidently, OrbiSIMS exhibits the required high sensitivity to discern changes during the infiltration of anti-cancer medications, an advantage that has proven advancement with conventional chromatographic techniques. This opens up an unprecedented avenue to study the behaviour of drugs relevant to cancer therapy. Acquiring such detailed data concerning drug response changes could help clarify the molecular mechanisms underlying pharmacological drug function and shed light on the impacts of both intrinsic and extrinsic influences.

The development of a dynamic SIMS approach for generating 3D chemical depth profiles of ex vivo breast cancer cells represent a significant advance in cancer research. The blend of high chemical specificity, mass resolution, and both lateral and axial resolution offered by this technology is unparalleled. This research has exhibited the potential of this method in mechanism investigations to deliver comprehensive data about a drug's distribution within cancer cells, particularly its profile across breast cancer cell membranes. However, the inability to acquire quantitative data is a fundamental limitation of this technique for monitoring substance penetration. Thus, to establish a relationship between ion intensity and relevant compound concentration, a calibration method needs to be developed. The creation of a unique approach to produce biological matrices holds considerable promise in this domain.

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Appendix

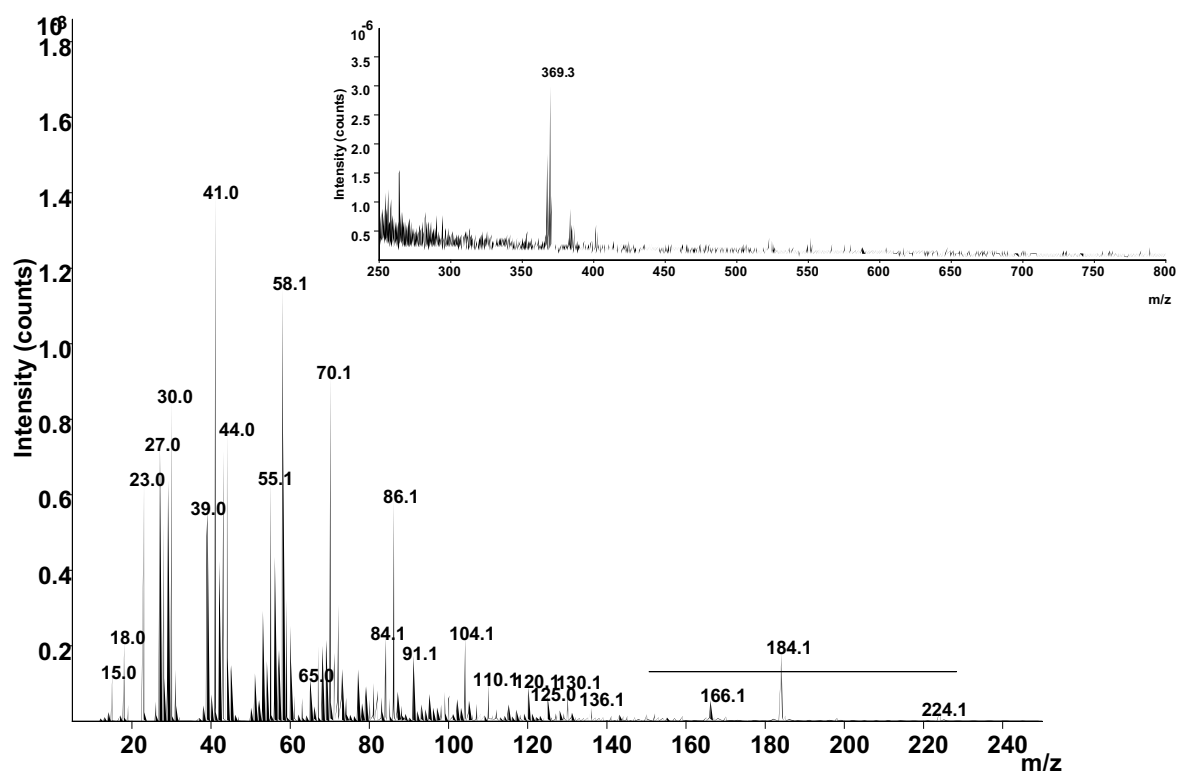


Figure S 1 Analysis of bovine liver homogenate BCR-185R using ToF-SIMS. Positive polarity was recorded using the Bi^{3+} as primary ion gun, Ar^{1700+} as sputter ion source. The significant peaks was labelled with assigned mass.

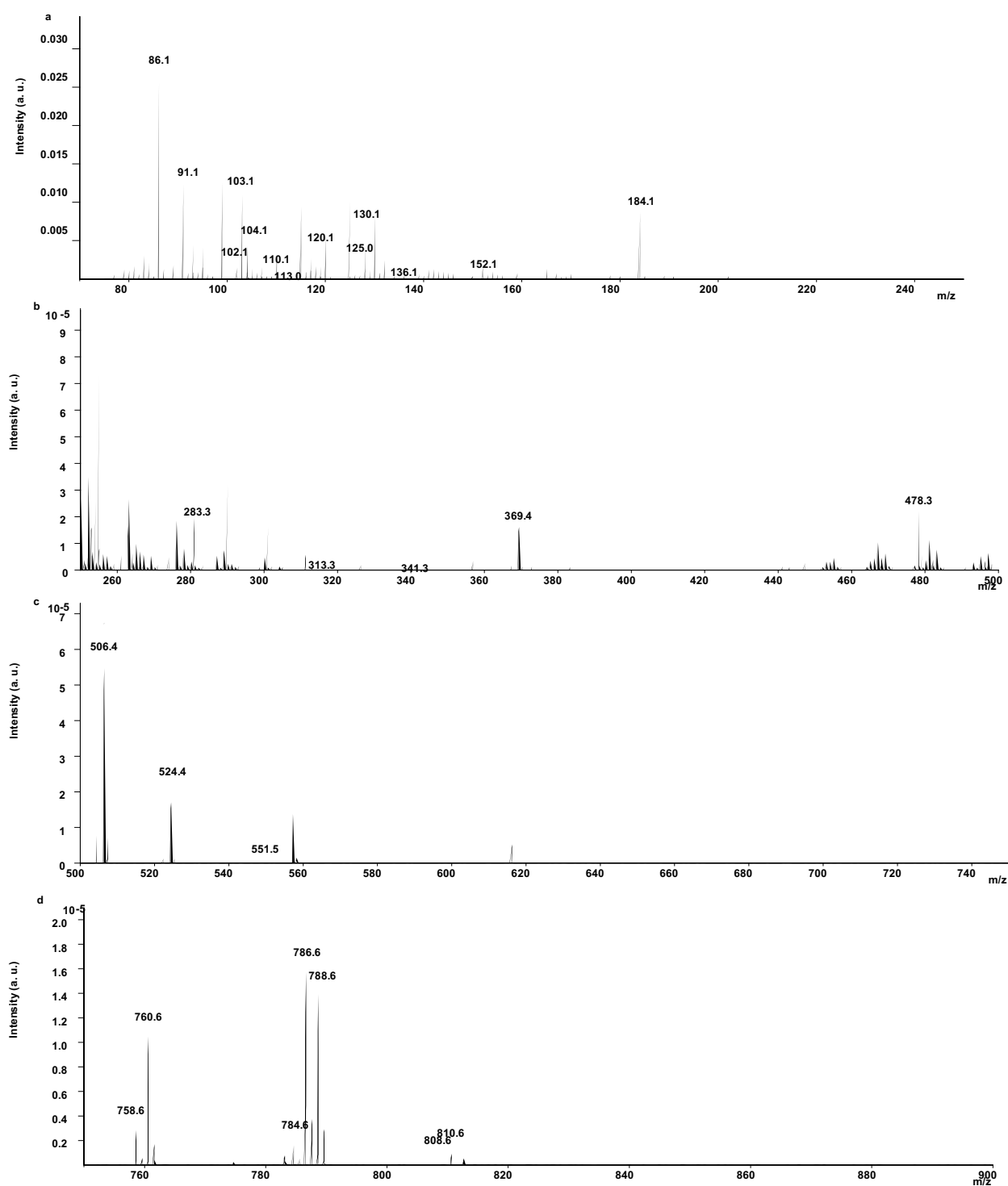


Figure S 2 Direct Analysis of bovine liver homogenate BCR-185R using OrbiSIMS. Positive polarity was recorded using Orbitrap analyser. Ar₃₀₀₀⁺ used as primary ion source. a) m/z 75-250; b) m/z 250-500; c) m/z 500-750; d) m/z 750-900

Table S 1. Endogenous metabolite identified by ToF-SIMS and OrbiSIMS

Metabolite	SIMS analysis		
	OrbiSIMS Ar ₃₀₀₀ ⁺	ToF-SIMS Bi ³⁺	
Adenine	+	Not detected	
Adenosine monophosphate	+	Not detected	
Arginine fragment	+	Not detected	
Asparagine	+	Not detected	
Aspartic acid fragment	+	Not detected	
C16 Taurine	+	Not detected	
Cytosine	+	Not detected	
Dopamine	+		
Gamma-aminobutyric acid	+	Not detected	
Glutamic acid fragment	+	Not detected	
Glutamine	+	Not detected	
Glycerol-3-phosphate	+	Not detected	
Guanosine monophosphate	+	Not detected	
Guanine	+	Not detected	
Histidine	+	Not detected	
Hypoanthine	+	Not detected	
Lactic acid	+	+	
Leucine	+	+	
Lysine	+	+	
Methylcytosine	+	Not detected	
Phenylalanine	+	Not detected	
Phosphocholine-CH3	+	Not detected	
Proline	+	+	
Pyruvic acid	+	Not detected	
Thymine	+	Not detected	
Tryptophan	+	Not detected	
Tyrosine	+	+	
Uracil	+	+	
Valine	+	+	
Lipid	Formula	OrbiSIMS Ar ₃₀₀₀ ⁺	ToF-SIMS Bi ³⁺
PC 18:0	C ₄₄ H ₈₉ NO ₈ P ⁺	+	Not detected
PC 32:1	C ₄₀ H ₇₉ NO ₈ P ⁺	+	Not detected
PC 32:0	C ₄₀ H ₈₁ NO ₈ P ⁺	+	Not detected
PE 36:1	C ₄₁ H ₈₁ NO ₈ P ⁺	+	Not detected
PC 34:2	C ₄₂ H ₈₁ NO ₈ P ⁺	+	+
PC 34:1	C ₄₂ H ₈₃ NO ₈ P ⁺	+	+

PC 34:0	C ₄₂ H ₈₅ NO ₈ P ⁺	+	+
PC 34:0+Na	C ₄₂ H ₈₄ NO ₈ PNa ⁺	+	Not detected
PC 36:2	C ₄₄ H ₈₅ NO ₈ P ⁺	+	Not detected
PC 36:1	C ₄₄ H ₈₇ NO ₈ P ⁺	+	Not detected
PC 36:1+Na	C ₄₄ H ₈₆ NO ₈ PNa ⁺	+	Not detected
PC 38:5	C ₄₆ H ₈₃ NO ₈ P ⁺	+	+
PI 34:1	C ₄₃ H ₈₀ O ₁₃ P ⁻	+	Not detected
PI 34:2	C ₄₃ H ₇₈ O ₁₃ P ⁻	+	Not detected
PI 34:3	C ₄₃ H ₇₆ O ₁₃ P ⁻	+	+
PI 36:4	C ₄₅ H ₇₈ O ₁₃ P ⁻	+	+
PI 38:1	C ₄₇ H ₈₈ O ₁₃ P ⁻	+	+
PI 38:2	C ₄₇ H ₈₆ O ₁₃ P ⁻	+	Not detected
PI 38:3	C ₄₇ H ₈₄ O ₁₃ P ⁻	+	+
PI 38:4	C ₄₇ H ₈₂ O ₁₃ P ⁻	+	Not detected
PI 38:5	C ₄₇ H ₈₀ O ₁₃ P ⁻	+	Not detected
PA 34:1	C ₃₇ H ₇₀ O ₈ P ⁻	+	Not detected
PA 34:2	C ₃₇ H ₆₈ O ₈ P ⁻	+	Not detected
PA 34:3	C ₃₇ H ₆₆ O ₈ P ⁻	+	Not detected
PA 36:1	C ₃₉ H ₇₄ O ₈ P ⁻	+	Not detected
PA 36:2	C ₃₉ H ₇₂ O ₈ P ⁻	+	Not detected
PA 36:3	C ₃₉ H ₇₀ O ₈ P ⁻	+	Not detected
PA 36:4	C ₃₉ H ₆₈ O ₈ P ⁻	+	Not detected
PA 36:5	C ₃₉ H ₆₆ O ₈ P ⁻	+	Not detected
PA 38:2	C ₄₁ H ₇₆ O ₈ P ⁻	+	Not detected
PA 38:3	C ₄₁ H ₇₄ O ₈ P ⁻	+	Not detected
PA 38:4	C ₄₁ H ₇₂ O ₈ P ⁻	+	Not detected
PS 36:3	C ₄₂ H ₇₅ NO ₁₀ P ⁻	+	Not detected
PS 36:2	C ₄₂ H ₇₇ NO ₁₀ P ⁻	+	Not detected
PS 36:1	C ₄₂ H ₇₉ NO ₁₀ P	+	Not detected
PS 38:3	C ₄₄ H ₇₉ NO ₁₀ P ⁻	+	Not detected
PS 40:6	C ₄₆ H ₇₇ NO ₁₀ P ⁻	+	Not detected

PS 40:5	C ₄₆ H ₇₉ NO ₁₀ P-	+	Not detected
PS 40:4	C ₄₆ H ₈₁ NO ₁₀ P-	+	Not detected
PE 34:1	C ₃₉ H ₇₅ NO ₈ P-	+	+
PE 34:2	C ₃₉ H ₇₃ NO ₈ P-	+	+
PE 36:1	C ₄₁ H ₇₉ NO ₈ P-	+	+
PE 36:2	C ₄₁ H ₇₇ NO ₈ P-	+	Not detected
PE 36:3	C ₄₁ H ₇₅ NO ₈ P-	+	Not detected
PE 38:3	C ₄₃ H ₇₉ NO ₈ P-	+	Not detected
PE 38:4	C ₄₃ H ₇₇ NO ₈ P-	+	Not detected
FA 12:0	C ₁₂ H ₂₃ O ₂ -	+	Not detected
FA 12:1	C ₁₂ H ₂₁ O ₂ -	+	Not detected
FA 12:2	C ₁₂ H ₁₉ O ₂ -	+	Not detected
FA 12:3	C ₁₂ H ₁₇ O ₂ -	+	Not detected
FA 12:4	C ₁₂ H ₁₅ O ₂ -	+	Not detected
FA 12:5	C ₁₂ H ₁₃ O ₂ -	+	Not detected
FA 12:0	C ₁₄ H ₂₇ O ₂ -	+	+
FA 12:1	C ₁₄ H ₂₅ O ₂ -	+	Not detected
FA 12:2	C ₁₄ H ₂₃ O ₂ -	+	Not detected
FA 14:0	C ₁₄ H ₂₁ O ₂ -	+	Not detected
FA 14:1	C ₁₄ H ₁₉ O ₂ -	+	Not detected
FA 14:2	C ₁₆ H ₃₁ O ₂ -	+	Not detected
FA 14:3	C ₁₆ H ₂₉ O ₂ -	+	Not detected
FA 14:4	C ₁₆ H ₂₇ O ₂ -	+	+
FA 16:0	C ₁₆ H ₂₅ O ₂ -	+	Not detected
FA 16:1	C ₁₈ H ₃₅ O ₂ -	+	Not detected
FA 16:2	C ₁₈ H ₃₃ O ₂ -	+	Not detected
FA 16:3	C ₁₈ H ₃₁ O ₂ -	+	Not detected
FA 18:0	C ₁₈ H ₂₉ O ₂ -	+	Not detected
FA 18:1	C ₁₈ H ₂₇ O ₂ -	+	Not detected
FA 18:2	C ₁₈ H ₂₅ O ₂ -	+	Not detected
FA 18:3	C ₂₀ H ₃₉ O ₂ -	+	Not detected
FA 18:4	C ₂₀ H ₃₇ O ₂ -	+	Not detected
FA 18:5	C ₂₀ H ₃₅ O ₂ -	+	Not detected
FA 20:0	C ₂₀ H ₃₃ O ₂ -	+	Not detected
FA 20:1	C ₂₀ H ₃₇ O ₂ -	+	Not detected
FA 20:2	C ₂₀ H ₃₅ O ₂ -	+	Not detected
FA 20:3	C ₂₀ H ₃₃ O ₂ -	+	+
FA 20:4	C ₂₀ H ₃₁ O ₂ -	+	Not detected
FA 20:5	C ₂₀ H ₂₉ O ₂ -	+	Not detected
FA 22:0	C ₂₂ H ₄₃ O ₂ -	+	Not detected
FA 22:1	C ₂₂ H ₄₁ O ₂ -	+	Not detected
FA 22:2	C ₂₂ H ₃₉ O ₂ -	+	Not detected

FA 22:3	$C_{22}H_{37}O_2^-$	+	Not detected
FA 22:4	$C_{22}H_{35}O_2^-$	+	+
FA 22:5	$C_{22}H_{33}O_2^-$	+	Not detected
FA 22:6 DHA	$C_{22}H_{31}O_2^-$	+	Not detected
FA 24:1	$C_{24}H_{45}O_2^-$	+	+
FA 24:0	$C_{24}H_{47}O_2^-$	+	+