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Review

Host cell environments and antibiotic efficacy in tuberculosis

Nathan J. Day,¹ Pierre Santucci,^{1,2} and Maximiliano G. Gutierrez^{1,*}

The aetiologic agent of tuberculosis (TB), *Mycobacterium tuberculosis* (Mtb), can survive, persist, and proliferate in a variety of heterogeneous subcellular compartments. Therefore, TB chemotherapy requires antibiotics crossing multiple biological membranes to reach distinct subcellular compartments and target these bacterial populations. These compartments are also dynamic, and our understanding of intracellular pharmacokinetics (PK) often represents a challenge for antitubercular drug development. In recent years, the development of high-resolution imaging approaches in the context of host–pathogen interactions has revealed the intracellular distribution of antibiotics at a new level, yielding discoveries with important clinical implications. In this review, we describe the current knowledge regarding cellular PK of antibiotics and the complexity of drug distribution within the context of TB. We also discuss the recent advances in quantitative imaging and highlight their applications for drug development in the context of how intracellular environments and microbial localisation affect TB treatment efficacy.

The challenge of targeting Mtb as an intracellular pathogen

TB, caused by Mtb, is primarily a pulmonary disease but it is not confined solely to the lungs. Extrapulmonary TB can manifest at multiple sites across the body, spreading via the bloodstream or lymphatic system to virtually any organ [1]. This can include the lymph nodes, causing lymphadenitis; the central nervous system, leading to tuberculous meningitis or brain abscesses; or the skeletal system resulting in Pott's disease or other bone and joint infections. Within this diverse array of tissue environments, the infection can establish itself either at the interface of host cells or within host cells, as an intracellular infection.

Mtb has evolved a wide variety of sophisticated strategies to evade the immune system and proliferate within diverse host cell types [2]. These strategies often involve colonising and exploiting the subcellular compartments and microenvironments that exist within host cells. The localisation of pathogens in specific subcellular compartments can facilitate their survival and replication, but it can also lead to heterogeneity in the host response to infection [3]. In this context, the distribution and action of antibiotics at these heterogeneous sites of infection is fundamental in determining whether chemotherapy will be successful.

The parameters that define antibiotic efficacy are conventionally defined by the **pharmacokinetics (PK)** (see Glossary) and **pharmacodynamics (PD)** of the drug, also known as PK/PD parameters [4]. The PD of an antibiotic determine how the treatment affects a bacterial target and can be studied *in vitro* by using **planktonic liquid cultures** to define conventional inhibitory parameters such as **minimal inhibitory concentrations (MICs)** and **minimal bactericidal concentrations (MBCs)** [5]. The PK of an antibiotic determine the intrinsic properties of the compound to be absorbed, distributed, and eliminated from an organism [5]. In the context of intracellular infections, PK parameters are complex, since they can display a heterogeneous distribution at multiple scales

Highlights

Intracellular pathogens such as *Mycobacterium tuberculosis* (Mtb) can evade the immune system and proliferate within host cells, making antibiotic treatment more challenging and potentially resulting in therapeutic failure and antimicrobial resistance.

Antibiotic efficacy is influenced by a range of factors across different spatio-temporal domains: tissue-level barriers and cell-specific responses can limit the pharmacokinetics of the drug; whilst different subcellular compartments and microenvironments can affect the pharmacodynamics of the drug.

This challenge is further complicated by the heterogeneity of tissue-level infection sites, host-cell types, and pathogenic cell subpopulations.

Further to this, this heterogeneity is dynamic: host cells will adopt different disease states dependent on infection, and the intracellular pathogen can possess a dynamic and facultative lifestyle.

Ergo, effective chemotherapy is incredibly complex and requires interdisciplinary investigation across varying spatiotemporal scales.

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such as organs and tissues, cell types, subcellular compartments, and ultimately individual bacteria. The pharmacokinetic/dynamic biodistribution and bioavailability of free antibiotic compounds at the site of infection have been extensively reviewed elsewhere over the past decades [4,6,7] and it is now commonly acknowledged that achieving **bacteriostatic** or **bactericidal** concentrations is critical to appropriately treat these infections.

This heterogeneous distribution of antibiotic throughout the body is known to subsequently influence the efficacy, with tissues that experience high blood flow, such as the liver and kidneys, receiving higher concentrations of antibiotics than tissues with lower blood flow, such as the bone. In addition to blood flow, the presence of barriers will also affect antibiotic distribution across different tissue types. For example, the blood–brain barrier or the placental barrier can limit penetration into the central nervous system and foetus, respectively [8]. The diverse ratios between extracellular concentrations of antibiotics (as measured in the epithelial lining fluid) versus intracellular concentrations in alveolar macrophages have been comprehensively reviewed by Kiem and Schentag [9].

Further to this, the disease state of a tissue will have a significant influence on the underlying structure and subsequent PK/PD parameters. Taking the complexity of diseased lung tissue as a case in point, the tuberculous lesions, and more particularly **granulomas**, have been extensively studied with regards to PK/PD. It is the tubercle-like appearance of these structures that gives TB its name and the composition of these structures has been reviewed previously [10,11]. Pulmonary TB will result in granulomatous structures that are highly heterogeneous in cell composition and have significantly modified internal microenvironments that influence the persistence capacity of Mtb. A detailed picture of this heterogeneous composition has been revealed in cynomolgus macaque lung granulomas, showing how different underlying, lesion-associated cell populations affect Mtb **persistence** [12]. Research into host microenvironmental conditions has shown that human TB granulomas develop hypoxic cores with decreasing oxygen tension gradients [13]. This central **hypoxia** results in more **lipid body** formation within the constituent granuloma cell-type, the **foamy macrophage (FM)** [14]. These FMs subsequently have diminished phagocytic and bactericidal activity [15] and can also act as a nutrient reservoir for intracellular Mtb populations [16]. During this complex interplay, active bacterial replication will cause cellular damage leading to host cell necrosis. This triggers the release of Mtb and FM-derived lipids that form a central ‘caseum’ of necrotic components [17]. Oxygen-starved planktonic cultures show a tolerance to many TB drugs [18], whilst the poorly-vascularised caseum of granulomas are known to provide a home to drug-tolerant subpopulations of Mtb [19] by preventing the free diffusion of antimicrobials via caseum binding [20]. However, other studies have shown that, whilst the hypoxic conditions within TB granulomas do induce greater lipid body formation, they do not result in any additional changes in intracellular Mtb drug tolerance [21], highlighting the complexity of these host environment–pathogen interactions. The granuloma is a complex site of spatially organised inflammatory signalling – with a core of **antimicrobial peptides** and **reactive oxygen species (ROS)** – whilst the external tissue has a comparatively anti-inflammatory nature [22]. These observations, validated in animal models [23–25], suggest that antimicrobial concentrations across the lung tissue are not necessarily reaching bactericidal concentrations, therefore leading to incomplete or nonoptimal treatments that may explain why long anti-TB therapies are required.

At the single-cell level, due to the diversity of host-cell types, heterogeneity of cellular states and subsequent complexity of intracellular microenvironments, the details of antibiotic distribution and PK are less well characterised. Mtb can be localised either extracellularly, in interstitial spaces, such as the meninges and blood vessels, or intracellularly within host cells. Many bacteria,

Glossary

Antimicrobial peptides: small proteins that can kill against bacteria, fungi, parasites and viruses.

Bacterial persistence: a nondividing state of bacteria that can occur when exposed to antibiotics.

Bactericidal: qualifies an antibiotic that kills bacteria.

Bacteriostatic: qualifies an antibiotic that inhibits the growth of bacteria.

Efflux capacity: the ability of bacteria to remove drugs from the cell.

Facultative localisation: the ability of bacteria to live either inside or outside cells.

Foamy macrophage (FM): a type of lipid-rich immune cell.

Granulomas: clusters of cells that form in response to infections or foreign substances.

Hypoxia: a condition in which there is a lack of oxygen in the body.

Lipid body: an organelle involved in the storage of lipids.

Lipophilicity: the tendency of a substance to dissolve in lipids.

MIC90: the minimum inhibitory concentration required to inhibit the growth of 90% of the bacteria.

Minimal bactericidal concentration (MBC): the lowest concentration of an antibiotic that kills bacteria.

Minimal inhibitory concentration (MIC): the lowest concentration of an antibiotic that inhibits the growth of bacteria.

Phagocytes: cells that engulf and destroy foreign particles such as bacteria.

Pharmacodynamics (PD): the study of how drugs affect the body.

Pharmacokinetics (PK): the study of the absorption, distribution, metabolism, and elimination of drugs in the body.

Planktonic liquid cultures: a method of growing bacteria in a liquid medium.

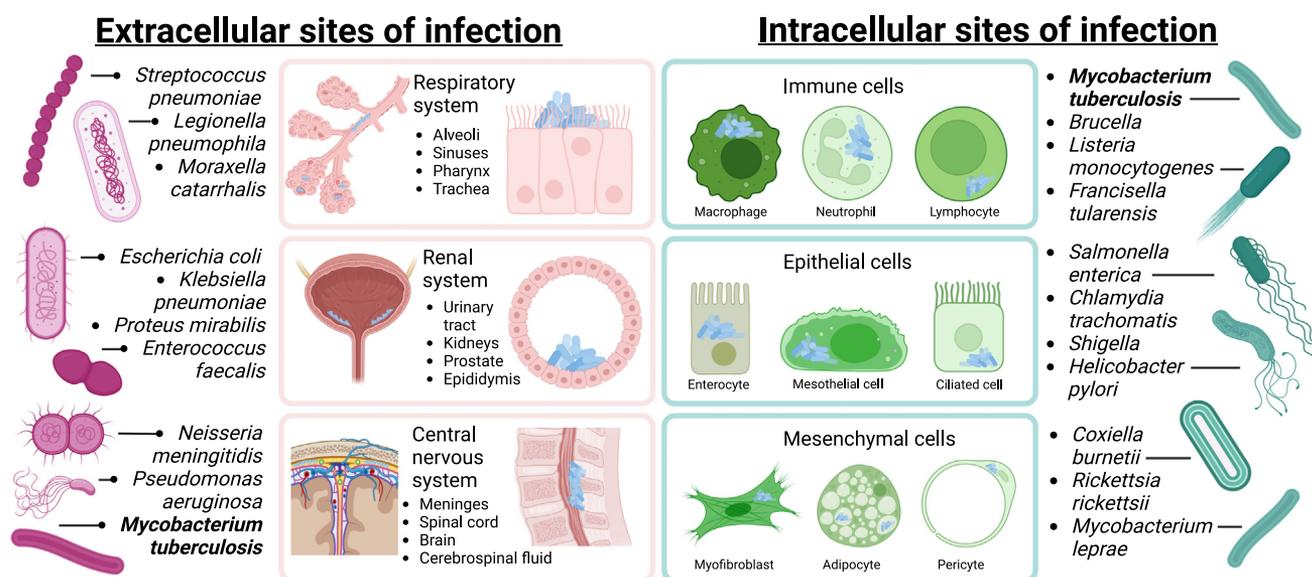
Reactive oxygen species (ROS): oxygen-derived chemical radicals that are natural metabolic by-products but also potent cell-damaging molecules.

Tropism: the ability of bacteria to infect specific types of cell, or the preferred cellular or subcellular localisation of a drug. In general, an organismal or biomolecular tendency to localise.

including Mtb, are capable of **facultative localisation**, either outside or inside cells depending on their pathogenic life cycles and the progress of the disease [2,26,27] (Figure 1). Given the various barriers that exist within a cell in the form of membranes, diffusive gradients, and active transport, reaching intracellular infections with an antibiotic presents a far more complex challenge from a pharmacokinetic perspective. Consequently, MICs and MBCs determined *in vitro* will likely not directly translate to the case where bacteria are within host cells. This has been recognised, and classical studies using radiolabelled antibiotics have shown differing penetration and accumulation in mammalian cells [28,29]. Within any given cell type, the PD and PK will be determined by the interplay between these tissue microenvironments, and the physicochemical properties of the drug. Therefore, there is a tight interconnection between single cell characteristics such as infection status, **efflux capacity**, metabolic rate, organelle-dependent sequestration and **tropism**, and the related biochemical properties of the antibiotic, such as **lipophilicity** ($\log P$) or acidity/alkalinity ($\log D$). Now that the challenge of targeting extra- and intracellular Mtb has been reviewed, the focus will shift to one of the primary hurdles in a comprehensive chemotherapy: the challenge of getting the antibiotic into the host cell. (See Box 1.)

How do antibiotics enter infected host cells?

Intracellular infections require adequate intracellular concentrations of antibiotics to be effective [30]. In that context, antibiotics that can accumulate within host cells and display high intracellular levels for prolonged periods of time are more likely to be highly effective against Mtb. Conversely, intracellular concentrations of antibiotics that are not able to achieve levels that are higher than the MIC/MBC parameters will be ineffective. Consequently, exposure to suboptimal antibiotic



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Figure 1. The pathways of antibiotics to reach extracellular and intracellular bacterial pathogens. Intracellular pathogens occupy a spatiotemporal domain with far greater heterogeneity than their extracellular counterparts. Extracellular and intracellular microbes occupy a diverse range of different microenvironments. The left side of this figure shows examples of some extracellular sites of infection and bacterial pathogens, and the right side of the figure shows examples of different cell types that can host intracellular bacterial pathogens. It is important to note that these categories are not mutually exclusive, as shown by the example of *Mycobacterium tuberculosis*. Unlike extracellular pathogens, antibiotics targeting intracellular pathogens must be able to penetrate host cells and multiple membranes. Furthermore, various cell types that harbour intracellular bacteria are localised in different tissue environments, which can impact the ability of antibiotics to penetrate and treat the infection.

Box 1. Methods for localising drug distribution at the tissue level

Autoradiography (ARG) is one of the earliest approaches to visualising the location of specific molecules within a tissue, capable of revealing the distribution of compounds across entire regions of tissue using whole-body autoradiography (WBA) [69–72], or across smaller regions of tissue at cellular resolutions using microautoradiography (MARG) [73–75]. ARG works by conjugating the molecule of interest with a radiolabelled nucleotide or amino acid, which subsequently reveals the spatial location of the underlying molecule when the sample is imaged. ARG approaches are limited in their capacity to quantify any specific compound's concentration within a tissue, providing data only on the concentration of radioactivity of the label, which can include parent drug, metabolites, and degradation products [76]. Conversely, other traditional approaches, such as liquid chromatography mass spectrometry, can yield a very high level of molecular-specific information [77–79] achieved by separating a sample into its individual components using chromatographic separation prior to a mass analysis. However, all spatial context of the molecular localisation will be lost during the necessary tissue homogenisation preparation step [80,81].

In the 1990s, Hashino *et al.* used immuno-gold electron microscopy (IEM) to detail the subcellular localisation of aminoglycoside antibiotics in sensory hair cells [82,83]. This technique relies on visualising the presence of colloidal gold particles in electron micrographs that are conjugated to the drug of interest using secondary antibody labelling [84]. The earliest of these studies demonstrated that the aminoglycoside kanamycin (KM) was found residing within vesicle structures beneath the apical surface of hair cells, and that this localisation was facilitated by receptor-mediated endocytosis [82]. A subsequent study showed that the internalised aminoglycosides were transported from these vesicle structures into lysosomes where they accumulate and end up disrupting lysosomal function, potentially leading to hair cell death [83]. Whilst this technique yields highly resolved, subcellular drug localisation, it is again limited by the lack of molecular specificity of the immunogold staining, lacking a direct quantification of the drug concentration from the secondary immunolabelling.

Mass spectrometry imaging (MSI) combines the specific compound identification of mass spectrometry with the spatial localisation of ARG/IEM, with the most widely applied approach being matrix-assisted laser desorption/ionisation mass spectrometric imaging (MALDI-MS) [85–87]. In MALDI-MS imaging, a laser probe is raster scanned across a matrix-coated section of the sample, acquiring a mass spectrum for each pixel in the *xy*-plane and subsequently yielding a label-free detection of key compounds and metabolites [85]. MALDI-MS has been used to demonstrate the distribution of moxifloxacin in TB-infected rabbit lungs [88] and granulomatous lesions, to illustrate the PK across complete rat sections [89], to reveal the heterogeneous drug distribution across tumours [90], and to reveal the levofloxacin distribution in TB-infected pulmonary lesions [91].

concentrations might have deleterious consequences by favouring tolerance or promoting the emergence of drug-resistant populations [31,32].

Both within the context of *Mtb* and the wider field of host–pathogen interactions, the process of how antibiotics enter mammalian cells is complex and is mainly driven by both the phenotypic state of the cell and physicochemical properties of the antibiotic. Among innate immune cells, macrophages form the first line of defence against pathogens, especially in the context of TB, where they constitute a preferential niche all along the infection process. Uninfected macrophages quickly released the fluoroquinolone Pefloxacin, whereas macrophages infected with *Legionella* retained approximately 20–30% of the drug, highlighting that the immune status of the cell can have an important impact on antibiotic penetration and retention [33]. Antibiotic discovery programs prioritise compounds likely to cross the cell membrane, leading to rules governing the size, polarity, and rigidity of chemical structures as a part of Lipinski's rule of 5 to define appropriate antibiotics [34,35]. Other studies show that streptomycin is unable to penetrate freely through the macrophage cell membrane and that, in its intracellular environment, sensitive *Escherichia coli* is protected from the antibiotic [36]. When considering the physicochemical properties of the antibiotic, aminoglycoside antibiotics have been shown to be too polar to reach sufficient intracellular concentrations [37], and β -lactam antibiotics have an acidity that prevents their intracellular accumulation [38].

In host cells, antibiotics can enter either via endocytosis or macropinocytosis [39,40] as well as passive or active transport [41]. There is evidence that both passive and active transport are important for antibiotic efficacy, but these processes may vary, depending on each molecule [42]. Antibiotic passive diffusion across membranes is driven by a concentration gradient and

the permeability coefficient (partition coefficient and diffusion coefficient), where non-polar, uncharged compounds, or antibiotics with a high permeability coefficient will be able to diffuse more easily across phospholipid bilayers. This process tends to be unspecific, does not require a carrier, and is energy-independent. In the context of facilitated diffusion, particular transporters are responsible for the gradient-dependent flux of antibiotics across the membrane. This process is more specific between substrate and the carrier and can be saturated or energy-dependent. For example, there are specific proteins in the plasma membrane, such as the interferon-induced transmembrane proteins (IFITMs), that assist in cellular uptake of chemotypes; this is achieved via modulation of the cell permeability of a prototypical biopic inhibitor of the mammalian target of rapamycin (mTOR) [43]. In many cases, antibiotics can be translocated via specific transporters; this includes amoxicillin, cefaclor, cefalexin, ceftibuten, cephaloridine, erythromycin, rifampicin, and tetracycline which can be imported by some specific solute carrier (SLC) family members [44]. There is also transporter-mediated uptake which is energy-dependent and operates against the gradient [45]. Here, the classical ATP-binding cassette (ABC) transporter family plays a predominant role. The semi-synthetic lincosamide antibiotic clindamycin has been one of the first antibiotics identified to be actively transported [46]. Indeed, clindamycin uptake is decreased when treated with potassium fluoride or sodium cyanide [28,47], suggesting an active transport [48]. However, successful uptake of an antibiotic should not be taken as the final point of consideration in the chemotherapy of an intracellular infection: indeed, it may constitute only an initial impediment in the wider challenge of eliminating a microbial threat.

The fate of antibiotics and their bacterial target within host cells

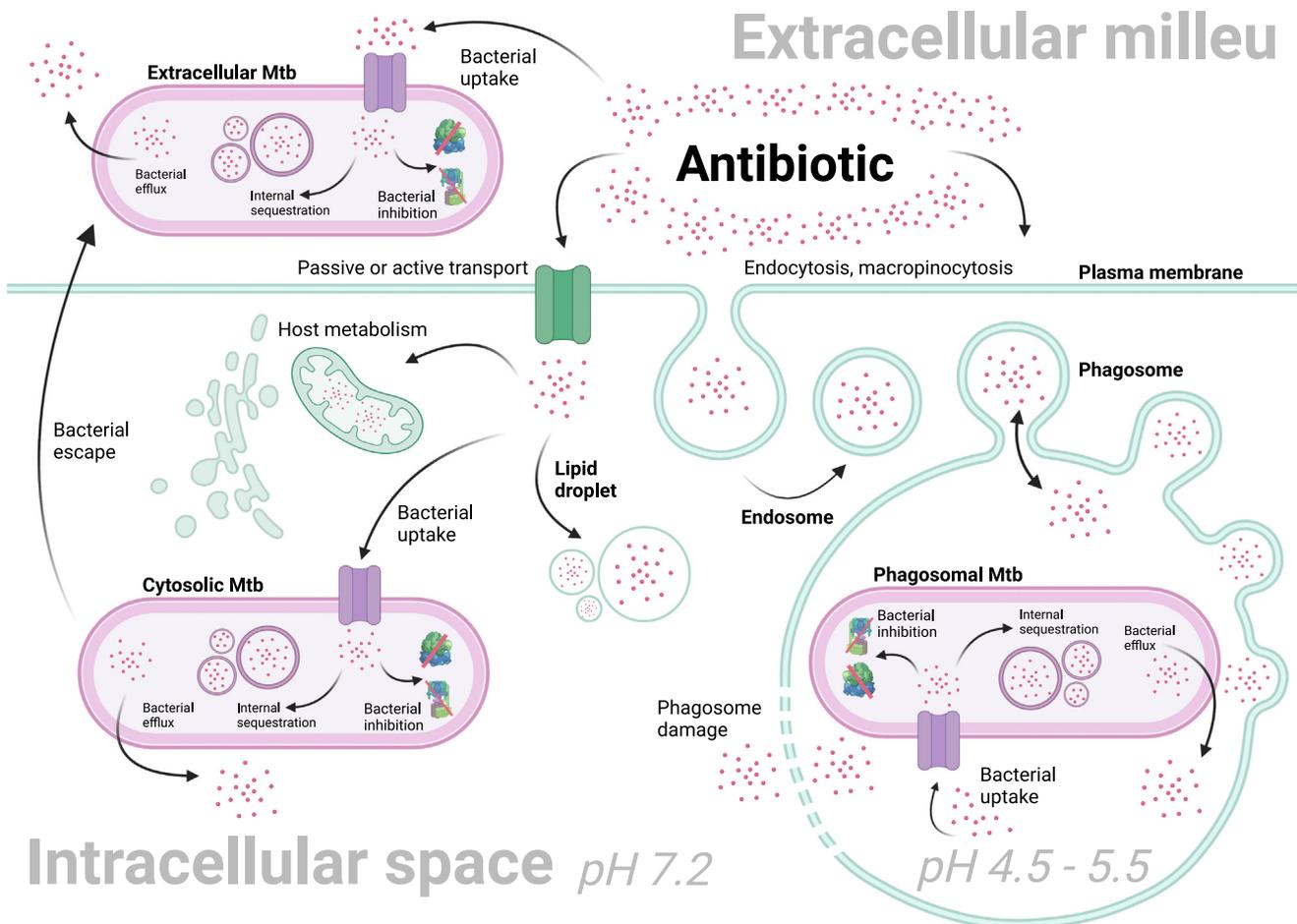
Efficient intracellular accumulation of antibiotics does not always translate to effective treatment of intracellular infections, suggesting that, within cells, there are further regulatory pathways of antibiotic efficacy and access to Mtb and other bacteria (reviewed in Table 1 of [49]). For example, there is evidence that *Staphylococcus aureus* and *Klebsiella pneumoniae* are poorly sensitive to antibiotics within **phagocytes** [49–51]. There is further evidence that the intracellular accumulation of antibiotics and the subsequently expected intracellular chemotherapy against *S. aureus* are not mutually inclusive phenomena [52]. One possible explanation for this is that *S. aureus*, as well as Mtb, can display bacterial persister cell behaviour when exposed to an antibiotic. This manifests as a nonresponsive, nondividing state that is reversible upon antibiotic removal [53,54]. Other studies have shown that a range of different antibiotics spanning seven different pharmacological classes were all less effective in the intracellular environment of macrophages than when compared with extracellular activities, regardless of the level of drug accumulation [55]. The importance of achieving sufficient intracellular concentrations of antibiotics is vital: it is well established that suboptimal levels of antibiotics can lead to persistent infections and the development of tolerant strains of pathogens [31,56,57].

Rifampicin, clindamycin, and erythromycin have been shown to achieve higher levels of concentration in alveolar macrophages from smoker patients, further highlighting the fact that external environmental influences and their subsequent effect at a single-cell level can significantly impact antibiotic PK [58].

Many studies have compared PD of planktonic Mtb versus intracellular Mtb cultures and found that in cells (mostly macrophages), the **MIC90** is very different between *in vitro* and *in cellulo* [21]. Fluorescent tetracycline has been one of the first examples of studies to investigate antibiotic subcellular location [59]. Additionally, seminal studies have shown that radiolabelled roxithromycin or erythromycin coupled with cell fractionation of mouse macrophages and human neutrophils demonstrated that approximately half of the drug that had been accumulated was associated with lysosomes [60]. Similar approaches were used by Carlier *et al.* with the fluoroquinolone

pefloxacin [33] and with the macrolide azithromycin [61], which revealed both lysosomal and cytosolic accumulation of these antibiotics.

Further studies have highlighted the importance of intracellular biodistribution of compounds, showing that intracellular tropism and accumulation of antibiotics affect their efficacy [62,63]. Organelles, pH, and cell dynamics are critical factors that contribute to the intracellular localisation of pathogens and associated antibiotic efficacy (Figure 2). Mtb has an ESX-1 type 7 secretion system, giving it the capacity to disrupt the membrane of the phagolysosomal compartment within which it is localised, subsequently preventing the pH of the immediate surroundings from becoming too acidic whilst also facilitating an escape into the cytosol. Pyrazinamide (PZA), an essential, first-line, antitubercular medication, is dependent on acidification within the endolysosomal compartment for optimal drug efficacy [62], so the capacity for Mtb to manipulate this further disrupts the PD of the antibiotic. It was also observed that macrophage necrosis drastically reduced PZA concentrations, suggesting that pathogen-mediated cell death impacts upon



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Figure 2. The pathways of antibiotics within cells. Antibiotics have several ways of reaching intracellular regions, such as endocytosis, macro/micropinocytosis, passive, or active transport. Once inside the cell, antibiotics can be either internalised and metabolised or sequestered into different organelles. This can affect the availability of antibiotics to target different bacterial subpopulations within diverse niches. For instance, some intracellular bacteria reside within phagolysosomes or other organelles, which can limit the effectiveness of antibiotics that cannot penetrate these compartments. Therefore, understanding the intracellular location and behaviour of bacteria is crucial for the successful treatment of intracellular infections. Abbreviation: Mtb, *Mycobacterium tuberculosis*.

antibiotic levels and therefore creates a replicative niche free of antimicrobial threat for Mtb [62]. Finofloxacin, a fluoroquinolone commonly used to treat *S. aureus* [64,65], and moxifloxacin, doxycycline, and rifampin treatment of *Coxiella burnetii* [66] have all been shown to have dramatically improved intracellular efficacy under acidic pH conditions, highlighting the influence of host microenvironment pH on PD. Considering the influence of intracellular organelles, the lipophilic bedaquiline (BDQ) has been shown to accumulate within host cell lipid droplets [62,63], acting as reservoirs of anti-TB molecules that are delivered to intracellular populations of bacteria during lipid consumption in macrophages. Finally, the cellular dynamics of immune cell activation has been shown to induce drug tolerance, with the degree of tolerance being proportional to the level of macrophage activation in the context of Mtb infection [67].

Concluding remarks and future perspectives

This review has focused on the journey, and associated challenges, an antibiotic must take to target Mtb and other intracellular pathogens. However, what has not been examined is the dynamic nature of the intracellular pathogens themselves, with subpopulations of bacteria exhibiting distinct phenotypic and functional characteristics that could potentially affect antibiotic access and efficacy. Furthermore, intracellular pathogens display diverse intracellular lifestyles, ranging from a replicative state to a persistent or dormant state. This diversity in intracellular lifestyles can also impact the accumulation of antibiotics within host cells. For instance, dormant or persistent bacteria may exhibit reduced metabolic activity, making them less susceptible to antibiotics that target actively growing bacteria [68].

Box 2. Methods for localising drugs at the subcellular level

Within the broader category of MSI there are several protocols with varying benefits mostly related to the level at which the drug can be resolved. For example, a typical MALDI-MS experiment will acquire at a resolution limit of around 10 μm , whereas secondary ion mass spectrometry (SIMS) approaches are favoured for increasing the spatial resolution to less than 250 nm [86]. SIMS approaches differ from MALDI-MS protocols as they employ a focused ion beam for the mass spectra acquisition [92].

For example, time of flight-SIMS (ToF-SIMS) uses a pulsed ion beam (typically from a primary ion source such as Bi^{3+}) and measures the resulting mass spectra using a time-of-flight mass spectrometer. This yields a high-resolution spatial distribution of the drug of the order of 10–100s of nanometres [86]. ToF-SIMS has been used to demonstrate the single-cell distribution of amiodarone in human embryonic kidney (HEK293), cervical cancer (HeLa), and liver cancer (HepG2) cells [93] and to visualise the distribution of ampicillin and tetracycline at the single-bacterium level, at ~ 300 nm spatial resolution [94].

NanoSIMS is a recent imaging modality which relies on a focused high-energy beam of ions (usually from a Cs^+ source) to scan the surface of the sample, with the resulting secondary ions being separated by a magnetic field, yielding highly specific single-pixel spectra for each xy coordinate on the scale of only a few nanometres [86]. This approach has shown, at a subcellular level, that amiodarone-treated macrophages accumulate the drug within their lysosomes, and that the antitubercular drug bedaquiline resides primarily within host macrophage lipid droplets, suggesting that these lipid droplets act as an antibiotic reservoir that bacteria take up during host lipid consumption [63].

Correlative light, electron, and ion microscopy (CLEIM) has been developed to visualise the subcellular localisation of antibiotics within fixed tissue [95]. In the first step of CLEIM, the antibiotic is labelled with a fluorescent marker and administered to the cells or tissue. A vibratome section of the labelled sample then undergoes nondestructive 3D imaging using confocal fluorescence microscopy to determine its location within the tissue. After fluorescence imaging, the section is then embedded in resin, and electron microscopy is used to obtain higher-resolution images of the sample to visualise the labelled antibiotic at the ultrastructural level. To correlate the electron micrographs with the fluorescence images, the sections are analysed with μCT 3D scanning. Finally, the same section is coated with 5 nm of gold for imaging in a NanoSIMS ion microscope to analyse the elemental composition of the antibiotic and its distribution within the cells or tissue. By combining these different imaging techniques, CLEIM can provide a comprehensive understanding of the subcellular localisation of antibiotics. This approach has been used to confirm the *in vivo* localisation of bedaquiline within lipid droplets in foamy macrophages [63], to reveal that bedaquiline also accumulates within polymorphonuclear cells [95] and to show the heterogeneous subcellular localisation of pyrazinamide within host cells [62].

Outstanding questions

How does tissue organisation and the specificity of lesions impact drug distribution at the site of infection?

What are the biophysical, chemical, or physiological factors that can enhance or alter the accumulation and/or effectiveness of antibiotics?

How do cellular states and key features influence the penetration, efflux, and accumulation of antibiotics?

What is the subcellular distribution of antibiotics within cells, and how does this affect their effectiveness?

What is the contribution of the endolysosomal network in antibiotic accumulation, distribution, and efficacy?

What contributions do host cell organelles make to intracellular chemotherapy?

What intracellular niches provide protective effects against antibiotic efficacy in the context of intracellular chemotherapy?

How can our knowledge of intracellular niches be used to design and develop appropriate combined therapies?

Can we identify or develop host-directed therapies that modulate host microenvironments or bacterial subcellular localisation to enhance antibiotic accumulation or efficacy?

Should we revisit the priorities of drug-discovery programs and redefine the rules for designing and developing new chemical entities based on the complexity of the infection site context?

Future investigations into effective chemotherapy of Mtb infection must consider each of these distinct spatiotemporal domains: from tissue-wide distribution to cellular penetration, to host intracellular microenvironments and finally intracellular bacterial dynamics. Each of these considerations act as a significant obstacle to overcome in the challenging race to eliminate deadly intracellular infections such as TB (see [Outstanding questions](#)).

Even though this review has focused on intracellular Mtb and antibiotic treatment, these concepts are directly applicable to other pathogens and drugs. *In vitro* evaluation/screening of drugs has been important in the 21st century, in both acellular and cellular systems, to improve initial assessments of pharmacological design. Understanding the relationship between extracellular and intracellular PK/PD properties, antibiotic physicochemical properties, and how they change over time and space, is crucial to enhance our understanding of antibiotic efficacy. New technologies such as matrix-assisted laser desorption/ionisation mass spectrometric imaging (MALDI-MS), nanoscale secondary ion mass spectrometry (NanoSIMS), and single-cell microscopy approaches are illuminating the localisation of antibiotics at very high spatial and temporal resolution (see [Box 2](#)). An emerging picture of the efficacy of antibiotics against intracellular targets that takes into consideration cell entry (e.g., efflux versus influx), intracellular distribution, partitioning, and impact of organelles, is important to design new combined therapies with multiple drugs that maximise the coverage of these environments. Addressing host cell and intracellular localisation heterogeneity, and how this affects antibiotic efficacy, is key for the development of drug combinations to target all the bacteria. In conclusion, understanding the diverse subcellular compartments, microenvironments, and intracellular lifestyles of pathogens is crucial for developing effective strategies to combat intracellular infections. The dynamics of intracellular pathogens highlight the need to consider the heterogeneity and dynamic nature of intracellular infections in the development of novel therapeutics.

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Declaration of interests

The authors declare no conflict of interest.

References

- Lee, J.Y. (2015) Diagnosis and treatment of extrapulmonary tuberculosis. *Tuberc. Respir. Dis. (Seoul)* 78, 47–55
- Gutierrez, M.G. and Enninga, J. (2022) Intracellular niche switching as host subversion strategy of bacterial pathogens. *Curr. Opin. Cell Biol.* 76, 102081
- Bussi, C. and Gutierrez, M.G. (2019) *Mycobacterium tuberculosis* infection of host cells in space and time. *FEMS Microbiol. Rev.* 43, 341–361
- Levison, M.E. and Levison, J.H. (2009) Pharmacokinetics and pharmacodynamics of antibacterial agents. *Infect. Dis. Clin. N. Am.* 23, 791–815, vii
- Aljayyousi, G. *et al.* (2017) Pharmacokinetic-pharmacodynamic modelling of intracellular *Mycobacterium tuberculosis* growth and kill rates is predictive of clinical treatment duration. *Sci. Rep.* 7, 502
- Liu, P. and Derendorf, H. (2003) Antimicrobial tissue concentrations. *Infect. Dis. Clin. N. Am.* 17, 599–613
- Nix, D.E. *et al.* (1991) Antibiotic tissue penetration and its relevance: impact of tissue penetration on infection response. *Antimicrob. Agents Chemother.* 35, 1953–1959
- Tetro, N. *et al.* (2018) The placental barrier: the gate and the fate in drug distribution. *Pharm. Res.* 35, 71
- Kiem, S. and Schentag, J.J. (2008) Interpretation of antibiotic concentration ratios measured in epithelial lining fluid. *Antimicrob. Agents Chemother.* 52, 24–36
- Ramakrishnan, L. (2012) Revisiting the role of the granuloma in tuberculosis. *Nat. Rev. Immunol.* 12, 352–366
- Williams, G.T. and Williams, W.J. (1983) Granulomatous inflammation—a review. *J. Clin. Pathol.* 36, 723–733

12. Gideon, H.P. *et al.* (2022) Multimodal profiling of lung granulomas in macaques reveals cellular correlates of tuberculosis control. *Immunity* 55, 827–846 e10
13. Belton, M. *et al.* (2016) Hypoxia and tissue destruction in pulmonary TB. *Thorax* 71, 1145–1153
14. Bostrom, P. *et al.* (2006) Hypoxia converts human macrophages into triglyceride-loaded foam cells. *Arterioscler. Thromb. Vasc. Biol.* 26, 1871–1876
15. Shim, D. *et al.* (2020) *Mycobacterium tuberculosis* infection-driven foamy macrophages and their implications in tuberculosis control as targets for host-directed therapy. *Front. Immunol.* 11, 910
16. Barisch, C. and Soldati, T. (2017) Breaking fat! How mycobacteria and other intracellular pathogens manipulate host lipid droplets. *Biochimie* 141, 54–61
17. Kim, M.J. *et al.* (2010) Caseation of human tuberculosis granulomas correlates with elevated host lipid metabolism. *EMBO Mol. Med.* 2, 258–274
18. Wayne, L.G. and Hayes, L.G. (1996) An *in vitro* model for sequential study of shutdown of *Mycobacterium tuberculosis* through two stages of nonreplicating persistence. *Infect. Immun.* 64, 2062–2069
19. Sarathy, J.P. *et al.* (2018) Extreme drug tolerance of *Mycobacterium tuberculosis* in caseum. *Antimicrob. Agents Chemother.* 62, e02266–17
20. Sarathy, J.P. *et al.* (2017) An *in vitro* caseum binding assay that predicts drug penetration in tuberculosis lesions. *J. Vis. Exp.* 123, 55559
21. Lanni, F. *et al.* (2023) Adaptation to the intracellular environment of primary human macrophages influences drug susceptibility of *Mycobacterium tuberculosis*. *Tuberculosis (Edinb)* 139, 102318
22. Marakalala, M.J. *et al.* (2016) Inflammatory signaling in human tuberculosis granulomas is spatially organized. *Nat. Med.* 22, 531–538
23. Kaya, F. *et al.* (2022) A rabbit model to study antibiotic penetration at the site of infection for nontuberculous mycobacterial lung disease: macrolide case study. *Antimicrob. Agents Chemother.* 66, e0221221
24. Irwin, S.M. *et al.* (2016) Bedaquiline and pyrazinamide treatment responses are affected by pulmonary lesion heterogeneity in *Mycobacterium tuberculosis* infected C3HeB/FeJ mice. *ACS Infect. Dis.* 2, 251–267
25. Irwin, S.M. *et al.* (2015) Presence of multiple lesion types with vastly different microenvironments in C3HeB/FeJ mice following aerosol infection with *Mycobacterium tuberculosis*. *Dis. Model. Mech.* 8, 591–602
26. Fredlund, J. and Enninga, J. (2014) Cytoplasmic access by intracellular bacterial pathogens. *Trends Microbiol.* 22, 128–137
27. Mitchell, G. *et al.* (2016) Strategies used by bacteria to grow in macrophages. *Microbiol. Spectr.* 4, Published online May 6, 2016. <https://doi.org/10.1128/microbiolspec.mchd-0012-2015>
28. Johnson, J.D. *et al.* (1980) Antibiotic uptake by alveolar macrophages. *J. Lab. Clin. Med.* 95, 429–439
29. Honeybourne, D. (1994) Antibiotic penetration into lung tissues. *Thorax* 49, 104–106
30. Steinberg, T.H. (1994) Cellular transport of drugs. *Clin. Infect. Dis.* 19, 916–921
31. Liu, J. *et al.* (2020) Effect of tolerance on the evolution of antibiotic resistance under drug combinations. *Science* 367, 200–204
32. Rybak, M.J. (2006) Pharmacodynamics: relation to antimicrobial resistance. *Am. J. Med.* 119, S37–S44; discussion S62–S70
33. Carlier, M.B. *et al.* (1990) Cellular uptake, localization and activity of fluoroquinolones in uninfected and infected macrophages. *J. Antimicrob. Chemother.* 26, 27–39
34. Lipinski, C.A. *et al.* (2001) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Deliv. Rev.* 46, 3–26
35. Lipinski, C.A. (2004) Lead- and drug-like compounds: the rule-of-five revolution. *Drug Discov. Today Technol.* 1, 337–341
36. Stevenson, M. *et al.* (1983) Enhanced activity of streptomycin and chloramphenicol against intracellular *Escherichia coli* in the J774 macrophage cell line mediated by liposome delivery. *Antimicrob. Agents Chemother.* 24, 742–749
37. Tulkens, P. and Trouet, A. (1978) The uptake and intracellular accumulation of aminoglycoside antibiotics in lysosomes of cultured rat fibroblasts. *Biochem. Pharmacol.* 27, 415–424
38. Renard, C. *et al.* (1987) Influence of conversion of penicillin G into a basic derivative on its accumulation and subcellular localization in cultured macrophages. *Antimicrob. Agents Chemother.* 31, 410–416
39. Tulkens, P.M. (1991) Pharmacokinetic and toxicological evaluation of a once-daily regimen versus conventional schedules of netilmicin and amikacin. *J. Antimicrob. Chemother.* 27, 49–61
40. Jiao, C.Y. *et al.* (2009) Translocation and endocytosis for cell-penetrating peptide internalization. *J. Biol. Chem.* 284, 33957–33965
41. Dockter, M.E. and Magnuson, J.A. (1974) Characterization of the active transport of chlorotetracycline in staphylococcus aureus by a fluorescence technique. *J. Supramol. Struct.* 2, 32–44
42. Smith, D. *et al.* (2014) Passive lipoidal diffusion and carrier-mediated cell uptake are both important mechanisms of membrane permeation in drug disposition. *Mol. Pharm.* 11, 1727–1738
43. Lou, K. *et al.* (2022) IFITM proteins assist cellular uptake of diverse linked chemotypes. *Science* 378, 1097–1104
44. Dobson, P.D. and Kell, D.B. (2008) Carrier-mediated cellular uptake of pharmaceutical drugs: an exception or the rule? *Nat. Rev. Drug Discov.* 7, 205–220
45. Surve, M.V. and Banerjee, A. (2019) Cell-to-cell phenotypic heterogeneity in pneumococcal pathogenesis. *Future Microbiol.* 14, 647–651
46. Hand, W.L. *et al.* (1983) Interactions of antibiotics and phagocytes. *J. Antimicrob. Chemother.* 12, 1–11
47. Prokesch, R.C. and Hand, W.L. (1982) Antibiotic entry into human polymorphonuclear leukocytes. *Antimicrob. Agents Chemother.* 21, 373–380
48. Hand, W.L. and King-Thompson, N.L. (1982) Membrane transport of clindamycin in alveolar macrophages. *Antimicrob. Agents Chemother.* 21, 241–247
49. Bongers, S. *et al.* (2019) Intracellular penetration and effects of antibiotics on *Staphylococcus aureus* inside human neutrophils: a comprehensive review. *Antibiotics (Basel)* 8, 54
50. Brummitt, W. *et al.* (1964) An assessment of ampicillin in the treatment of urinary tract infection. *Postgrad. Med. J.* 40, 55–62
51. Rungelrath, V. and DeLeo, F.R. (2021) *Staphylococcus aureus*, antibiotic resistance, and the interaction with human neutrophils. *Antioxid. Redox Signal.* 34, 452–470
52. Sandberg, A. *et al.* (2009) Intracellular activity of antibiotics against *Staphylococcus aureus* in a mouse peritonitis model. *Antimicrob. Agents Chemother.* 53, 1874–1883
53. Peyrusson, F. *et al.* (2020) Intracellular *Staphylococcus aureus* persists upon antibiotic exposure. *Nat. Commun.* 11, 2200
54. Torrey, H.L. *et al.* (2016) High persister mutants in *Mycobacterium tuberculosis*. *PLoS One* 11, e0155127
55. Barcia-Macay, M. *et al.* (2006) Pharmacodynamic evaluation of the intracellular activities of antibiotics against *Staphylococcus aureus* in a model of THP-1 macrophages. *Antimicrob. Agents Chemother.* 50, 841–851
56. Liu, X. *et al.* (2020) Sublethal levels of antibiotics promote bacterial persistence in epithelial cells. *Adv. Sci. (Weinh)* 7, 1900840
57. Bush, K. *et al.* (2011) Tackling antibiotic resistance. *Nat. Rev. Microbiol.* 9, 894–896
58. Hand, W.L. *et al.* (1985) Antibiotic uptake by alveolar macrophages of smokers. *Antimicrob. Agents Chemother.* 27, 42–45
59. Dubuy, H.G. *et al.* (1964) Tetracycline fluorescence in permeability studies of membranes around intracellular parasites. *Science* 145, 163–165
60. Carlier, M.B. *et al.* (1987) Cellular uptake and subcellular distribution of roxithromycin and erythromycin in phagocytic cells. *J. Antimicrob. Chemother.* 20, 47–56
61. Carlier, M.B. *et al.* (1994) Accumulation, release and subcellular localization of azithromycin in phagocytic and non-phagocytic cells in culture. *Int. J. Tissue React.* 16, 211–220
62. Santucci, P. *et al.* (2021) Intracellular localisation of *Mycobacterium tuberculosis* affects efficacy of the antibiotic pyrazinamide. *Nat. Commun.* 12, 3816
63. Greenwood, D.J. *et al.* (2019) Subcellular antibiotic visualization reveals a dynamic drug reservoir in infected macrophages. *Science* 364, 1279–1282
64. Lemaire, S. *et al.* (2011) Activity of fleroxacin, a novel fluoroquinolone with increased activity at acid pH, towards extracellular and intracellular *Staphylococcus aureus*, *Listeria monocytogenes* and *Legionella pneumophila*. *Int. J. Antimicrob. Agents* 38, 52–59

65. Chalhoub, H. *et al.* (2020) Influence of pH on the activity of flinofloxacin against extracellular and intracellular *Burkholderia thailandensis*, *Yersinia pseudotuberculosis* and *Francisella philomiragia* and on its cellular pharmacokinetics in THP-1 monocytes. *Clin. Microbiol. Infect.* 26, 1254 e1–1254 e8
66. Smith, C.B. *et al.* (2019) The effect of pH on antibiotic efficacy against *Coxiella burnetii* in axenic media. *Sci. Rep.* 9, 18132
67. Liu, Y. *et al.* (2016) Immune activation of the host cell induces drug tolerance in *Mycobacterium tuberculosis* both *in vitro* and *in vivo*. *J. Exp. Med.* 213, 809–825
68. Akela, A.K. and Kumar, A. (2021) Bioenergetic heterogeneity in *Mycobacterium tuberculosis* residing in different subcellular niches. *mBio* 12, e0108821
69. Ullberg, S. (1954) Studies on the distribution and fate of S35-labelled benzylpenicillin in the body. *Acta Radiol. Suppl.* 118, 1–110
70. Luckey, G.W. (1975) *Apparatus and Method for Producing Images Corresponding to Patterns of High Energy Radiation*, Eastman Kodak Company, Rochester, N.Y., USA
71. Berlin, M. and Ullberg, S. (1963) Accumulation and retention of mercury in the mouse. I. An autoradiographic study after a single intravenous injection of mercuric chloride. *Arch. Environ. Health* 6, 589–601
72. Kutzim, H. (1962) The quantitative determination of the distribution of S35-sulfate in mice using autoradiography. *Nucl. Med. (Stuttg)* 3, 39–50
73. Stumpf, W.E. and Roth, L.J. (1964) Vacuum freeze drying of frozen sections for dry-mounting, high-resolution autoradiography. *Stain. Technol.* 39, 219–223
74. Stumpf, W.E. (2003) *Drug Localization in Tissues and Cells: Receptor Microscopic Autoradiography: a Basis for Tissue and Cellular Pharmacokinetics, Drug Targeting, Delivery, and Prediction*, IDDC-Press, Chapel Hill, NC, p. 170
75. Caro, L.G. (1961) Electron microscopic radioautography of thin sections: the Golgi zone as a site of protein concentration in pancreatic acinar cells. *J. Biophys. Biochem. Cytol.* 10, 37–45
76. Solon, E.G. *et al.* (2010) Autoradiography, MALDI-MS, and SIMS-MS imaging in pharmaceutical discovery and development. *AAPS J.* 12, 11–26
77. Tal'roze, V.L.K. *et al.* (1968) Capillary systems for the introduction of liquid mixtures into an analytical mass spectrometer. *Russ. J. Phys. Chem.* 42, 1658–1664
78. Arpino, P. *et al.* (1974) Liquid chromatography-mass spectrometry. II. Continuous monitoring. *Biomed. Mass Spectrom.* 1, 80–82
79. Tal'roze, V.L.G. *et al.* (1978) Capillary system for continuous introducing of volatile liquids into analytical MS and its application. *Adv. Mass Spectrom.* 7, 858
80. Ryan, D.M. and Cars, O. (1980) Antibiotic assays in muscle: are conventional tissue levels misleading as indicator of the antibacterial activity? *Scand. J. Infect. Dis.* 12, 307–309
81. Mouton, J.W. *et al.* (2008) Tissue concentrations: do we ever learn? *J. Antimicrob. Chemother.* 61, 235–237
82. Hashino, E. and Shero, M. (1995) Endocytosis of aminoglycoside antibiotics in sensory hair cells. *Brain Res.* 704, 135–140
83. Hashino, E. *et al.* (1997) Lysosomal targeting and accumulation of aminoglycoside antibiotics in sensory hair cells. *Brain Res.* 777, 75–85
84. Faulk, W.P. and Taylor, G.M. (1971) An immunocolloid method for the electron microscope. *Immunochemistry* 8, 1081–1083
85. Caprioli, R.M. *et al.* (1997) Molecular imaging of biological samples: localization of peptides and proteins using MALDI-TOF MS. *Anal. Chem.* 69, 4751–4760
86. Prideaux, B. *et al.* (2018) Imaging and spatially resolved quantification of drug distribution in tissues by mass spectrometry. *Curr. Opin. Chem. Biol.* 44, 93–100
87. Stoeckli, M. *et al.* (1999) Automated mass spectrometry imaging with a matrix-assisted laser desorption ionization time-of-flight instrument. *J. Am. Soc. Mass Spectrom.* 10, 67–71
88. Prideaux, B. *et al.* (2011) High-sensitivity MALDI-MRM-MS imaging of moxifloxacin distribution in tuberculosis-infected rabbit lungs and granulomatous lesions. *Anal. Chem.* 83, 2112–2118
89. Hopfgartner, G. *et al.* (2009) Matrix-assisted laser desorption/ionization mass spectrometric imaging of complete rat sections using a triple quadrupole linear ion trap. *Rapid Commun. Mass Spectrom.* 23, 733–736
90. Giordano, S. *et al.* (2016) Heterogeneity of paclitaxel distribution in different tumor models assessed by MALDI mass spectrometry imaging. *Sci. Rep.* 6, 39284
91. Prideaux, B. *et al.* (2015) Mass spectrometry imaging of levofloxacin distribution in TB-infected pulmonary lesions by MALDI-MSI and continuous liquid microjunction surface sampling. *Int. J. Mass Spectrom.* 377, 699–708
92. McDonnell, L.A. *et al.* (2006) Higher sensitivity secondary ion mass spectrometry of biological molecules for high resolution, chemically specific imaging. *J. Am. Soc. Mass Spectrom.* 17, 1195–1202
93. Newman, C.F. *et al.* (2017) Intracellular drug uptake—a comparison of single cell measurements using ToF-SIMS imaging and quantification from cell populations with LC/MS/MS. *Anal. Chem.* 89, 11944–11953
94. Tian, H. *et al.* (2017) Subcellular chemical imaging of antibiotics in single bacteria using C(60)-secondary ion mass spectrometry. *Anal. Chem.* 89, 5050–5057
95. Fearn, A. *et al.* (2020) Correlative light electron ion microscopy reveals *in vivo* localisation of bedaquiline in *Mycobacterium tuberculosis*-infected lungs. *PLoS Biol.* 18, e3000879