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High-Sensitivity MALDI-MRM-MS Imaging of Moxifloxacin Distribution in Tuberculosis-Infected Rabbit Lungs and Granulomatous Lesions

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Abstract

MALDI-MSI is a powerful technology for localizing drug and metabolite distributions in biological tissues. To enhance our understanding of tuberculosis (TB) drug efficacy and how efficiently certain drugs reach their site of action, MALDI-MSI was applied to image the distribution of the second-line TB drug moxifloxacin at a range of time points after dosing. The ability to perform multiple monitoring of selected ion transitions in the same experiment enabled extremely sensitive imaging of moxifloxacin within tuberculosis-infected rabbit lung biopsies in less than 15 min per tissue section. Homogeneous application of a reference standard during the matrix spraying process enabled the ion-suppressing effects of the inhomogeneous lung tissue to be normalized. The drug was observed to accumulate in granulomatous lesions at levels higher than that in the surrounding lung tissue from 1.5 h postdose until the final time point. MALDI-MSI moxifloxacin distribution data were validated by quantitative LC/MS/MS analysis of lung and granuloma extracts from adjacent biopsies taken from the same animals. Drug distribution within the granulomas was observed to be inhomogeneous, and very low levels were observed in the caseum in comparison to the cellular granuloma regions. In this experiment the MALDI-MRM-MSI method was shown to be a rapid and sensitive method for analyzing the distribution of anti-TB compounds and will be applied to distribution studies of additional drugs in the future.

Tuberculosis (TB) is a common and frequently deadly disease caused by *Mycobacterium tuberculosis* (MTB). According to the World Health Organization, approximately 1 in 3 people worldwide is latently infected with MTB, while 8–10 million new active cases are detected each year, resulting in 1.3 million deaths in 2008.¹ It takes 6 to 9 months of combination therapy to cure drug-sensitive tuberculosis (TB) despite the availability of antibiotics that have proven activity in vitro. In contrast, other pulmonary infectious diseases can be cured with similar drugs used as monotherapy for a few days to a few weeks. One

potential reason for this poor in vivo efficacy is lack of penetration of existing drugs into the granulomatous lesions that characteristically form in the lungs of TB-infected patients. TB granulomas are formed when inhaled TB bacteria are phagocytosed by alveolar macrophages in the lungs, which then recruit additional leukocytes to the site of infection. Early stage granulomas contain a core of infected macrophages enclosed by foamy macrophages and other mononuclear phagocytes, surrounded by lymphocytes. Later stages develop a fibrous capsule and necrosis in the lesion center, which may liquefy and cavitate, releasing bacilli into the airways and facilitating MTB transmission.²

Traditionally, TB drug concentrations are measured in plasma. However, as they exert their effects in defined tissues and lesions, it would be of great value to develop a method of directly measuring their levels in tissues. Relatively few studies have been performed on the pharmacokinetics of standard TB drugs, including their penetration into granuloma lesions. Studies conducted on non-TB drugs in lung and abscess fluid have indicated that penetration into diseased tissue is both drug-specific and lesion-specific.^{3,4}

Moxifloxacin (MXF), a fourth generation fluoroquinolone, is considered one of the most efficacious second-line agents against multidrug resistant (MDR) TB⁵ and is in several clinical trials aimed at shortening TB treatment.^{6,7} In addition to their demonstrated in vitro bactericidal activity, fluoroquinolones have been shown to penetrate into macrophages and display bactericidal activity within.^{8,9} Because of the combination of this bactericidal activity and ability to accumulate within tissues and macrophages they achieve superior early bactericidal activity to other secondline anti-TB agents evaluated.^{10,11}

Since its introduction over 10 years ago,¹² MALDI mass spectrometric imaging (MALDI-MSI) has emerged as a valuable method of mapping the distribution of drugs and metabolites in biological tissues.^{13–15} Briefly, sections of selected tissue, biopsies, or dosed whole animals are prepared and coated with a solution of UV absorbing matrix. A laser is rastered across the surface of the tissue causing ionization of the matrix and the cocrystallized analyte. Full spectra, MS/MS scans, or selected ion transitions are recorded at selected points during the scan corresponding to individual pixels in the resulting ion image.

There are several key advantages of utilizing MALDI-MSI over traditional autoradiography methods for compound localization in tissue, namely the relinquishing of the requirement to label the administered drug (which is identified by its mass and fragmentation pattern) and the ability to acquire a vast amount of data from one experiment either as whole spectra (with full mass range capabilities) or the monitoring of multiple selected ion transitions.¹⁶ Drugs can therefore be distinguished from their respective metabolites in the extracted ion images as well as colocalized with endogenous species of interest such as lipids and peptides.

When using the MALDI-MSI technique to measure compound localization in tissue there are certain inherent problems concerning the analysis of low molecular weight species directly from biological tissue, and these are defined by the sample itself and the choice, and method of application, of the requisite matrix. Common MALDI matrixes such as α -cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB), and 3,5-dimethoxy-4-hydroxycinnamic acid (SA) produce spectra with lots of peaks in the low mass regions where compound peaks would be expected to be observed. Many compounds will only ionize well using a few or even one single matrix, and therefore it may be necessary to use a matrix that could potentially result in significant isobaric interference. In addition to the matrix, the tissue itself may also contribute high levels of spectral interference. Because of the complex nature of a tissue sample there will be many competing endogenous peaks present in each spectrum acquired from it. Specifically, lipids and lipid fragments are

present in high quantities in the mass range m/z 150–1000, which is where most drug peaks of interest are located.

MALDI-MS utilizing multiple reaction monitoring (MRM), as demonstrated on commercial triple quadrupole and ion trap mass spectrometers, allows for measurement of selected ion transitions and has been shown to be a viable alternative method to traditional electrospray ionization methods for the sensitive and selective high throughput analysis of low molecular weight compounds at extremely high speed.^{17,18} Single reaction monitoring (SRM) MALDI-MS imaging of compound distribution in whole rat tissue sections using a triple quadrupole linear ion trap instrument has been previously described.¹⁹ The specificity of monitoring selected ion transitions enables absolute identification of the compounds and metabolites of interest as well as circumventing interfering MALDI matrix or tissue signals in the low mass range.

However, significant problems of suppression and matrix effects are encountered when imaging a whole body section or organ containing many different tissue structures. One cannot assume that the matrix crystallization over such a tissue section will be homogeneous even if the matrix application is. Specific organs will have differing physical properties that will affect the formation of matrix crystals, their size, and their quality. The extraction efficiencies for selected analytes will differ from tissue to tissue, and the presence of high levels of blood and competing endogenous species such as lipids may lead to significant suppression of the desired compound signal. This ion suppression is due to the fact that the MALDI ionization process does not result in equal ionization efficiencies for each species located on the tissue surface.¹³ Therefore, the analyte with the highest signal intensity in an image is not necessarily the most abundant in the tissue.

A given signal for an analyte of interest can be normalized against a reference peak (or peaks) in an attempt to counter suppression and/or matrix effects. A range of reference peaks have been utilized including selected matrix peaks or the total ion current (TIC); however, all have their limitations and we propose normalizing against a reference standard which has a homogeneously applied distribution across the tissue surface and similar ionization properties to the analyte of interest. For this reason a structurally and chemically similar compound or preferably isotope-labeled version of the same drug applied homogeneously across the tissue surface provides the information required for successful normalization against tissue-specific suppression and matrix localization and ionization effects (such as hot-spots).

In this investigation we applied MALDI-MSI utilizing the sensitivity, selectivity, and speed of MRM to map the localization of MXF and reference standard in infected rabbit lung biopsies following oral dosing to determine the penetration of the drug into granulomas. The use of an established fluoroquinolone compound displaying similar ionization properties to the analyte as a reference standard is also described.

METHODS

Animals

Experiments utilizing New Zealand White (NZW) rabbits were performed with the approval of the animal care and use committee (ACUC) of NIH/NIAID under assurance #A-4149-01 and protocol # LCID-3. Female rabbits used in infection studies were housed in individual cages in a biosafety level 3 (BSL3) animal facility approved for the containment of MTB. All MTB infected rabbit tissues were processed in a certified BSL3 facility until the viable organisms had been inactivated. Tissues for MSI analysis (maximum size 2 cm × 2 cm × 0.5

cm deep) were sterilized in a single vertical layer in dry ice and exposed to γ -irradiation in a ^{60}Co irradiator using the nearest position and all three rods until 3 MRad was delivered.

Aerosol Infection of Rabbits

MTB strain HN878 was a kind gift from J. Musser. For rabbit infection, the aerosol inocula were prepared by diluting frozen stocks to 1×10^9 CFU/L in phosphate-buffered saline (PBS). The aerosol was generated using a BANG nebulizer delivering 18 L/min of filtered air and 6.4 L/min of aerosol to the CH Technologies inhalation system (Westwood, NJ) designed for rabbits, and the inoculum was calibrated to generate 50 to 100 granulomas per rabbit lung. The infection was allowed to develop for 7 to 9 weeks prior to initiation of drug distribution studies.

Drug Treatment and Blood Sampling

Animals were habituated to accepting suspensions of raspberry syrup as described previously.²⁰ Drug suspensions for oral administration were compounded as directed by the National Institutes of Health (NIH) Veterinary pharmacy by mixing powdered MXF with Oraplus suspending vehicle (Paddock Laboratories; Minneapolis, MN) in a mortar and pestle and adding an equal volume of Orasweet flavored syrup vehicle containing 1 mL of raspberry flavor (LorAnn Oils Inc.; Lansing, MI) for a final concentration of 25 mg/mL MXF. The animals were randomly assigned to necropsy time between 1 h and 8 h after drug administration. Serial blood samples were drawn into heparinized tubes at practical times between drug administration and sacrifice. Immediately prior to necropsy, rabbits were sedated with an intramuscular injection of ketamine (25 mg/kg) and acepromazine (0.5 mg/kg); once each animal was observed to be flaccid and calm, a final 2 mL blood sample was collected and the animal was euthanized.

Necropsy and Dissection

Rabbits were euthanized after the selected dwelled time for the drug cocktail as rapidly as possible with Beuthanasia-D (200 mg/mL) injected through the marginal ear vein. Death was verified by the absence of breathing and heartbeat, and necropsy was immediately performed with the pluck removal occurring no more than 10 min after the last blood draw (Figure 1A). The lung was photographed, and location, number, and size in the largest dimension of the lesions were recorded. Three to six small pieces (0.1 to 0.2 g) of grossly uninvolved lung were obtained from each animal, weighed, and snap frozen for determination of average drug concentration in grossly normal lung. Lesions ranging from 1.5 to 10 mm in diameter were identified, the tissue surrounding them was cut so that the lesions were bisected near the center, and at least two samples were prepared from each region. One sample (one-half of the identified lesion) was sliced with the surrounding normal tissue remaining intact and placed into a plastic tissue mold for cryopreservation in liquid nitrogen vapor (Figure 1A, inset arrow 1). The normal lung parenchyma was dissected away from remaining portion of the lesion so that only lesion remained (Figure 1A, inset arrow 2), and it was weighed and snap frozen on dry ice for later drug quantitation and bacterial burden quantitation. Procedures for histopathology were described previously.²⁰

A necropsy log describing the collection and division of lesions into matched samples for MSI, biochemical drug quantitation, histology, and bacterial burden was prepared for each animal. Lesions were initially categorized based on gross appearance at dissection, and the classification was confirmed upon histological examination of either stained cryosections or paraffin sections.

Preparation of Samples for MALDI-MSI

Serial 12 μm thick sections from each biopsy were cut directly onto 44 $\times$ 44 mm stainless steel MALDI target plates using a cryotome (Leica Microsystems, Wetzlar, Germany) and stored at -80°C until required for analysis. Subsequent sections for each biopsy were cut onto standard glass microscope slides and tissue fixation and H&E staining was performed.

Stainless steel targets were removed from deep freeze and immediately transferred to a desiccator. Following 15 min desiccation time, the plates were scanned using a flatbed scanner (HP Scanjet 6300C) and weighed.

CHCA matrix solution was prepared at 10 mg/mL in 50% acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA). Levofloxacin (LEV) (Sigma) as reference standard in a stock solution of 1 nmol/ μL (50% ACN) was added to produce a final concentration of 2 pmol/ μL .

Seven milliliters of matrix/LEV standard solution was applied to each plate using a TLC sprayer (VWR, Dietikon, Switzerland) operated at 0.5 bar pressure and held at a distance of 20 cm from the plate. Approximately 30 passes were performed per plate with 30 s drying time between cycles. The homogeneity of the matrix coverage was evaluated using a stereomicroscope (Leica Wild M3C, Wetzlar, Germany), and the plate was weighed to determine the total amount of matrix/LEV standard applied.

For the use of a sprayed reference compound as standard to be successful, the spray procedure itself had to prove reproducible. The amount of matrix applied to each plate (as defined by weight) had a mean of 4.69 mg with a low standard deviation across all plates analyzed of 3.37% (0.16 mg). This meant that the spray procedure, in one given experiment, was highly reproducible across all the plates sprayed and thus the amount of matrix (incorporating the LEV standard) on each plate was consistent.

MALDI-MSI Analysis

Standards of moxifloxacin HCl (Bayer HealthCare AG, Wuppertal, Germany) and levofloxacin (Sigma-Aldrich, Buchs, Switzerland) were analyzed using the FlashQuant QTRAP mass spectrometer (AB Sciex) operated in Q1 (full scan) mode to determine the ability of both compounds to ionize.

MS instrument parameters were optimized for MXF signal. Product ion scans of the protonated precursor ions (m/z 402.2 MXF, m/z 362.2 LEV) revealed a clear fragmentation pattern for both fluoroquinolones with the transitions m/z 402.2 $\rightarrow$ 384.2 (MXF) and m/z 362.2 $\rightarrow$ 344.2 (LEV) dominating the spectra. Increasing the laser power and collision energy resulted in a greater range of product ions produced but markedly reduced sensitivity.

FlashQuant 1.1 beta imaging software (AB Sciex), fitted with a 1 kHz ND:YAG laser, was used for image acquisition. The instrument was operated in MRM mode monitoring positive ions with the laser rastering across the tissue at a continuous scan speed of 1.5 mm per second and at a raster dimension of 200 μm in a serpentine pattern. This method setup resulted in a dwell time of 62 ms for each of the two transitions. Both Q1 and Q3 were set to transmit a 1 Da window centered on the specific masses. Image acquisition windows varied in size between 1.5 $\times$ 1.5 cm and 2 $\times$ 2 cm depending upon the dimensions of the biopsy section, and image acquisition time was approximately 15 min per tissue section.

In-house-developed software (Stoeckli, Novartis, Switzerland) was used to convert the standard FlashQuant data format (.wiff) into the BioMap readable Analyze 7.5 format

(Mayo Foundation, Rochester, MN). BioMap (Novartis, Basel, Switzerland) was used for data visualization. Normalization was performed by dividing the MXF distribution image by the LEV distribution image using the divide scans function within the BioMap software.

Quantitative Analysis of Extracts

Drug-free tissue infected with MTB was sterilized using γ -irradiation as previously described, shipped frozen on dry ice, and stored at -20°C until use for analysis. This material was used as a naive matrix for building calibration curves. Drug-containing material was processed at source with the sample preparation procedure described below, lyophilized, shipped on dry ice, and stored at -20°C until they were reconstituted in mobile phase and analyzed.

Frozen drug-free tissue for use as matrix for calibration standards was thawed at room temperature and homogenized in PBS using a Qiagen TissueLyzer beat beater. One milliliter of PBS was added for every 0.2 g of tissue.

Plasma and tissue levels of MXF were quantified by LC/MS/MS following protein precipitation with a 9:1 extractant (acetonitrile with 0.2% acetic acid) to plasma or tissue homogenate ratio. Standards, quality control samples, and blanks in the matrix of interest were used.

The analysis was performed on an AB Sciex API4000 LC/MS/MS mass spectrometer coupled to Spark Symbiosis Pharma HPLC system. Sample analysis was accepted if the low level quality control samples were within $\pm 20\%$ of nominal concentration and $\pm 15\%$ for mid and high level quality control samples. Gradient elution conditions with an Agilent Zorbax Phenyl $4.6 \times 75\text{ mm } 3.5\text{ }\mu\text{m}$ column were used. The mobile phase A was 0.2% acetic acid, and the mobile phase B was 0.2% acetic acid in acetonitrile.

Multiple reaction monitoring of parent/daughter transitions in electrospray positive ionization mode was used to track presence of analyte, and quantitation of drug levels was performed using the transitions m/z 402.2 $\rightarrow$ 261.2 and 402.2 $\rightarrow$ 384.3 for moxifloxacin. Warfarin was used as internal standard, and transitions m/z 309.2 $\rightarrow$ 163.1 and 309.2 $\rightarrow$ 251.1 were monitored. Mass spectrometer source conditions were optimized for the compound.

Data processing was performed using Analyst software 1.4.2.

RESULTS AND DISCUSSION

Aerosol infection of rabbits with MTB proved highly successful at producing multiple granulomas in each animal.²⁰ Clearly visible granulomas were observed in all the biopsies taken from the infected animal lungs (Figure 1). A range of granuloma types were observed including granulomas which contained large (1–2 mm diameter) areas of caseous necrosis in several biopsies (an example of which is shown in Figure 1B).

The MALDI-MS product ion spectra for MXF and LEV are shown in Figure 2. The most intense peak observed in both spectra corresponds to a loss of water (-18 Da). These two ions were chosen for each of the respective transitions for the measurement of each compound. As the transition corresponding to loss of water is relatively common among molecules, this may reduce the selectivity of the method. While small levels of background noise were sometimes observed in the acquired images (Figure 3 prenormalization) these were effectively minimized following normalization (Figure 3 postnormalization). Efforts to increase the yield of alternative products ions by increasing the collision gas, collision

energy, and exit potential resulted in many highly fragmented species all at relatively low intensity. Comparisons with tissue sections taken from lung biopsies from an untreated rabbit were performed, and it was clear that very few background signals were observed in the untreated control for MXF (Figure 4) and thus the selected transitions were suitable for the experiment.

The MALDI-MRM-MSI method produced high quality MS images of the distribution of MXF in the lung and granuloma tissue (Figures 3 and 4). The images were far superior to those acquired during previous experiments conducted by our group using Q-TOF technology²¹ and highlight the benefits of the additional selectivity and sensitivity of the MRM imaging method. Normalizing the MXF signal against the distribution of the homogeneous sprayed LEV standard appeared to compensate well for the inhomogeneity of the tissue sections (as shown in Figure 3). A minimum of three sections were imaged for each biopsy (within 100 μm distance of the H&E reference tissue) to ensure that the method was reproducible, and the data for the 1.5 h biopsy is shown in Table 1. Reproducibility was shown to be high, as only low levels of deviation (between 6.83 and 13.70%) were observed for the MXF ion signal recorded over the three repeat images.

MXF was clearly observed in all of the lung biopsies imaged from the dosed rabbits as shown in Figure 4. At the earliest time point (0.5 h) the distribution of MXF appeared to be relatively homogeneous throughout the tissue, and there was no evidence of accumulation of MXF within the large granulomas in comparison to the surrounding normal lung tissue. From the 90 min time point onward, clear accumulation of the compound was observed within granulomas with particularly strong signals at the 1.5, 2.17, and 3.25 h time points.

The mean postnormalization MXF ion signal intensities for all granulomas in each tissue section plotted against defined areas of normal lung are shown in Figure 5. The highest signal quantity within granulomas was observed between 1.5 and 3.25 h postdose, and little difference was observed in the signal intensity in the three biopsies covered over that time period. Granuloma MXF intensities were still 2-fold higher than the normal lung at the final time point recorded.

MXF distribution within the granulomas was not observed to be homogeneous. A donut-shaped distribution is present in several of the large granulomas (1.5 and 2.17 h) in which the highest signal for MXF is located in the periphery of the granuloma. The center of these granulomas consists of caseous necrosis and necrotic debris (as shown as a pink-colored area in the center of the H&E-stained granulomas shown in Figures 3 and 4). It appears from these images that MXF is not present at high levels within the necrotic granuloma areas. The highest MXF signal was observed in the cellular (non-necrotic) granuloma regions, and this is where the highest amount of activated macrophages, lymphocytes, and other immune cells is situated. In the large necrotic central granuloma located in the 1.5 h biopsy tissue the mean MXF signal in the cellular region of the granuloma was 2.2 fold higher than that in the necrotic core (Table 1). Our findings are supported by the published literature concerning the high uptake levels of MXF into active macrophages.⁵

The imaging data was validated by the quantitative analysis of homogenates of dissected granulomas and normal lung tissue taken from the same rabbits. Between three and five granuloma and healthy lung samples from each animal were used, and there were clearly high variance of MXF concentration between the samples taken from the same animal, particularly in the normal lung tissue (as reflected by the large standard deviation observed in Figure 6). The observed variance in granuloma drug concentrations was expected due to the inhomogeneity within individual granulomas and their many different types which may affect the degree of drug penetration and accumulation within them.

MXF absolute concentrations within granulomas were observed to be lower than in the surrounding lung tissue after 0.5 h postdose. A sharp increase occurred between 0.5 and 1.5 h at which point the concentration of MXF in granulomas was more than 2-fold higher than in normal lung. The highest concentration of MXF was recorded at the 2.17 h time point, and both the 2.17 and 3.25 h time points contained granulomas with 2-3-fold higher concentrations of drug within granulomas in comparison to surrounding lung tissue. Even at the last time point, higher levels of MXF were found in the granulomas than in the normal lung although by this point the ratio was much lower, and this is indeed reflected in the corresponding MALDI images of the selected biopsy (Figure 4). This quantitative data is in agreement with the MALDI-MSI MXF signal data (Figure 5). In both cases the highest MXF levels are observed within granulomas at the 1.5–3.25 h time range. However, the granuloma $t_{\max}$ at 2.17 h observed from the LC-MS data is not conclusively reproduced in the MALDI-MSI data in which the granuloma $t_{\max}$ cannot be clearly distinguished between the three samples covering the 1.5–3.25 h postdose time range.

MXF plasma levels, taken from each animal immediately prior to sacrifice, are displayed in Figure 6. Overall, MXF levels in plasma were considerably lower than in both normal lung and granulomas, except at 0.5 h postdose. The absolute plasma level should be interpreted with caution, as they originate from a single animal at each time point.

CONCLUSIONS

The MALDI-MRM-MSI method has been shown to produce high quality images, enabling the penetration and accumulation of MXF in TB granulomas to be rapidly and reproducibly determined. The use of the MRM method improved the sensitivity and selectivity of the analysis, enabling detailed drug localization within lung and granuloma that could not be determined by alternative methods. The use of another fluoroquinolone compound as an externally applied reference standard was described and proved effective at reducing compound specific ion suppression due to heterogeneity of the lung and granuloma tissues.

MXF was observed to penetrate into the granulomas and accumulate from the 1.5 h time point until the final time point measured. Highest levels were observed between 1.5 and 3.25 h postdose. The imaging data correlated well with quantitative PK LC/MS/MS data of MXF in normal lung, granuloma, and plasma extracts. Distributions within the granulomas themselves were inhomogeneous with highest levels observed in the areas of the granulomas known to contain active macrophages, while relatively low levels were detected in the central necrotic caseum. This observation could be of clinical relevance since extracellular MTB populations are thought to reside in this lesion compartment.

Further experiments concerning additional TB drug uptake into granulomas are currently being conducted. Increasing the number of MRM transitions in the experiment would enable simultaneous monitoring of more drugs (or metabolites) although with increasing number of transitions either dwell time or spatial resolution would have to be reduced to ensure each ion signal was being acquired from fresh tissue.

The methods developed thus far have significant potential for application to a number of pharmaceutical compounds in a range of biological tissues. The MS images and data acquired thus far are being utilized to aid the interpretation of published and ongoing fluoroquinolone efficacy studies, providing a unique and valuable insight into the drug localization at the site of action and potential explanations for the very long therapy required to cure TB. This methodology also provides a potentially valuable screening technique for comparing and prioritizing lead candidates within a chemical series.

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Figure 1.

Tuberculomas as observed 6–7 weeks post aerosol infection of a NZW rabbit with MTB strain HN878. A. Dorsal aspect of the lung showing cream-colored lesions (arrows); inset, cross-section of the left upper lobe prepared for cryosectioning (arrowhead 1) and half of one matched lesion (arrowhead 2) prior to processing for drug quantitation by LC/MS/MS analysis. B. Small necrotizing lesions identified by central necrosis with peripheral macrophages and lymphocytes in ball-like lesions stained with H&E. C. Cellular lesion containing epithelioid macrophages but little or no central necrosis, similarly stained. Scale bar = 0.5 mm in length.

Figure 2. MALDI-MS/MS spectra for levofloxacin (A) and moxifloxacin (B). The dominant product ions at m/z 344 and m/z 384 correspond to the $[M + H - H_2O]^+$ fragments of each respective fluoroquinolone.

Figure 3.

Figure showing the MALDI-MS images at each stage of the normalization process. Lower LEV reference standard signals were observed from the granulomas (large central granuloma indicated by the arrow in the H&E reference image) compared to that of the surrounding normal lung tissue. Greater LEV signal suppression occurred in the viable granuloma compared to that in the caseum (identifiable as the light pink center of the large central granuloma). Ion signal intensities were individually scaled for each image. Scale bar = 5 mm.

Figure 4.

MS images showing MXF distributions within the rabbit lung biopsy sections at a defined range of postdose times. A subsequent H&E stained reference tissue section is displayed below these images. MXF is uptaken rapidly into the lung, and accumulation within granulomas occurs from 1.5 h. Granuloma drug levels remain higher than surrounding lung tissue over the remaining time points monitored. Lower levels of MXF are observed within the central caseous necrotic areas of the granulomas in the 1.5 and 2.17 h tissues (necrosis is visible as a light pink center in the H&E stained reference tissue). Signal intensities are shown as a fixed scale. Scale bar = 5 mm.

Figure 5.

Mean postnormalization granuloma ($n = 2-6$) and normal lung ($n = 3$) MXF ion signal intensities taken from a selected MALDI-MS image for each postdose time point. Clear accumulation of MXF within granulomas above surrounding lung begins to occur between 0.5 and 1.5 h. Granuloma MXF levels are consistently higher than those in normal lung over the 1.5–5.14 h period. Viable granuloma and necrotic granuloma areas were not resolved when defining the granuloma region of interest.

Figure 6.

Graph showing the concentrations of MXF in plasma, lung, and granuloma lesion tissues at a range of time points postdose. Plasma was sampled immediately prior to euthanasia, lesion, and lung extracts were taken from several ($n = 2-5$) biopsies from the lungs of each animal. From 1.5 h postdose lesion, concentrations of MXF were higher than that of normal lung. The highest concentration of MXF in granuloma was recorded at 2.17 h postdose.

Table 1Actual MXF Signal Intensity Values in Infected Lung, Granuloma, and Necrosis^a

biopsy section no.	viable granuloma	necrotic core	mean granuloma (<i>n</i> = 5)	mean lung (<i>n</i> = 3)
1	4.41	2.02	4.39 (±0.34)	1.68 (±0.15)
2	4.01	1.69	3.93 (±0.30)	1.38 (±0.21)
3	3.88	1.84	3.70 (±0.19)	1.31 (±0.20)
mean (<i>n</i> = 3)	4.10 (±0.28)	1.85 (±0.17)	4.01 (±0.35)	1.46 (±0.20)

^aMean postnormalization MXF signal intensities (a.u.) from defined regions of interest of three MALDI-MSI imaging experiments on adjacent sections from the 1.5 h postdose biopsy shown in Figure 3. The regions were defined as follows: viable area of large central granuloma (not including necrotic core), necrotic core of large central granuloma, mean intensity over all five granulomas in the tissue, and mean intensity of three distinct normal lung regions. Viable granuloma signal levels were at least 2-fold higher than that in the necrotic core in all three experimental repeats. Reproducibility of the method is confirmed by the low (<14%) standard deviation of MXF signal in the defined tissue regions over the three experimental repeats.