Radiosynthesis and Biodistribution of $^{18}$F-Linezolid in *Mycobacterium tuberculosis*-Infected Mice Using Positron Emission Tomography

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Supporting Information

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Experimental methods; HPLC traces; intracellular accumulation of $^{18}$F-linezolid in live and heat-killed *M. tuberculosis*; CT scan; $^{18}$F-linezolid PET derived AUC ratios in tissues (PDF)

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Abstract

Oxazolidinones are a novel class of antibacterials with excellent activity against resistant Gram-positive bacteria including strains causing multidrug-resistant tuberculosis (TB). Despite their excellent efficacy, optimal dosing strategies to limit their toxicities are still under development. Here, we developed a novel synthetic strategy for fluorine-18-radiolabeled oxazolidinones. As proof-of-concept, we performed whole-body 18F-linezolid positron emission tomography (PET) in a mouse model of pulmonary TB for noninvasive in situ measurements of time–activity curves in multiple compartments with subsequent confirmation by ex vivo tissue gamma counting. After intravenous injection, 18F-linezolid rapidly distributed to all organs with excellent penetration into Mycobacterium tuberculosis-infected lungs. Drug biodistribution studies with PET can provide unbiased, in situ drug measurements, which could boost efforts to optimize antibiotic dosing strategies.

Graphical Abstract

Keywords

PET; pharmacokinetics; tuberculosis; fluoride-18

Oxazolidinones are a novel group of synthetic drugs that are active against a wide-spectrum of Gram-positive bacteria. They display antibacterial activity by inhibition of protein synthesis through binding to the prokaryotic ribosome, thus preventing mRNA translation. Linezolid was the first of the oxazolidinone class of antibacterials to be approved by the U.S. Food and Drug Administration (FDA). It is highly active against most clinically relevant Gram-positive bacteria, orally bioavailable, and also efficacious in the treatment of multidrug-resistant (MDR) and extensively drug-resistant tuberculosis (XDR-TB). Recently, a shorter (six months) all-oral three drug regimen utilizing bedaquiline, pretomanid, and linezolid (BPaL) was approved by the FDA for patients with MDR-TB. However, linezolid treatment has been linked to mitochondrial toxicity and adverse effects such as neuropathy and myelosuppression. For example, in the recent Nix-TB trial in
which patients received BPaL treatment, 81% and 37% of patients demonstrated neuropathy and myelosuppression, respectively, with only a minority of patients being able to remain on the full dose of linezolid for the entire six months.\textsuperscript{5} Because of this and despite its excellent efficacy, the optimal dosing regimen for linezolid and several other oxazolidinones (e.g., sutezolid, tedizolid, TBI-223) is still under debate.\textsuperscript{5,7,8} Therefore, methods to identify drug dosing strategies that could optimize drug levels at the target site (infection) while minimizing toxicity and accumulation at off-target sites are needed.

Drug pharmacokinetic (PK) parameters are conventionally derived from easily available biological samples such as blood, but this provides limited information regarding the drug concentration at the site of infection, which is often characterized by complex and heterogeneous biochemistry that affects drug delivery. Conversely, current methods used to quantify local drug concentrations require tissue biopsies or resections, which limit their applications.\textsuperscript{9,10} Therefore, detailed intralesional PK data at infection sites, as well as other organs subject to toxicity, could substantially boost the optimization of new antibacterial treatments. Positron emission tomography (PET), a clinically translatable technology, can provide noninvasive, whole-body measurements of radiolabeled drugs with high sensitivity (nano- to picomolar concentrations).\textsuperscript{11} The PET-captured time–activity curves can be used to calculate the area under the curve tissue to plasma ($\text{AUC}_{\text{tissue/plasma}}$) ratios in different organs, particularly at the site(s) of infection.\textsuperscript{12–14} Here, we report a novel strategy to synthesize fluorine-18-radiolabeled oxazolidinones that was used in the development of $^{18}$F-linezolid, which is chemically identical to the parent drug. Whole-body $^{18}$F-linezolid PET was utilized for noninvasive \textit{in situ} measurements of $^{18}$F-linezolid $\text{AUC}_{\text{tissue/plasma}}$ ratios and supplemented by \textit{ex vivo} tissue gamma counting in a mouse model of pulmonary TB, which replicates key features of human pathology.\textsuperscript{15–17} Combined, these data provide a holistic view of the biodistribution of the radiolabeled drug in \textit{M. tuberculosis}-infected mice and detailed information regarding its penetration into privileged areas such as infected lung lesions where bacteria reside.

\textbf{Results and Discussion.}

\textbf{Radiosynthesis of $^{18}$F-Linezolid.}

Since linezolid contains a fluorine atom, we devised a synthetic route that would allow the incorporation of $^{18}$F (physical half-life = 109.8 min, $\beta^+ 97\%$), the most commonly used PET isotope, and could be used in the radiolabeling of other relevant oxazolidinones (e.g., sutezolid, tedizolid, TBI-223). Radiofluorination of arenes and heteroarenes not amenable to nucleophilic aromatic substitution ($\text{S}_\text{N} \text{Ar}$) has recently become more accessible with the development of novel metal-catalyzed radiochemical methods. For the radiosynthesis of $^{18}$F-linezolid, we optimized a copper-mediated method for the radiofluorination of boronates, which was achieved using the synthetic route illustrated in Figure 1. Aniline (3) was prepared from the commercial boronate ester (1) via $\text{S}_\text{N} \text{Ar}$ with morpholine, followed by catalytic hydrogenation. Aniline (3) was subsequently treated with (S)-N-glycidylphthalimide to afford an intermediate secondary alcohol, which was cyclized to oxazolidinone (4) using 1,1-carbonyldimidazole in dichloromethane. During the formation of oxazolidinone (4), boronate ester hydrolysis to the corresponding boronic acid was
observed. We are unaware of a literature precedent for this hydrolysis, which we found unavoidable. Deprotection of the phthalimide to provide the methylamine (5) was followed by acylation to afford amide (6). The boronic acid intermediate (6) was further treated with pinacol to form the desired boronate ester (7).

Although reproducibility and radiochemical yields are dependent on the position of the aryl boronate and additional functional groups present, most successful copper-mediated methods for radiofluorinations of boronates react the precursor with K\(^{18}\)F and a copper reagent in N,N-dimethylformamide for 20 min at 110 °C.\(^ {18–21}\) The nature and amount of base required to elute \(^{18}\)F\(^{−}\) from quaternary methylammonium (QMA) ion exchange cartridges are known to influence the subsequent copper-mediated reactions.\(^ {22,23}\) Similarly, the concentration of the boronic acid precursor in radiolabeling protocols also affects the yields, with higher amounts being found to inhibit the reaction. Initial attempts to obtain \(^{18}\)F-linezolid through nucleophilic fluorination of boronic acid precursor (6) following literature protocols were unsuccessful. We found, however, that \(^{18}\)F\(^{−}\) elution with a solution of potassium acetate and Kryptofix 222 and a reaction with copper(II) triflate in the presence of pyridine allowed us to generate 18F-linezolid in 1.5–4% \( (n = 3) \) radiochemical conversion (RCC; % integrated area corresponding to product versus \(^{18}\)F\(^{−}\) and side products in a radio-HPLC trace). Using the same conditions, radiofluorination of the pinacol boronate ester (7) yielded the same result. Further optimization of the radiolabeling conditions allowed the biosynthesis of 18F-linezolid to proceed in 2–15% RCC and 1–3% \( (n = 4) \) radiochemical yield (RCY) in the presence of tetrakis-(pyridine)copper(II) triflate \( (\text{Cu(OTf)}_2(\text{py})_4) \), following \(^{18}\)F\(^{−}\) elution with potassium carbonate instead of potassium acetate. 18F-Linezolid was isolated by preparative HPLC, and the identity of the radiotracer was confirmed by the coinjection of cold standard linezolid with 18F-linezolid (Supplementary Figure 1).

\(^{18}\)F-Linezolid PET.—Linezolid inhibits protein synthesis through binding to the prokaryotic ribosome; thus, we investigated the bacterial uptake of 18F-linezolid. In vitro studies did not demonstrate any significant uptake of 18F-linezolid in \textit{M. tuberculosis} cultures (Supplementary Figure 2), suggesting lack of bacterial accumulation.

Following aerosol infection with \textit{M. tuberculosis}, mice developed heterogeneous pulmonary lesions visible by computed tomography (CT) (Supplementary Figure 3). \(^{18}\)F-Linezolid (at subpharmacological levels) was administered to the animals intravenously, and time–activity curves and AUC\textsubscript{tissue/plasma} ratios were measured in infected pulmonary lesions, control unaffected pulmonary regions, and other organs (Figures 2 and 3). The plasma PK parameters for linezolid after oral dosing are already established in animals and humans; therefore, the primary goal of our current study was to establish AUC\textsubscript{tissue/plasma} ratios in \textit{M. tuberculosis}-infected animals using PET. These PET-derived measurements in conjunction with plasma PK would allow accurate quantification of the intralesional linezolid concentrations.

Since the elimination half-life of linezolid is between 0.4 and 0.9 h in mice,\(^ {24}\) we performed dynamic PET scans for the first 60 min after intravenous injection to capture the initial biodistribution of the radiolabeled drug into all tissues. 18F-Linezolid rapidly distributed to all major organs and, as expected, eliminated via renal and hepatobiliary routes (Figure 3).
Penetration into both infected and unaffected lung areas was high with $AUC_{\text{tissue}}/AUC_{\text{plasma}}$ ratios of $>1$ and consistent with prior data. \textit{Ex vivo} tissue gamma counting matched well with the PET data (Figure 4). PET-derived $AUC_{\text{tissue}}/AUC_{\text{plasma}}$ for all compartments measured are provided in Supplementary Figure 4. While we performed imaging over 60 min, the physical half-life of $^{18}\text{F}$ (109 min) allows PET imaging up to 4–6 h (2–3 physical half-lives). Therefore, accurate drug PK measurements for oxazolidinones with longer elimination half-lives could also be performed. Future studies in relevant animal models \cite{12,25} are needed to further investigate our preliminary findings.

In summary, we have developed a novel strategy to synthesize fluorine-18-radiolabeled oxazolidinones and demonstrated proof-of-concept with $^{18}\text{F}$-linezolid. PET provided unbiased, noninvasive \textit{in situ} measurements of linezolid AUC in multiple compartments. While additional studies are needed to establish the role of $^{18}\text{F}$-linezolid PET, this technology is clinically translatable and could allow cross-species animal and human studies to measure tissue drug levels with several advantages over current tools. It should also be noted that $^{18}\text{F}$-linezolid was administered at subpharmacological doses (microdose). While there have been concerns that data from microdose studies may not accurately reflect drug PK at full therapeutic doses, current evidence suggests that, in most cases, microdosing is an excellent predictive tool.\textsuperscript{14,26} In the future, this approach could be utilized to measure drug levels of novel oxazolidinones in development (e.g., sutezolid, TBI-223) and efficiently conduct complex studies in larger and more expensive animal models (e.g., rabbits, nonhuman primates). It could also be applied to other infections by pathogens such as \textit{Staphylococcus aureus} and non-TB mycobacteria, which have similar challenges.

**MATERIALS AND METHODS**

All protocols were approved by the Johns Hopkins Biosafety, Radiation Safety, and Animal Care and Use Committees.

**General Materials and Methods.**

All chemicals and solvents were reagent grade and purchased from a commercial supplier. Radio-HPLC was performed on an Agilent 1260 HPLC coupled to an Ecklert and Ziegler flow-count radio detector. The syntheses of the precursors (6 and 7) and radiofluorination of boronic acid (6) are described in the Supplementary Methods. The \textit{in vitro} assay used to determine $^{18}\text{F}$-linezolid uptake in \textit{M. tuberculosis} is also described in the Supplementary Methods.

**Radiosynthesis of $^{18}\text{F}$-Linezolid.**

$^{18}\text{F}$ (target wash) was obtained from the JHU PET Center. Aqueous $^{18}\text{F}$-fluoride was trapped on a QMA cartridge pre-equilibrated with Trace-Select Water (10 mL). K$^{18}\text{F}$ was eluted from the cartridge with a solution of potassium carbonate (1.4 $\mu$mol) and Kryptofix 222 (1.6 $\mu$mol) in a mixture of acetonitrile (80%) and water (1 mL) into a Wheaton v-vial and dried under a constant stream of N$_2$ for 15 min at 110 °C. K$^{18}\text{F}$ was dried by azeotropic distillation with acetonitrile (400 $\mu$L, twice) at 90 °C. Precursor 7 (18 $\mu$mol) was dissolved in DMF (200 $\mu$L) and added to a solution of Cu(OTf)$_2$(py)$_4$ (5 $\mu$mol) in DMF (200 $\mu$L).
resulting mixture was then added to a vial containing the K\(^{18}\)F redissolved in DMF (100 \(\mu\)L) and reacted for 20 min at 110 °C. The reaction was quenched with water, and upon cooling, the crude mixture was injected onto the radio-HPLC and purified using a Luna Phenomenex C-18(2) 10 \(\mu\)m, 250 × 10 mm column at a flow rate of 3 mL/min, using water and acetonitrile as mobile phases. The following gradient was applied: 5% acetonitrile for 5 min; 5–50% for 10 min; 50–95% for 3 min; 95% for 4 min; 95–5% for 6 min. \(^{18}\)F-Linezolid eluted at 18 min and was dissolved in water to a total volume of 10 mL, trapped into a preactivated Sep-Pak C-18 Light cartridge, and eluted with 1 mL of ethanol. The ethanol was evaporated under \(N_2\), and the radiotracer was formulated in saline containing less than 10% ethanol.

**Animal Infection.**

Four- to six-week-old female C3HeB/FeJ mice (Jackson Laboratory) were aerosol-infected with frozen stocks of *M. tuberculosis* (H37Rv), using the Middlebrook Inhalation Exposure System (Glas-Col) as described previously.

**PET Imaging.**

After an incubation period of 10 weeks postinfection with *M. tuberculosis*, anesthetized animals \((n = 4)\) were injected with 3.52 ± 1.27 MBq of \(^{18}\)F-linezolid via tail vein and imaged within a sealed biocontainment bed (Minerve), as described previously. The injection time coincided with the start of PET acquisition to obtain dynamic tracer uptake information over 60 min. PET scans were followed by computed tomography (CT) scans and performed using the nanoScan PET/CT (Mediso). The images were analyzed using VivoQuant 3.0 (Invicro) for visualization and quantification of the biodistribution of the radiolabeled drug by drawing regions of interest (ROI) on the basis of the CT. Data for blood activities were obtained by placing an ROI in the left ventricle of the heart and then converted to plasma using the hematocrit values. PET data was adjusted for mass using the density of each ROI (obtained from the CT as Hounsfield units) and is expressed as % injected dose (ID)/mass of tissue (g).

**Ex Vivo Biodistribution.**

After an incubation period of 10 weeks postinfection with *M. tuberculosis*, anesthetized animals were injected with 1.9 ± 0.1 MBq of \(^{18}\)F-linezolid via tail vein and sacrificed 2 and 30 min after injection \((n = 3\) per time point). Organs and tissues of interest were harvested, and the associated radioactivity was measured using an automated gamma counter (1282 Compugamma CS Universal gamma counter; LKB Wallac). Data are represented as % ID/g.

**Statistical Analysis.**

Data was analyzed using Prism 8 version 8.1.0 (GraphPad). AUCs were calculated using the linear trapezoidal rule. Comparisons were made using a two tailed, Mann–Whitney \(U\) test. \(P\) values of less than 0.05 were considered statistically significant.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.
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Figure 1.
Synthetic approach to $^{18}$F-linezolid. Reagents and conditions: (i) morpholine, toluene, 110 °C, 12 h; (ii) 10% Pd/C, H$_2$, methanol, rt, overnight; (iii) (a) (S)-N-glycidylphthalimide, MeOH, 65 °C, (b) 1,1-carbonyldiimidazole, DCM, rt, 12 h; (iv) hydrazine hydrate, MeOH, reflux, 1 h; (v) acetic anhydride, triethylamine, DCM, 0 °C to rt, 12 h; (vi) pinacol, 4 Å molecular sieves, THF, rt, 12 h; (vii) K$^{18}$F, potassium carbonate, Kryptofix 222, Cu(OTf)$_2$(py)$_4$, DMF, 110 °C, 20 min.
Figure 2.
$^{18}$F-Linezolid PET of *M. tuberculosis-*infected mice. (A) Three-dimensional maximum intensity projections of $^{18}$F-linezolid PET/CT from a representative mouse at 2 and 30 min post-injection, showing rapid distribution of $^{18}$F-linezolid to the heart (H), lungs (L), and liver (Liv) and clearance through the bladder (B), gallbladder (GB), and intestine (G). (B) Time–activity curves in plasma and lung (infected and unaffected). Data represented as median ± interquartile range, *n* = 4 animals per group.
Figure 3.
Dynamic PET of $^{18}$F-linezolid. (A) Time–activity curves for liver and kidneys demonstrate both renal and hepatobiliary excretion. (B) Uptake in brain and bone. Data represented as median ± interquartile range, $n = 4$ animals per group.
**Figure 4.**
*Ex vivo* biodistribution of $^{18}$F-linezolid into *M. tuberculosis*-infected mouse tissues. Data represented as median ± interquartile range, *n* = 3 animals per time point.