Study of the Cellular Uptake and Subcellular Distribution of the Novel Oxazolidinone Tedizolid in Murine J774 Macrophages: Lack of Association With Mitochondria

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ABSTRACT

INTRODUCTION. Linezolid (LZD) causes mitochondrial dysfunction resulting in potentially severe adverse effects (hyperlactacidemia, metabolic acidosis, and peripheral neuropathy). Tedizolid (TZD) is a novel oxazolidinone antibacterial with potent activity against a wide range of Gram-positive pathogens, including resistant strains such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci, and a favorable tolerability profile. It accumulates about 10-fold more in macrophages compared with LZD, with commensurate larger activity against intraphagocytic *S. aureus* (phagolysosomes), *Legionella pneumophila* (phagosomes), and *Listeria monocytogenes* (cytosol). However, this also raises questions about the potential association of TZD with mitochondria.

METHODS. Murine J774 macrophages were exposed to TZD (2·50 mg/L) for 2 hours, collected, and homogenized for fractionation by differential (pelleting) and isopycnic (sucrose gradient) centrifugation. After extraction with $CHCl_3$:CH_3OH (8:4), TZD was quantified by liquid chromatography (reverse phase) coupled with mass spectrometry (electrospray; selective ion monitoring at MW 370·372, with LZD as the internal standard). TZD distribution was compared with that of marker enzymes for mitochondria (cytochrome *c*-oxidase), lysosomes (*N*-acetyl- β-hexosaminidase), and cytosol (lactate dehydrogenase).

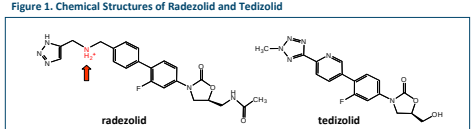
RESULTS. After differential and isopycnic cell fractionation, TZD was consistently recovered in fractions corresponding to the cell cytosol and not detected in those enriched in mitochondria or lysosomes.

CONCLUSIONS. Despite a higher accumulation in eukaryotic cells compared with LZD, TZD does not seem to associate in a stable fashion to mitochondria. Observing TZD in the cytosol after cell fractionation may point to its ability to diffuse throughout the cell, explaining its higher activity against organisms harbored in distinct subcellular compartments, while at the same time not necessarily implying higher potential mitochondrial toxicity.

INTRODUCTION

- There is a continuous need to discover and develop new antibacterials to treat infections caused by multidrug-resistant pathogens. In this context, new oxazolidinones such as tedizolid have emerged that show higher intrinsic activity (lower minimum inhibitory concentrations [MICs]) than linezolid against Gram-positive pathogens from clinical origin, including methicillin-resistant *Staphylococcus aureus*, as well as activity against linezolid-resistant, *cfr*+ strains without chromosomally-mediated linezolid resistance.[1,2]
- Tedizolid also accumulates about 10-fold more than linezolid in human THP-1 macrophages in vitro[3] and accumulates about 20-fold more than in serum concentrations in healthy adult volunteers.[4] Although this may confer an advantage in terms of activity against intracellular forms of target bacteria,[3] it also raises concerns with respect to potential toxicity. Indeed, one of the main likely mechanisms for the long-term toxicity of linezolid probably relates to its ability to impair mitochondrial DNA-dependent protein synthesis,[5,6] owing to structural similarities between mitochondrial and prokaryotic ribosomes.[7]
- Surprisingly, there are no published results of studies evaluating the subcellular localization of linezolid. Conversely, we know that another advanced oxazolidinone, radezolid, which also accumulates more in human THP-1 cells than linezolid, is mainly localized in lysosomes.[8] This can be rationalized based on the presence of an amino function with a pKa-7.4 (**Figure 1**) resulting in a proton-trapping of its ionized form in acidic membrane-bounded organelles.[9] However, tedizolid has no such amino function and remains essentially uncharged between pH 2 and 11, making prediction of its subcellular localization difficult.

Figure 1. Chemical Structures of Radezolid and Tedizolid

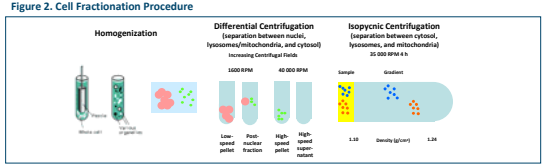


- The current study is aimed at studying the subcellular distribution of tedizolid.

METHODS

- Cell fractionation (**Figure 2**), which consisted of collecting cells incubated with the drug of interest, was used, disrupting the pericellular membrane without damaging the integrity of the subcellular organelles (homogenization) and separating these organelles by subjecting the suspension to centrifugation at increasing speeds (differential pelleting, in which organelles are mainly separated based on their size) or by centrifugation through sucrose gradients (isopycnic centrifugation, in which organelles are mainly separated based on their buoyant density).
- Fractions were thereafter assayed for the presence of markers of the organelles of interest and for drug content.

Figure 2. Cell Fractionation Procedure



- These methods, originally developed to discover and characterize subcellular organelles,[10] have been previously used and validated for study of the subcellular localization of aminoglycosides (lysosomes), macrolides (2/3 in lysosomes; 1/3 in cytosol), and fluoroquinolones (cytosol).[11]
- In the current study, murine J774 macrophages were selected because these cells can easily be fractionated and mitochondria can be satisfactorily separated from lysosomes by isopycnic centrifugation.
- Cells were incubated for 2 hours with 20 mg/L tedizolid before being harvested and subjected to fractionation.
- Marker enzymes included:
  - Lysosomes: *N*-acetyl- β-hexosaminidase (NABgase)
  - Mitochondria: cytochrome *c*-oxidase (cyt-oxidase)
  - Cytosol: lactate dehydrogenase (LDHase)

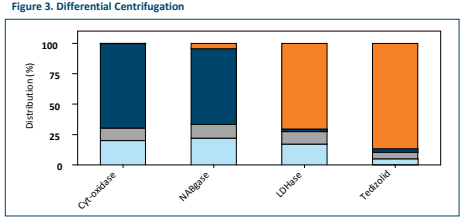
Analytic Method

- Tedizolid was extracted from cell fractions by phase partition and protein precipitation in chloroform:methanol (8:4), recovered in methanol, and quantified by liquid chromatography (reverse phase) coupled to mass spectrometry (LTQ-orbitrap).
- The gradient (0.4 mL/min) was designed as follows: from methanol-water (1:1) to methanol in 20 minutes, then 5 minutes with methanol, and subsequent equilibration in the initial conditions.
- Typical tedizolid elution time was 7 minutes.
- Acetic acid (0.5%) was used to assist ionization in the positive mode.
- Linezolid was used as an internal standard, with linear response obtained in a 10- to 1000-ng/mL range.

RESULTS

- Fractionation of cells by differential centrifugation showed that the bulk of tedizolid is associated with the high-speed supernatant that also contained the largest part of the LDHase. Conversely, there was minimal association of tedizolid with the granules fraction that contained the bulk of cyt-oxidase and NABgase (**Figure 3**).

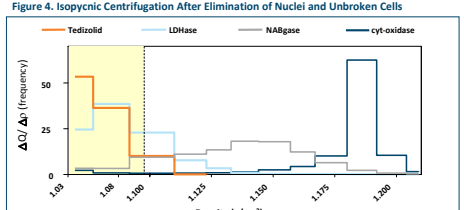
Figure 3. Differential Centrifugation



Cyt-oxidase, cytochrome *c*-oxidase; LDHase, lactate dehydrogenase; NABgase, *N*-acetyl- β-hexosaminidase.

- After isopycnic centrifugation, the bulk of tedizolid was recovered in the top fractions corresponding to where material was deposited (**Figure 4**, zone highlight in yellow), indicating no association with organelles that have moved into the gradient to equilibrate at their buoyant density. The separation from mitochondria (cyt-oxidase) is strikingly complete. There was also no sign of association with lysosomes (NABgase). The slight migration of LDHase was caused by the size of this protein that allows for migration because of the high centrifugation field used (**Figure 4**).

Figure 4. Isopycnic Centrifugation After Elimination of Nuclei and Unbroken Cells



Cyt-oxidase, cytochrome *c*-oxidase; LDHase, lactate dehydrogenase; NABgase, *N*-acetyl- β-hexosaminidase.

CONCLUSIONS

- There is no evidence of a stable association of tedizolid with mitochondria despite large cellular accumulation of tedizolid.
- Although we cannot exclude posthomogenization desorption of tedizolid from an intracellular storage site, the data suggest that the intracellular drug might be essentially free, which might explain its enhanced activity against intracellular bacteria but does not exclude a reversible inhibitory activity against mitochondrial protein synthesis.
- Additional studies with longer incubation times, measurement of mitochondrial protein synthesis, and comparison with linezolid and other oxazolidinones might help to better elucidate the significance of the current data.

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