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Study of the Cellular Uptake and Subcellular Distribution of the Oxazolidinone Tedizolid (TZD) in Murine J774 Macrophages: Lack of Association with Mitochondria

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Abstract (edited)

Background: Linezolid (LZD) causes mitochondrial dysfunction resulting in potentially severe adverse effects (hyperlactacidemia, metabolic acidosis, and peripheral neuropathy). Tedizolid, (TZD, formerly known as torezolid, TR700 or DA7157), retains activity against LZD-resistant isolates. It accumulates about 10-fold more in macrophages compared to LZD, with commensurate larger activity against intraphagocytic *Staphylococcus aureus* (phagolysosomes), *Legionella pneumophila* (phagosomes), and *Listeria monocytogenes* (cytosol) [Lemaire et al., JAC, 2009; 64:1035-1043]. The large accumulation of tedizoid, however, raises questions about potential association with mitochondria.

Methods: Murine J774 macrophages were exposed to TZD (2-50 mg/L) for 2h, collected and homogenized for fractionation by differential (peletting) and isopycnic (sucrose gradient) centrifugation (Tulkens *et al.* J Cell Biol 1974; 63:383-401; Renard *et al.* AAC 1987; 31:410-6). TZD was quantified after extraction with $CHCl_3$:CH_3OH (8:4) by liquid chromatography (reverse phase) coupled with mass-spectrometry (LTQ-orbitrap; ESI; selective ion monitoring, with LZD as internal standard). TZD distribution was compared to that of marker enzymes for mitochondria (cytochrome *c*-oxidase [CYTOX]), lysosomes (N-acetyl-β-hexosaminidase [NaBGase]) and cytosol (lactate dehydrogenase [LDH]).

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

Conclusions: In spite of a higher accumulation in eukaryotic cells compared to LZD, TDZ seems not associated in a stable fashion to mitochondria. Observing TDZ in the cytosol after cell fractionation may point to its ability to diffuse throughout the cell, explaining it higher activity against organisms harbored in distinct subcellular compartments while at the same time not necessarily implying a higher potential mitochondrial toxicity.

References

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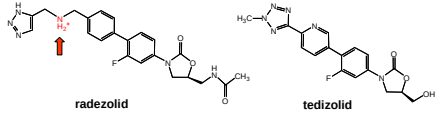
Background and Aim

There is an continuous need of discovering and developing new antibiotics to treat infections caused by multidrug-resistant pathogens. In this context, new oxazolidinones have emerged such as tedizolid (also referred to as torezolid or TR-700) that show higher intrinsic activities (lower MICs) than linezolid against Gram-positive cocci from clinical origin, including MRSA, and unimpaired activity against *cfr*+ linezolid-resistant strains [1,2].

Tedizolid also accumulates about 10-fold more than linezolid in human THP-1 macrophages *in vitro* [3] and accumulates about 20-fold over serum concentrations in alveolar macrophages of healthy adult volunteers [4]. While this may confer an advantage in terms of activity against intracellular forms of target bacteria [3], it also raises concerns with respect to potential toxicities. Indeed, one of the main likely mechanisms for the long-term toxicities of linezolid probably relates to its ability to impair mitochondrial DNA-dependent protein synthesis [5,6], due to structural similarities between mitochondrial and procaryotic ribosomes [7].

Surprisingly, however, there are no published studies examining 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 aminofunction with a pKa ~7.4 (see structure) resulting in a proton-trapping of its ionized form in acidic membrane-bounded organelles [9]. Tedizolid, however, has no such aminofunction and remains essentially uncharged at pHs 2 to 11, making prediction of its subcellular localization difficult.

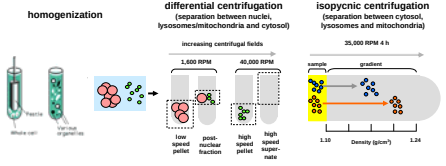


radezolid tedizolid

The present study, therefore, is aimed at studying the subcellular distribution of tedizolid.

Methods

We used the method of cell fractionation, consisting in collecting cells incubated with the drug of interest, disrupting their 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 pelletting, in which organelles are mainly separated based on their size) or by centrifugation through sucrose gradients (isopycnic equilibration) in which organelles are mainly separated based on their buoyant density). Fractions are thereafter assayed for the presence of markers of the organelles of interest and for drug content.



These methods, originally developed to discover and characterize subcellular organelles [10], have been previously used and validated for the study of the subcellular localization of aminoglycosides (lysosomes), macrolides (2/3 in lysosomes; 1/3 in cytosol) and fluoroquinolones (cytosol) [11].

Murine J774 macrophages were selected because these can easily be fractionated and mitochondria satisfactorily separated from lysosomes by isopycnic centrifugation.

Cells were incubated for 2 h with 20 mg/L tedizolid before being harvested and subjected to fractionation.

Marker enzymes were:

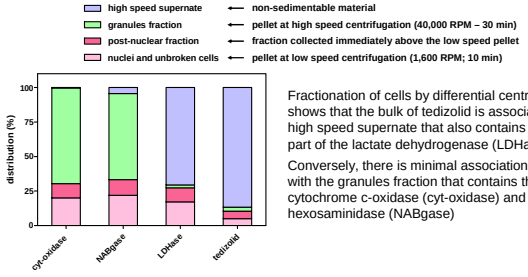
- for lysosomes: N-acetyl-β-hexosaminidase (NABgase)
- for mitochondria: cytochrome *c*-oxidase (cyt-oxidase)
- for cytosol: lactate-dehydrogenase (LDHase)

Analytical 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 min, then 5 min with methanol, and subsequent equilibration in the initial conditions. (typical tedizolid elution time: 7 min). 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 10-1,000 ng/mL range.

Results

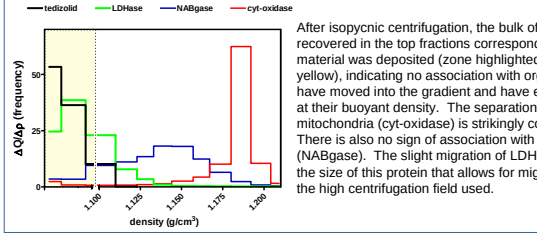
1. Differential centrifugation



Fractionation of cells by differential centrifugation shows that the bulk of tedizolid is associated with the high speed supernate that also contains the largest part of the lactate dehydrogenase (LDHase).

Conversely, there is minimal association of tedizolid with the granules fraction that contains the bulk of cytochrome c-oxidase (cyt-oxidase) and N-acetyl-β-hexosaminidase (NABgase)

2. Isopycnic centrifugation (after elimination of nuclei and unbroken cells)



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

Conclusions

1. There is no evidence of any stable association of tedizolid with mitochondria of eukaryotic cells in spite of its large cellular accumulation (compared to linezolid). Tedizolid, rather, seems to be free in cytosol.
2. While we cannot exclude post-homogenization desorption of tedizolid from an intracellular storage site, the data rather suggest that the intracellular drug may be essentially free, which may explain its enhanced activity against intracellular bacteria but does not exclude a reversible inhibitory activity against mitochodrial protein synthesis.
3. Additional studies with longer incubation times, measurement of mitochondrial protein synthesis, and comparison with linezolid and other oxazolidinones may help in better defining the significance of the present data.

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