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Mitochondrial Metabolism Impairment and Ultrastructural Alterations Induced by Linezolid (LZD) and Tedizolid (TZD) at Clinically-relevant Concentrations: Studies with two human cell lines (HL-60 [Promyelocytes] and THP-1 [Monocytes])

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Abstract (edited and abridged)

Background
Oxazolidinones inhibit mitochondrial DNA-encoded protein synthesis causing mitochondrial dysfunction, which has been associated with the development of severe side effects (myelosuppression, lactic acidosis and neuropathies). Our aim was to compare LZD to TZD for mitochondrial metabolism impairment and ultrastructural alterations in two human cell lines exposed to equitherapeutic concentrations of these drugs.

Methods
HL-60 and THP-1 cells were tested for (i) oxidative metabolism (lactate release/glucose consumption molar ratio [enzymatic analysis CMA600]; cytochrome c-oxidase activity [decrease in OD_{550} of cytochrome c/mg protein]; mitochondrial spare oxidative capacity [MSOC; Seahorse XF bioanalyzer]); (ii) expression of CYTox I, a mitochondrial DNA-encoded subunit of cytochrome c-oxidase, compared to nuclear DNA-encoded succinate dehydrogenase (western blot, normalization to TOM20); (iii) intracellular drug accumulation (HPLC-MS); and (iv) mitochondrial ultrastructure (electron microscopy). Treatments: continuous exposure to 2.5 mg/L or 15 mg/L for LZD and 0.5 mg/L or 3 mg/L for TZD (typical respective human C_{min} and C_{max}, following approved doses and schedules) for up to 3 days, followed by transfer to drug-free medium for 3 additional days (assessment of recovery).

Results
HL-60 were less oxidative than THP-1 cells (lactate/glucose ratio: 1.76±0.09 vs 1.06±0.23). The poster presents the results of the other analyses at day 3. Both LZD and TZD at C_{max} and after 3 days induced major mitochondrial ultrastructural alterations (decrease of inner membrane cristae and swelling of the matrix with preservation of the outer membrane) that were more pronounced in HL-60 than in THP-1 cells. The biochemical alterations were fully reversed within 3 days after drug removal (tested in HL-60 cells only).

Conclusions
Both LZD and TZD cause mitochondrial metabolism impairment and ultrastructural alterations consistent with a downregulation of the proteins encoded by the mitochondrial genome.
While TZD is globally more inhibitory than LZD at equitherapeutic concentrations (potentially due to its higher accumulation), its toxic effects may be mitigated by fast recovery following shorter courses.

References

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Background and Aims

LZD inhibits bacterial protein synthesis. Due to the high degree of homology between bacterial and mitochondrial ribosomes, LZD also inhibits mitochondrial protein synthesis [1-2]. Common undesired effects of LZD (anemia, thrombocytopenia, lactic acidosis, neuropathies) are thought to result from impairment of mitochondrial protein synthesis and ensuing mitochondrial dysfunction [3]. TZD shows lower MICs than linezolid, due to the increased binding to bacterial ribosomes [4] and is also a more potent inhibitor of mitochondrial protein synthesis than LZD [5]. However, TZD is effective and approved worldwide at a lower daily dose and short treatment duration (200 mg qD; 6 days) than LZD (600 mg BID; 10 days).

The aims of our study were to compare TZD and LZD for (i) inhibition of the expression of a protein encoded by the mitochondrial genome, (ii) effects on mitochondrial metabolic activities and morphology in two types of cultured human cell lines (HL-60 [promyelocytes] and THP-1 [monocytes]) at microbiologically and clinically pertinent concentrations.

Methods

1. ***Expression of CYTox I*** *(encoded by the mitochondrial genome; Western blot analysis)*: Electrophoresed mitochondrial extracts were reacted with mouse anti-cytochrome c-oxidase subunit I (CYTox I) monoclonal antibody, and revealed by HRP-coupled anti-antibodies (band intensities measured with Image J software).
2. ***Cytochrome c-oxidase activity***: assayed on cell lysates treated with 0.5% digitonin by measuring the rate of oxidation of reduced cytochrome c [6]
3. ***Oxygen consumption rate measurements (OCR)***: measured on cells seeded in poly-L-Lysine-coated microplates with OCR measured using the XF Cell Mito stress test kit on a Seahorse XF96 analyzer (Seahorse Bioscience; see diagram in the Results section for the successive additions of respiration inhibitors and interpretation of results).
4. ***Electron microscopy:*** cells fixed in 2% glutaraldehyde in 0.1M sodium cacodylate, post-fixed in 1% osmium tetroxide, stained *en bloc* with 0.5 % uranyl acetate, and ultrathin sections stained with lead citrate and uranyl acetate and observed at 80 kV.
5. ***Oxazolidinone cellular concentrations:*** sonicated cell lysates extracted with acetonitrile:methanol (21:4), dried, solubilized in methanol, and subjected to LC-MS analysis (LTQ-Orbitrap mass spectrometer) with [$^2H_8$]-LZD and [^{13}C, $^2H_3$]-TZD (Alsachim SAS, Illkirch, France) as internal standards, respectively.
   → **Results are not shown but demonstrated a ~ 5-fold larger accumulation of TZD over LZD.**
6. ***Concentrations used and expression of results:*** to ensure the microbiological and clinical pertinence of our data, most experiments used concentrations corresponding to or covering the human C_{min} - C_{max} range of LZD and TZD (2-15 and 0.5-3 mg/L). Concentrations causing inhibition of protein expression or activity are also presented as multiples of the modal EUCAST MICs of LZD and TZD against *S. aureus* (2 and 0.5 mg/L)

Main messages and Key Conclusion

- Both LZD and TZD cause an impairment of the expression of CYTox I, an inhibition of the activity of cytochrome *c*-oxidase activity, and a decrease of the spare capacity of the mitochondrial respiration in HL-60 and THP-1 exposed to concentrations pertinent of their microbiological activity and clinical use.
- TZD is globally more inhibitory than LZD even at microbiologically equipotent concentrations, potentially due to its higher intracellular accumulation (see above and [7]).

➢ **Fast recovery upon drug withdrawal, and once-daily regimen [5] combined with shorter treatment duration for TZD compared to LZD may mitigate its toxic effects in patients.**

Typical Results (data expressed by reference to microbiological or human pharmacokinetic pertinent concentrations [see Methods])

1. CYTox I expression and cytochrome c-oxidase activity: effect of concentration (HL-60)

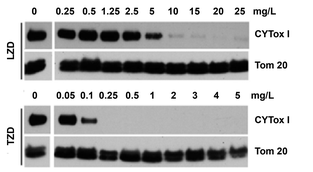


Figure 1: CYTox I Western blot analysis (Tom20 as control)

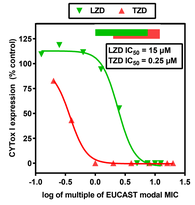


Figure 2: quantitative of data of Fig 1 (green and red bars: C_{min}-C_{max} ranges of LZD and TZD in humans).

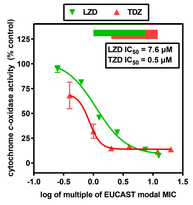


Figure 3: cytochrome c-oxidase activity in cell lysates (green and red bars as in Fig.2)

Both LZD and TZD inhibit the expression of a protein encoded by the mitochondrial genome and the activity of a key enzyme complex in mitochondria. TZD is a more potent inhibitor than LZD for CYTox I expression and equipotent to LZD for cytochrome *c*-oxidase activity inhibition when compared at concentrations corresponding to the respective EUCAST modal MICs of these antibiotics for *S. aureus* (https://mic.eucast.org/Eucast2/)

4. Ultrastructural studies (HL-60 cells)

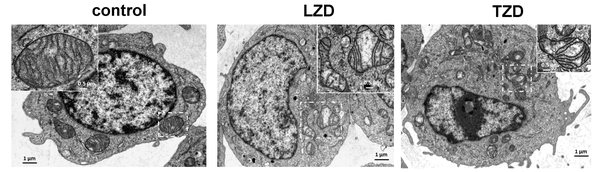


Figure 7: ultrastructural appearance of whole cells and of mitochondria after 3 days incubation without (control) or with LZD or TZD at C_{max} (15 and 3 mg/L)

Both LZD and TZD induce marked morphological changes in mitochondria with the development of larger electron-lucent zones between cristae (tightly packed in controls), consistent with an impairment of inner membrane proteins encoded by the mitochondrial genome

2. CYTox I and cytochrome c-oxidase activity: time and cells

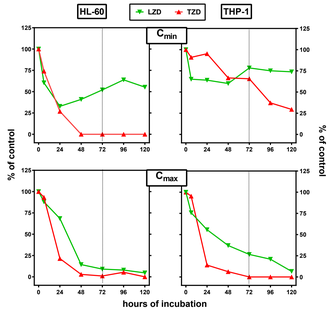


Figure 4: Change of CYTox I expression over time of incubation (C_{min}/$_{max}$: see Methods)

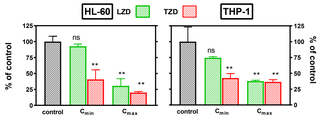
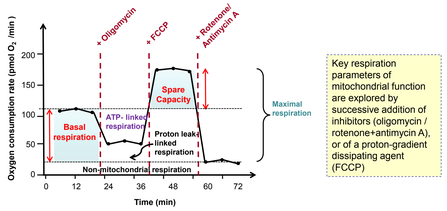


Figure 5: Activity of cytochrome *c*-oxidase at 72h (C_{min}/$_{max}$: see Methods)

A fast inhibition of CYTox I expression and of cytochrome c-oxidase activity is observed at C_{max}, with lesser (and slower) effects for LZD at C_{min}. THP-1 (monocytes) are less susceptible than HL-60 (promyelocytes).

3. Mitochondrial respiration after 72h incubation



Key respiration parameters of mitochondrial function are explored by successive addition of inhibitors (oligomycin / rotenone+antimycin A), or of a proton-gradient dissipating agent (FCCP)

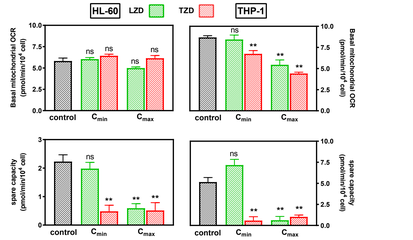


Figure 6: Basal oxygen consumption rate and spare capacity at 72h (C_{min}/$_{max}$: see Methods)

LZD and TZD do not affect the basal respiration but almost completely block the spare capacity (at C_{max} for LZD and C_{max} and C_{min} for TZD) consistent with the inhibition of CYTox I expression and of cytochrome *c*-oxidase activity.

5. Recovery upon drug withdrawal (CYTox I, cytochrome c-oxidase activity and spare capacity)

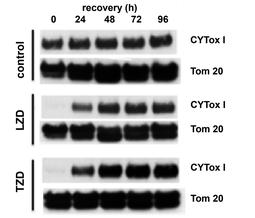


Figure 8: CYTox I Western blot analysis (Tom20 as control)

Cells were exposed to C_{max} of LZD (15 mg/l) or TZD (3 mg/L) for 72 h and then transferred to drug-free medium for up to 96 h (recovery)

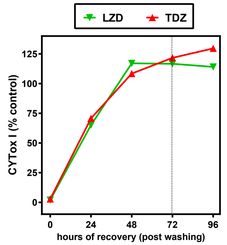


Figure 9: quantitative analysis of data of Fig 8

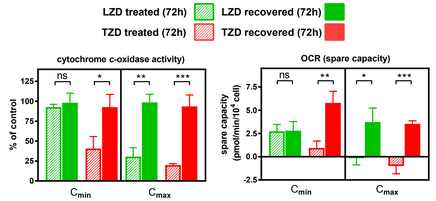


Figure 10 : Recovery of cytochrome c-oxidase activity and of spare capacity upon drug withdrawal (72h) in comparison with values observed after 72 h incubation at C_{min} and C_{max} of LZD or TZD

All tested parameters (for both LZD and TZD) returned to normal values upon drug withdrawal.

This poster will be made available after the meeting at http://www.facm.ucl.ac.be/posters