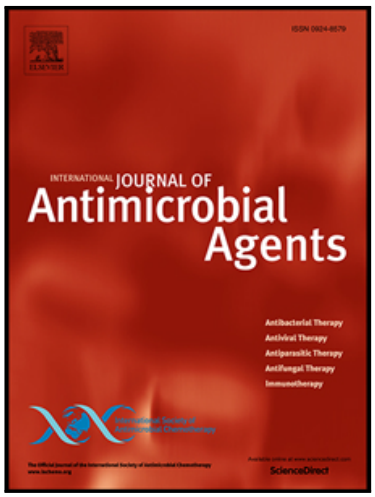

Prolonged inhibition and incomplete recovery of mitochondrial function in oxazolidinone-treated megakaryoblastic cell lines

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Highlights

- Oxazolidinones (linezolid, tedizolid, …) reversibly impair mitochondrial protein synthesis and metabolic functions in various cell types, but the link with thrombocytopenia (a common untoward effect seen in patients treated for >14 days) has not been established
- In megakaryoblastic cell lines exposed to oxazolidinones, inhibition of cytochrome *c*-oxidase activity and of mitochondrial spare capacity was readily obtained as in other cell types but was not fully reversed during drug washout.
- These protracted effects may explain why thrombocytopenia develops as a most frequent side effect of oxazolidinones during treatment.

Prolonged inhibition and incomplete recovery of mitochondrial function in oxazolidinone-treated megakaryoblastic cell lines

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Running title: Mitochondrial functions and oxazolidinones

ABSTRACT

Thrombocytopenia is commonly seen in patients receiving linezolid for >14 days. Linezolid is also a reversible inhibitor of mitochondrial functions in various cell types. We have studied the inhibitory effects of linezolid and tedizolid and their potential recovery on (i) CYTox I expression (subunit I of cytochrome *c*-oxidase; encoded by the mitochondrial genome), (ii) cytochrome *c*-oxidase activity, and (iii) mitochondrial respiration (Seahorse bioanalysis) in two megakaryocytic cell lines (UT-7 WT [human acute megakaryoblastic leukemia cells] and UT-7 MPL [transduced to stably express the thrombopoietin (TPO) receptor]). Cells were exposed to linezolid (0.5-25 mg/L) or tedizolid (0.1-5 mg/L) for up to 5 days and recovery followed after drug removal. Both oxazolidinones caused a concentration- and time-dependent inhibition of CYTox I expression, cytochrome *c*-oxidase activity and mitochondrial spare capacity. In the electron microscope, mitochondria appeared dilated with a loss of cristae. Globally, tedizolid exerted stronger effects than linezolid. While the expression of CYTox I was completely recovered after 6 days of drug washout, we observed only a partial (linezolid) or no (tedizolid) recovery of cytochrome *c*-oxidase activity, and no rescue of mitochondrial spare capacity (after 3 days). Thus, and in contrast with previous studies using a variety of cell lines unrelated to megakaryocytic lineages, the inhibitory effects exerted by oxazolidinones on mitochondrial functions of megakaryoblastic cells appear to be particularly protracted. Given the dynamics of platelet production and destruction, these results may explain why oxazolidinone-induced thrombocytopenia emerges as one of the most frequent untoward effects in patients exposed to these antibiotics.

Keywords: Linezolid; tedizolid; oxazolidinone; megakaryocyte; megakaryoblast; UT-7 cells; mitochondria; cytochrome c-oxidase; CYTox I; Seahorse; thrombocytopenia

1. Introduction

Oxazolidinones are the latest class of antibiotics with a true novel mode of action having reached widespread clinical use. They inhibit bacterial protein synthesis by binding to ribosomal 50S subunit and impairing the formation of the initiation complex [1]. They are active against most multi-resistant *Staphylococci* and *Enterococci* from clinical origin [2]. Due to their excellent oral bioavailability, they are also often selected for early discharge of patients. Unfortunately, treatments with linezolid (the first approved oxazolidinone) for > 14 days and/or in patients with renal insufficiency are frequently associated with hematological untoward effects, with thrombocytopenia affecting up to 30% of patients [3-5]. Although about 2 to 8-fold more potent than linezolid [2], tedizolid was reported to cause less thrombocytopenia, based on the results of dose-escalating phase I trials and of fixed dose, 6-days treatment phase III clinical trials [6]. However, this has been challenged based on FDA registry data covering an 18 months clinical use of tedizolid [7].

Given the similarity between bacterial and mitochondrial ribosomes, oxazolidinones also bind to mitochondrial ribosomes, impair the expression and synthesis of mitochondrial proteins encoded by the mitochondrial genome [8;9], and inhibit the activity of cytochrome *c*-oxidase and the mitochondrial oxidative activity [9]. These effects are rationalized by structural similarities between bacterial and mitochondrial ribosomes, with common binding sites for oxazolidinones [10]. Most notably, tedizolid possesses additional interactions with the oxazolidinone target site in bacterial ribosomes [11]. It also causes a higher inhibition than linezolid for (i) mitochondrial protein synthesis in isolated cardiac mitochondria [12], and (ii) CYTox I expression (a protein encoded by the mitochondrial genome) and cytochrome *c*-oxidase activity in human HL-60 promyelocytes and THP-1 monocytes [9]. These cell lines, however, have no or only a weak link to the megakaryocytic lineage, making their predictive value to assess the impact of oxazolidinones on platelet precursors questionable. In this context, we examined, and report here, the impact of linezolid and tedizolid on the expression of CYTox I and on cytochrome *c*-

oxidase activity in UT-7 cells considered as a useful *in vitro* surrogate of megakaryoblasts. We compared them with UT-7 transduced to stably express a functional thrombopoietin (TPO) receptor, corresponding to more differentiated megakaryocytes.

2. Methods

Linezolid (potency 99.2%) was obtained as RX-0366-00-005 from Rib-X Pharmaceuticals (presently Melinta Therapeutics, New Haven, CT), and tedizolid (potency 101.4%) as microbiological standard from Trius Pharmaceuticals (San Diego, CA) and thereafter from Cubist Pharmaceuticals GmbH (Zürich, Switzerland), now both parts of Merck & Co. (Kenilworth, NJ). Stock solutions were prepared in DMSO, and thereafter diluted to bring the final DMSO concentration in the culture fluid to 0.5% (no cytotoxicity observed). Granulocyte-macrophage colony-stimulating factor (GM-CSF) and human TPO were from Miltenyi Biotec GmbH, Bergisch Gladbach, Germany.

UT-7 WT human acute megakaryoblastic leukemia cells were obtained from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSMZ], Braunschweig, Germany) [13]. UT-7 MPL cells [14] are UT-7 WT cells transduced with viral particles derived from packaging cell lines transfected with pMex-ires-GFP HA huMPL vector that codes for the human TPO receptor (TpoR or c-MPL). These cells were grown in MEM-alpha GlutaMAX^{TM} medium (Gibco-Life Technologies, Thermo-Fisher Scientific Inc., Waltham, MA) supplemented with 10% fetal bovine serum (Gibco-Life Technologies) and GM-CSF at 10 ng/mL for UT-7 WT or TPO at 10 ng/mL for UT-7 MPL. They were incubated at 37°C in a 5% CO_2-air atmosphere. Their proliferation was evaluated using an automated cell counter (Beckman Coulter Life Science, Indianapolis, IN).

Mitochondria-enriched preparations were obtained as previously described [9]. Protein concentrations were measured by the bicinchoninic acid assay (BCA Protein Assay Reagent, Pierce, Thermo Fisher, Waltham, MA), with equal amounts of proteins separated by NuPAGE™ using 10% Bis-Tris precasted gels. The following primary antibodies were used: mouse anti-cytochrome *c*-oxidase subunit I (CYTox I) monoclonal antibody (Anti-OxPhos Complex IV Subunit I Monoclonal Antibody; Invitrogen cat. no. 459600) at 0.4 µg/mL and rabbit anti-Tom20 polyclonal antibody (Invitrogen, cat. no. PA5-39247) at 0.2 µg/mL. Secondary antibodies (rabbit

anti-mouse IgG [Invitrogen cat.no A27025] and goat anti-rabbit IgG antibody [Invitrogen cat.no A27036]) were used at a concentration of 0.4 µg/mL. Band intensity was quantified on membranes scanned by the Chemiluminescence Imaging- Fusion PULSE apparatus (Analis, Namur, Belgium), using the Image J software (National Institutes of Health, Bethesda, MD; available from https://imagej.nih.gov/ij/).

Cytochrome *c*-oxidase activity was assayed on sonicated cells as previously described [9] after exposure to 0.2 % digitonin, and calculated as the slope of the change in absorption of reduced cytochrome *c* over time after logarithmic linearization and normalized based on the protein content of the cell lysate, determined by Lowry's method.

For mitochondrial oxygen consumption rate measurements, cells were collected by low speed centrifugation, and resuspended at a suitable dilution in Dulbecco′s Modified Eagle′s Medium (Sigma-Aldrich cat. no. D5030, Sigma Aldrich, St-Louis, MO) supplemented with 1.85 g/L NaCl, 10 mM D-glucose and 2 mM L-glutamine, and seeded in a poly-L-Lysine-coated (Sigma-Aldrich cat. no. P6282) Seahorse XF96 V3 PS cell culture microplate (Agilent cat no. 101085-004, Agilent technologies, Santa Clara, CA) at densities of 250,000 cells/well. Oxygen consumption rate (OCR) and its various stages (basal respiration; ATP-linked respiration; spare capacity; maximal respiration was then measured on a Seahorse XF96 analyzer as previously described in [9] (see the supplementary material of this paper). Data were normalized by cell count using a SpectraMax i3 plate imager (Molecular Devices, Sunnyvale, CA) and the SoftMax Pro software (Molecular Devices).

Preparation of samples for electron microscopy was performed as previously described [9].

Products not described above were obtained from Sigma-Aldrich or Merck KGaA (Darmstadt, Germany).

Curve fitting and statistical analyses were performed using GraphPad Prism® version 8.1.1. (330) and GraphPad InStat® version 3.10 both for Windows® (GraphPad Software, Inc., San Diego, CA, www.graphpad.com).

3. Results

Oxazolidinones at increasing concentrations inhibit CYTox I expression and cytochrome *c*-oxidase activity

CYTox I (one of 14 proteins of mitochondrial respiratory chain encoded by mitochondrial genome) expression was examined in UT-7 WT and UT-7 MPL megakaryocytic cell lines incubated during 5 days with linezolid or tedizolid over a wide range of concentrations chosen to cover the range of clinically relevant concentrations (see caption of Figure 1). The expression of Tom20, a translocase from outer mitochondrial membrane encoded by the nuclear genome, was measured in parallel. While the expression of Tom20 was not affected by oxazolidinones, both drugs inhibited CYTox I expression in a concentration-dependent manner. The protein became almost undetectable for concentrations of linezolid > 15 mg/L and of tedizolid of 0.3 mg/L (Figure 1A). Calculations of the concentration of antibiotic causing 50% activity inhibition (IC_{50}) showed that tedizolid was a 17 to 20 times more potent inhibitor than linezolid in UT-7 WT and UT-7 MPL cells, respectively (Figure 1B [upper panel]; see caption for IC_{50} values).

The enzymatic activity of cytochrome *c*-oxidase was measured in parallel in the same conditions (Figure 1B [lower panel]). Both oxazolidinones induced a concentration-dependent decrease in enzymatic activity, with again tedizolid being more potent than linezolid (9 to 24 times in UT-7 MPL and UT-7 WT, respectively).

UT-7 WT were thus slightly more susceptible to linezolid treatment than UT-7 MPL cells (1.3- fold decrease in CYTox I protein expression and 1.2- fold decrease in enzyme activity). Concerning tedizolid, the ratio of IC_{50} values for UT-7 WT to UT-7 MPL was roughly 1 for CYTox I expression, but 3 for the enzyme activity.

Influence of the time of incubation with oxazolidinones on CYTox I protein expression, cytochrome *c*-oxidase activity, mitochondrial respiration and ultrastructure

We followed the changes in CYTox I protein expression over time in cells exposed to a fixed concentration of both drugs corresponding to their human C_{max} (linezolid: 15 mg/L; tedizolid: 3 mg/L). Data are presented in Figure 2A,B. Both drugs completely inhibited CYTox I protein expression after 72 h in UT-7 WT cells, as well as linezolid in UT-7 MPL cells. However a residual activity of about 20% was observed in UT-7 MPL cells treated with tedizolid. In order to assess the potential functional impact of this decrease, we measured cytochrome *c*-oxidase activity and the mitochondrial respiration of UT-7 WT and UT-7 MPL cells (Figure 2C,D). Cells were incubated during 72 h at a concentration corresponding to the C_{max} of oxazolidinones, at which maximal inhibition of CYTox I expression was obtained. As shown in Figure 2C, enzyme activity was significantly reduced to about 50 to 75% in both cell lines. Figure 2D shows that basal mitochondrial OCR and the spare capacity (i.e., the ability to increase mitochondrial OCR from a basal to a maximal level) were reduced by either oxazolidinones in UT-7 WT, while only the spare capacity was impaired in UT-7 MPL cells. Typical images of mitochondria under control conditions and after incubation with oxazolidinones at their C_{max} for 72 h are shown in Figure 2E. Mitochondria appeared less dense in UT-7 MPL cells than in their WT counterparts, with fewer cristae. In oxazolidinone-exposed cells, mitochondria were dilated in both cell types and harbored scarce, disorganized cristae.

Reversibility of the inhibition CYTox I expression, cytochrome *c*-oxidase activity and mitochondrial spare capacity

Cells were treated during 72 h with oxazolidinones at their C_{max} (to obtain maximal inhibition of cytochrome *c*-oxidase subunit I expression) and then transferred to drug-free media for up to 144 h. The expression of CYTox I recovered completely in both cell lines after 72 h of washing (Figure 3A,B), but this period of time was insufficient to completely restore cytochrome

c-oxidase activity in both cell lines. Prolonging the washout period to 144 h allowed for an increased recovery of enzyme activity after linezolid but not after tedizolid treatment (Figure 3C). We also tested whether drug washout would allow for recovery of mitochondrial spare capacity, and found that after 3 days it was variable but only partial in most cases (data not shown).

4. Discussion

Inhibition of mitochondrial protein synthesis by oxazolidinones, due to the existence of common binding sites for oxazolidinones between bacterial and human mitochondrial ribosomes [10], has already been reported in both preclinical and clinical studies [8;15]. It has been convincingly associated with impairment of mitochondrial oxidative metabolism in human promyelocytic (HL-60) and monocytic (THP-1) cell lines [9]. We present here data using cell lines that are more directly related to platelets-generating cells *in vivo*, namely megakaryoblasts and megakaryocytes. Our key observations are that (i) UT-7 cells are as susceptible as other cell lines to the inhibitory effects of oxazolidinones on key mitochondrial parameters, namely CYTox I expression (encoded by mitochondrial genome), cytochrome *c*-oxidase activity and oxidative metabolism; (ii) but that some of these inhibitory effects, especially those caused by tedizolid, are less easily reversed compared to what has been observed with the cell lines examined so far. Interactions between oxazolidinones and bacterial ribosomes are non-covalent and, therefore should be reversible. In UT-7 cells, CYTox I expression was readily reversed in oxazolidinone-free medium but the recovery of cytochrome *c* oxidase activity was slower and incomplete, especially in cells exposed to tedizolid. Of interest, the effects on UT-7 MPL cells, which are more differentiated megakaryoblasts/megakaryocytes and have a maximal TpoR cell-surface expression, were more pronounced than on UT-7 parental cells. Possibly, basal signaling by the TpoR itself might activate pathways that regulate mitochondrial size and components, which are relevant for toxicity and recovery in the context of oxazolidinones. We may also speculate that the perturbations induced by oxazolidinones on mitochondrial structure are so severe that they alter the correct assembly of the cytochrome *c*-oxidase complex. Cytochrome *c*-oxidase is made of 14 subunits, 3 of which (CYTox I, II and III) are encoded by the mitochondrial genome. Specific inhibition of their synthesis by oxazolidinones would create a disbalance between all the subunits necessary for cytochrome *c*-oxidase expression and activity [16], resulting in a disassembly of complex IV that may not be easily reversed. Also, we

know that phorbol myristate-induced differentiation of megakaryocytes is accompanied by a reduction of their mitochondrial activity and alteration of mitochondria ultrastructure (diminished matrix density, disorganized cristae) [17] reminiscent of what was observed here for UT-7 cell lines when exposed to oxazolidinones. Mitochondrial fragmentation has been shown to physiologically occur during megakaryoblats differentiation, probably as a compensatory response to restore an adequate mitochondrial function [17]. This may become impossible and result in irreversible loss of function if oxazolidinones are present. An increase in mitochondrial DNA content and RNA expression after linezolid therapy has also been suggested as a compensatory mechanism to prevent further damage [18]. Although calling for further studies, our data study may already identify megakaryocytes as a privileged target for expression of toxicity *in vivo*. The non-recoverable impairment of their mitochondrial functions would lead to progressive thrombocytopenia, as is observed in patients who then require another full round of megakaryoblasts and megakaryocytes formation from hematopoietic precursors/stem cells before correcting for their platelets deficit. The dynamics of platelet formation and destruction would favor negative clinical effects. This is largely in line with the recent pharmacokinetic modelling approaches describing how oxazolidinones interfere with the whole process of megakaryocyte differentiation and platelet release [19].

Lastly, we observed that tedizolid caused more extensive and sustained mitochondrial alterations than linezolid, even when comparing equitherapeutic concentrations. While this may describe an *in vitro* reality (also observed with isolated mitochondria [20]), the clinical situation could be different since the conditions of use (dosages and frequency of administration) as well as the extent of protein binding of tedizolid and linezolid are quite different (see discussion in [9] and [12]). Thus, true toxicity ranking will require more detailed comparative *in vitro* studies, as well as more extensive clinical experience.

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Declarations

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Ethical Approval: Not applicable.

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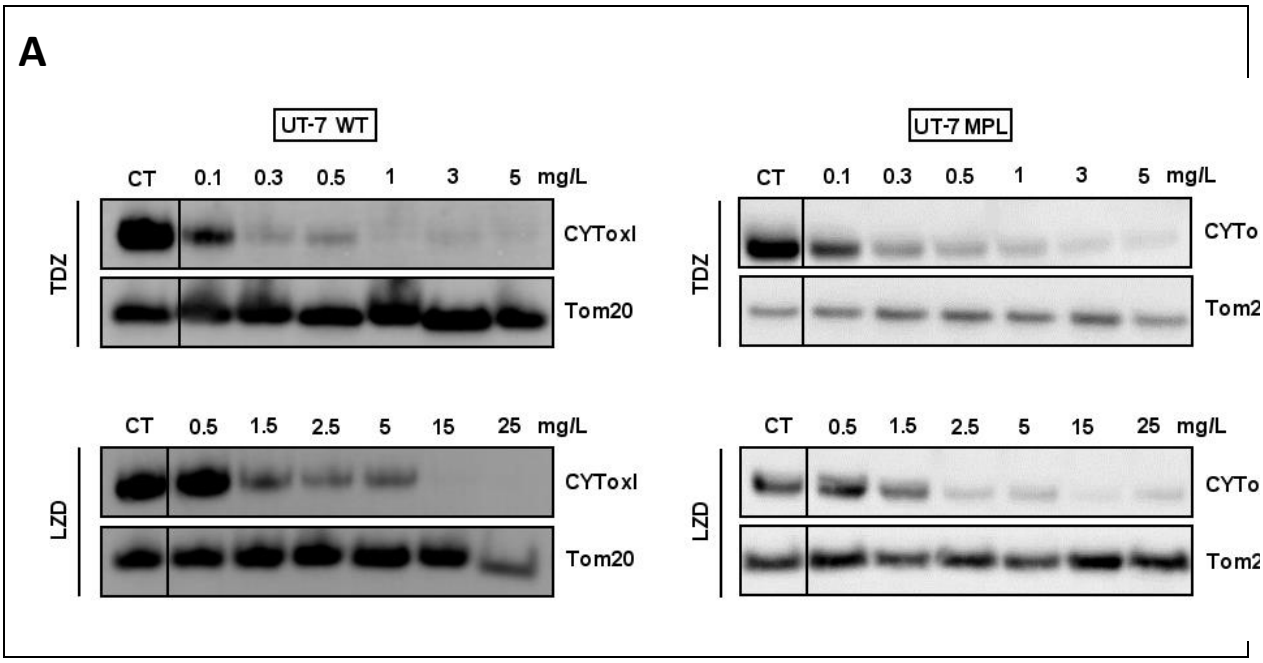
Figure 1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UT-7 WT
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LZD

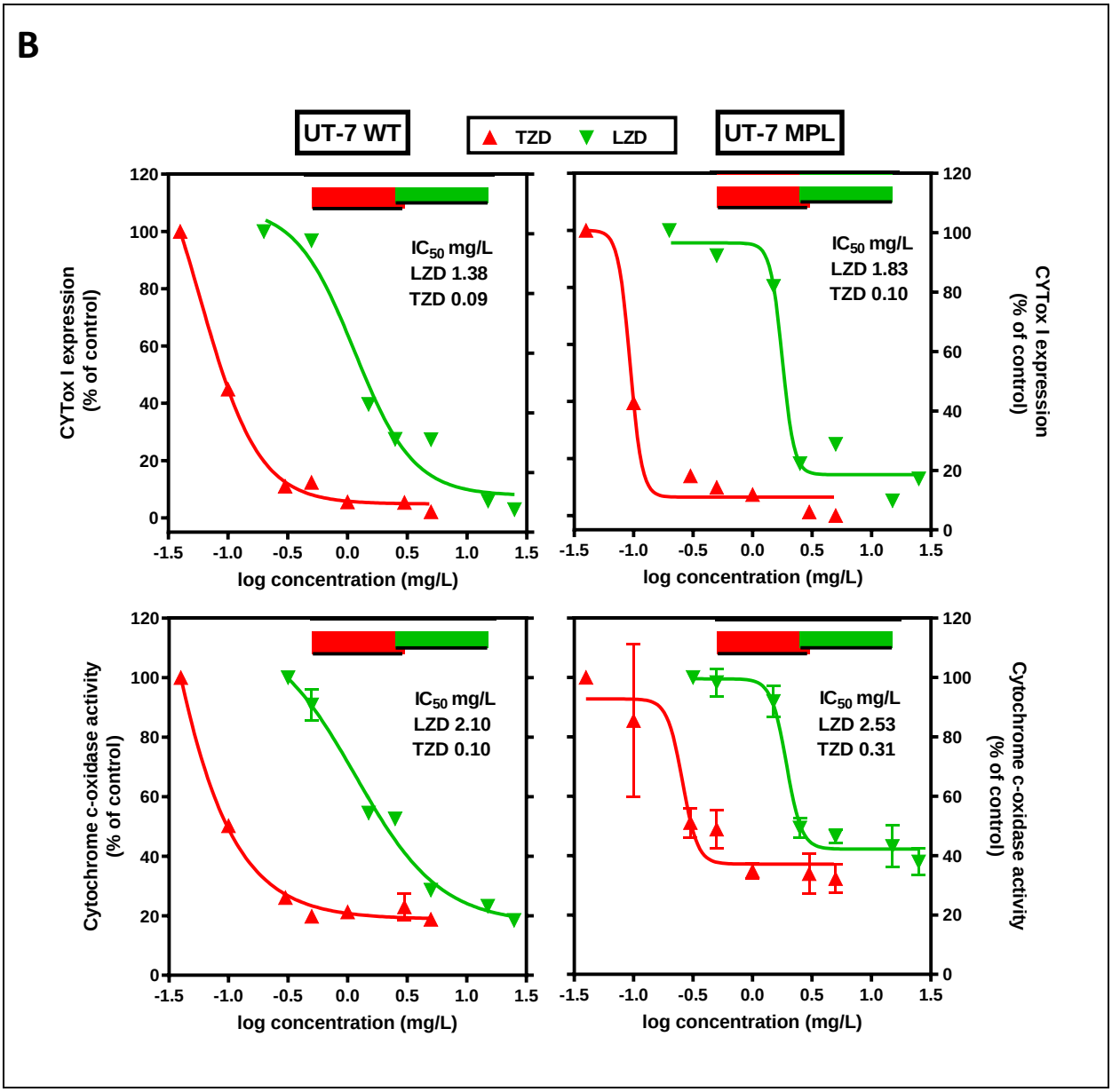
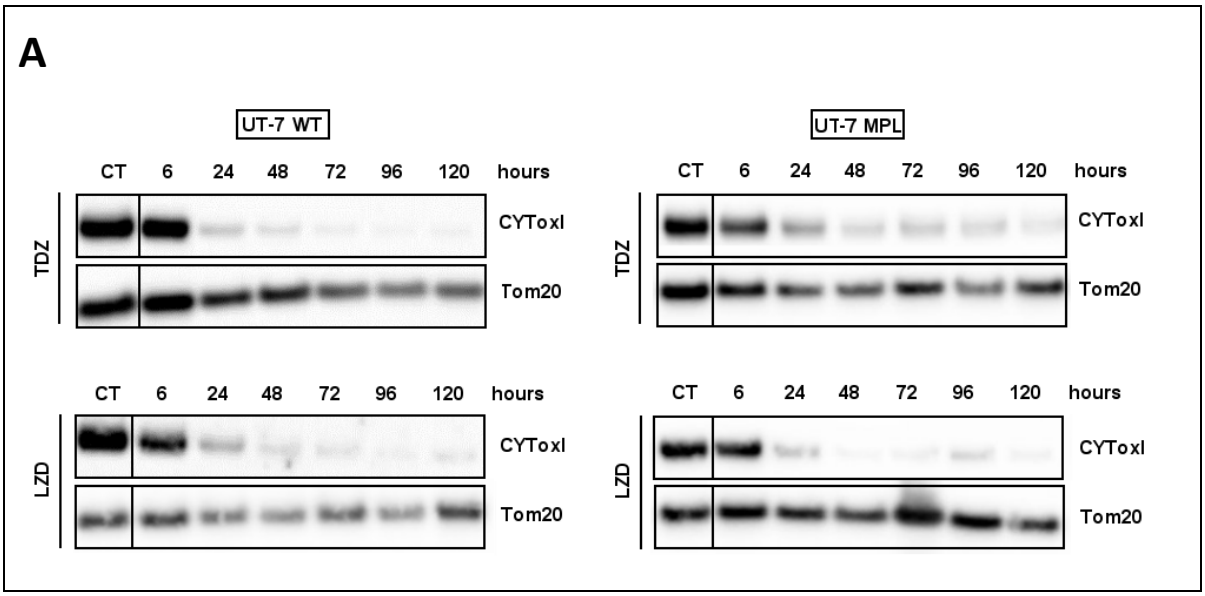
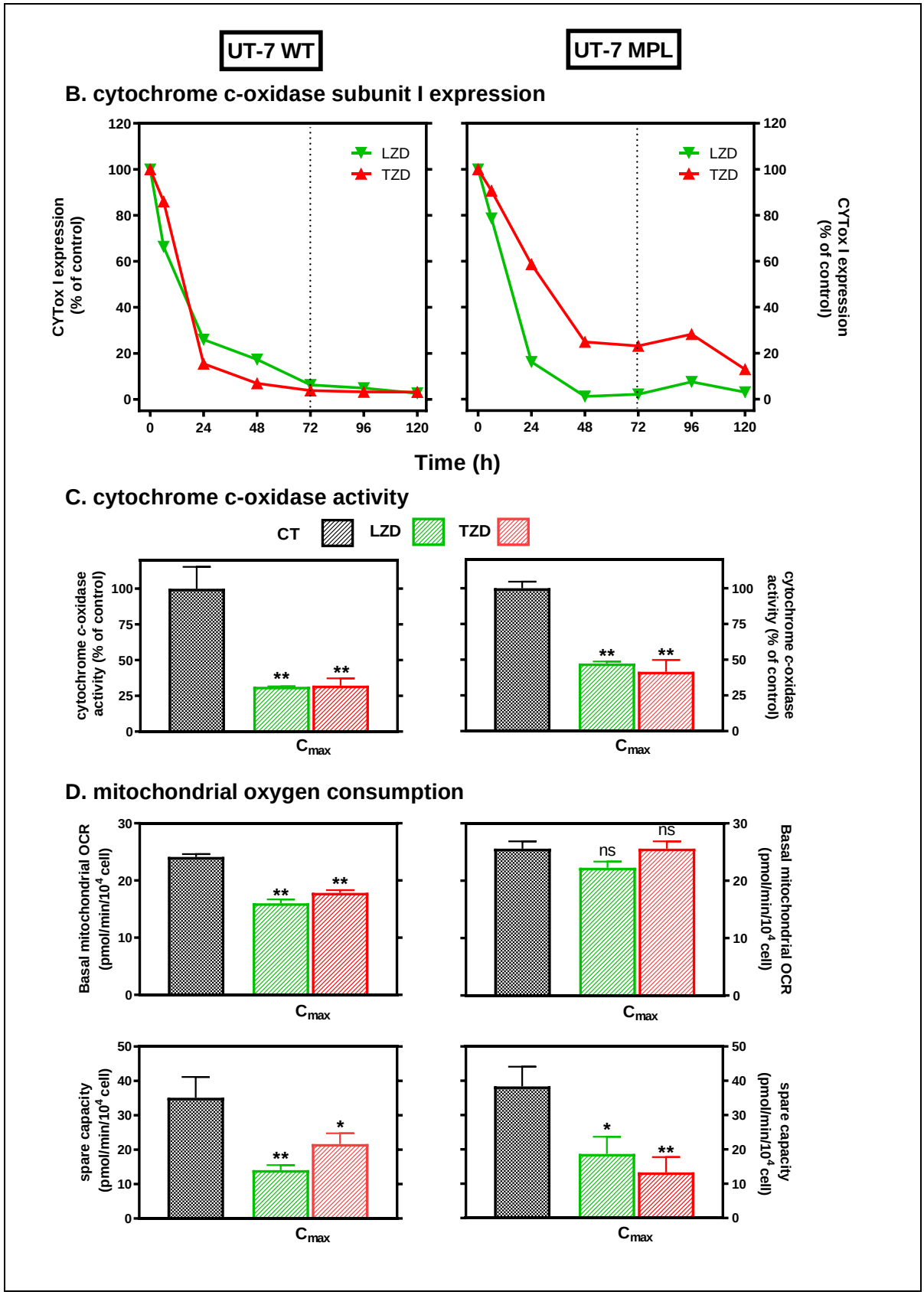


Figure 1: Influence of increasing concentrations of tedizolid (TZD) and linezolid (LZD) on CYTox I protein expression and on of cytochrome *c*-oxidase activity of UT-7 WT and UT-7MPL cells incubated for 5 days. **A**, Western blots of CYTox I and of Tom 20 (used as a loading control) in mitochondrial fractions with controls (vehicle only) on the left and increasing concentrations (from 0.1 to 5 mg/L and 0.5 to 25 mg/L for TZD and LZD, respectively). **B**, quantitative measurements: change in cytochrome *c*-oxidase activity presented as means ± SD of triplicates in a single experiment (when not visible, the SD bars are smaller than the symbols); for both graphs, the abscissa is expressed as the $\log_{10}$ of the concentration of each

drug. IC_{50} values (calculated from the Hill functions) for UT-7 WT upper panel (LZD 1.38 mg/L or 4.09 µM; TZD 0.09 mg/L or 0.24 µM); lower panel (LZD 2.10 mg/L or 6.23 µM; TZD 0.1 mg/L or 0.27 µM) and for UT-7 MPL upper panel (LZD 1.83 mg/L or 5.43 µM; TZD 0.1 mg/L or 0.27 µM); lower panel (LZD 2.53 mg/L or 7.51 µM; TZD 0.31 mg/L or 0.83 µM). The colored rectangles on the top graphs refer to interval between the C_{min} and C_{max} values commonly observed in the serum humans receiving conventional does of TZD of LZD (0.5 to 3 mg/L and 2 to 15 mg/L, respectively).

Figure 2




UT-7 WT
UT-7 MPL
B. cytochrome c-oxidase subunit I expression
CYTox I expression (% of control)
LZD
TZD
Time (h)
C. cytochrome c-oxidase activity
CT
LZD
TZD
cytochrome c-oxidase activity (% of control)
Cmax
D. mitochondrial oxygen consumption
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spare capacity (pmol/min/10^4 cell)
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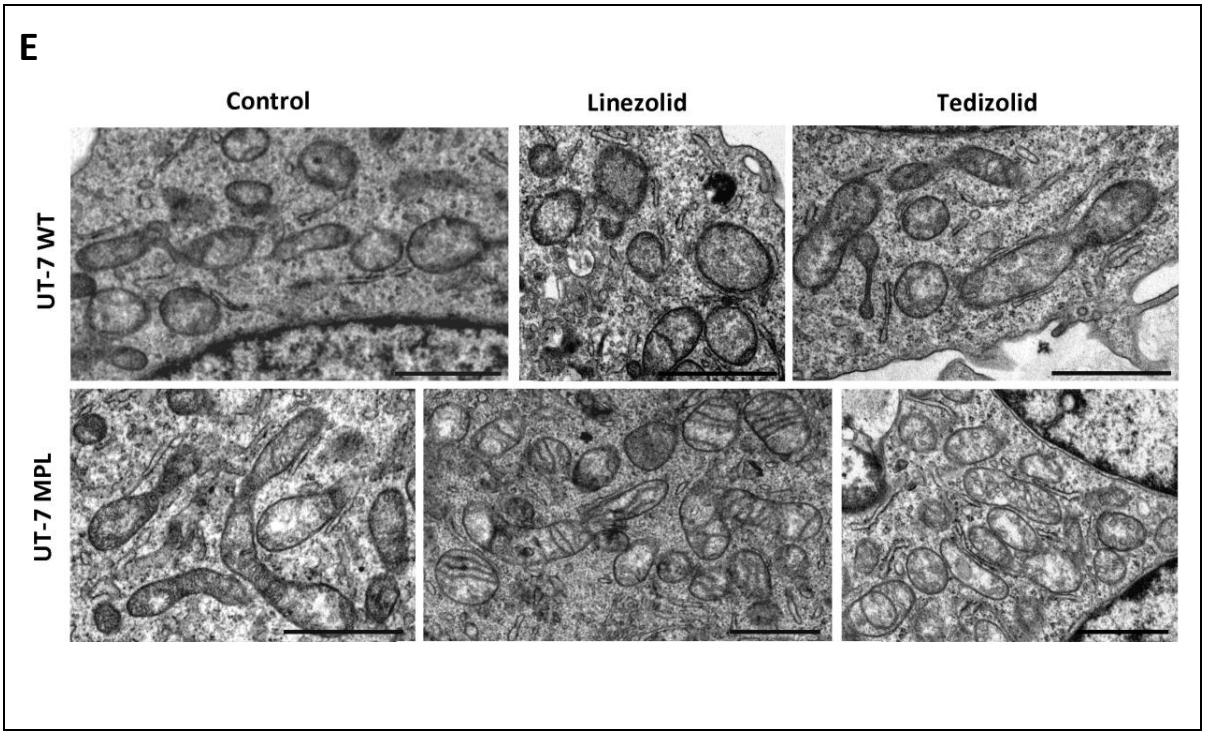
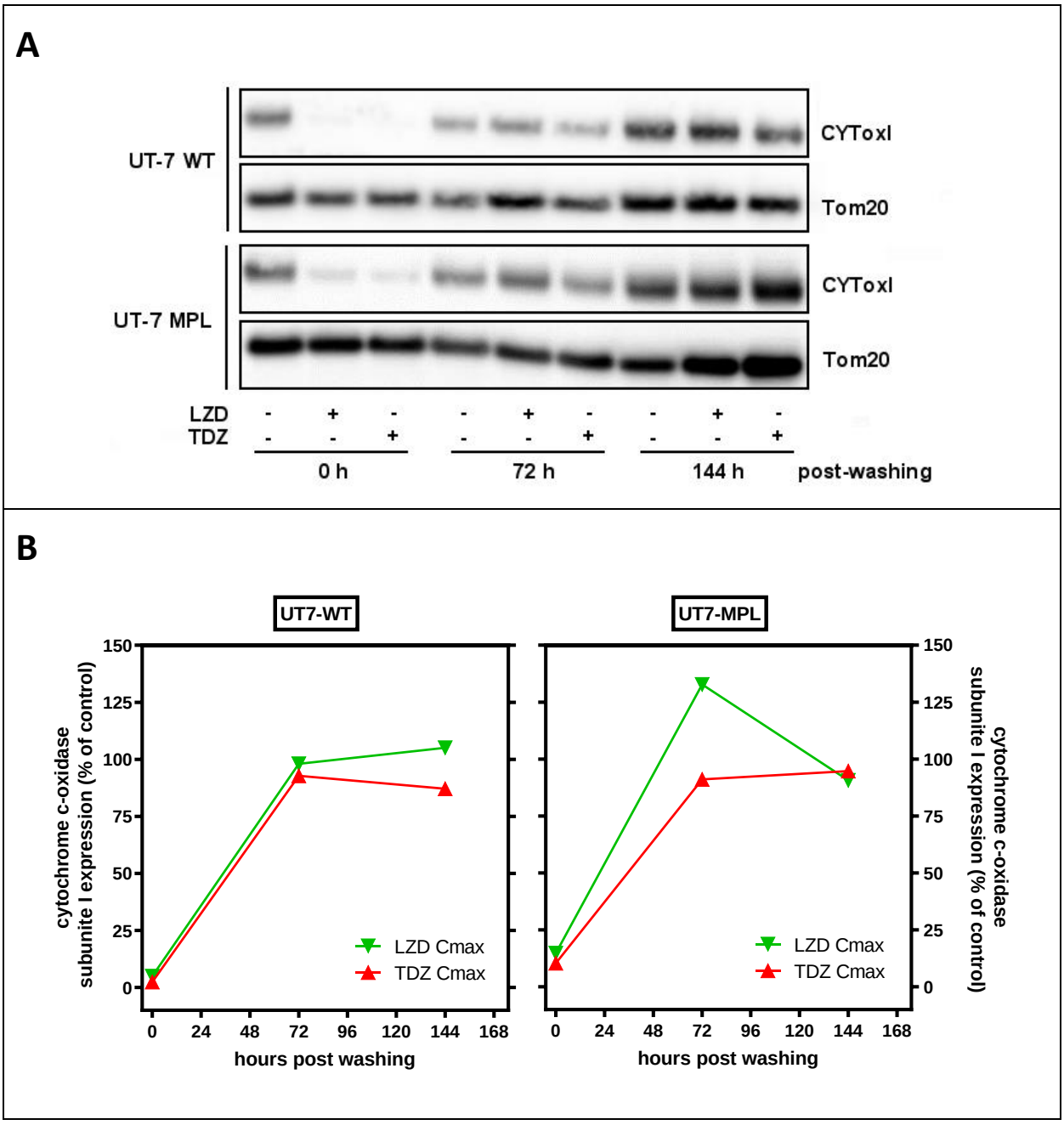


Figure 2: Influence of time of incubation with linezolid (LZD) or tedizolid (TZD) on CYTox I expression, cytochrome *c*-oxidase activity, mitochondrial respiration and mitochondrial ultrastructure in UT-7 WT (left) and UT-7 MPL (right) megakaryocytes after 72 h of incubation. **A**, Western blots of CYTox I and of Tom 20 (used for normalization) in mitochondrial protein fractions, with controls (vehicle only) on the left and increasing times of incubation at a concentration corresponding to their peak (C_{max}) serum concentrations most commonly observed for each antibiotic when administered to humans at conventional dosages (TZD: 3 mg/L; LZD: 15 mg/L) **B**, Quantitative measurements (band density) of CYTox I to Tom 20 ratio (the vertical dotted line at 72 h refers to the time of incubation selected in further experiments). **C,** Changes of cytochrome *c*-oxidase activity and **D**, mitochondrial respiration in UT-7 WT and UT-7 MPL cells incubated for 72 h in control conditions (white hatched bars) or in the presence of LZD (LZD; green hatched bars) or TZD (TZD; red hatched bars). Values are shown as percent of control ± SD of triplicates for cytochrome *c*-oxidase activity and as means of absolute values ± SEM of 6 to 8 wells for mitochondrial oxygen consumption. Statistical analysis: one-way ANOVA with Dunnett's multiple comparison of each treatment *vs*. control (ns: $p > 0.05$, * $p < 0.05$, ** $p < 0.01$). **E**, morphological appearance of mitochondria in control and oxazolidinone-treated UT-7 WT and UT-7 MPL cells. Representative transmission electron micrographs of

cells incubated for 72 h in control conditions or with linezolid or tedizolid at the extracellular concentration corresponding to their human total C_{max}. Bars: 1 µm.

Figure 3


A
UT-7 WT
CYToxI
Tom20
UT-7 MPL
CYToxI
Tom20
LZD
TDZ
0 h
72 h
144 h
post-washing
B
UT7-WT
UT7-MPL
cytochrome c-oxidase subunite I expression (% of control)
hours post washing
LZD Cmax
TDZ Cmax
0
24
48
72
96
120
144
168
0
25
50
75
100
125
150

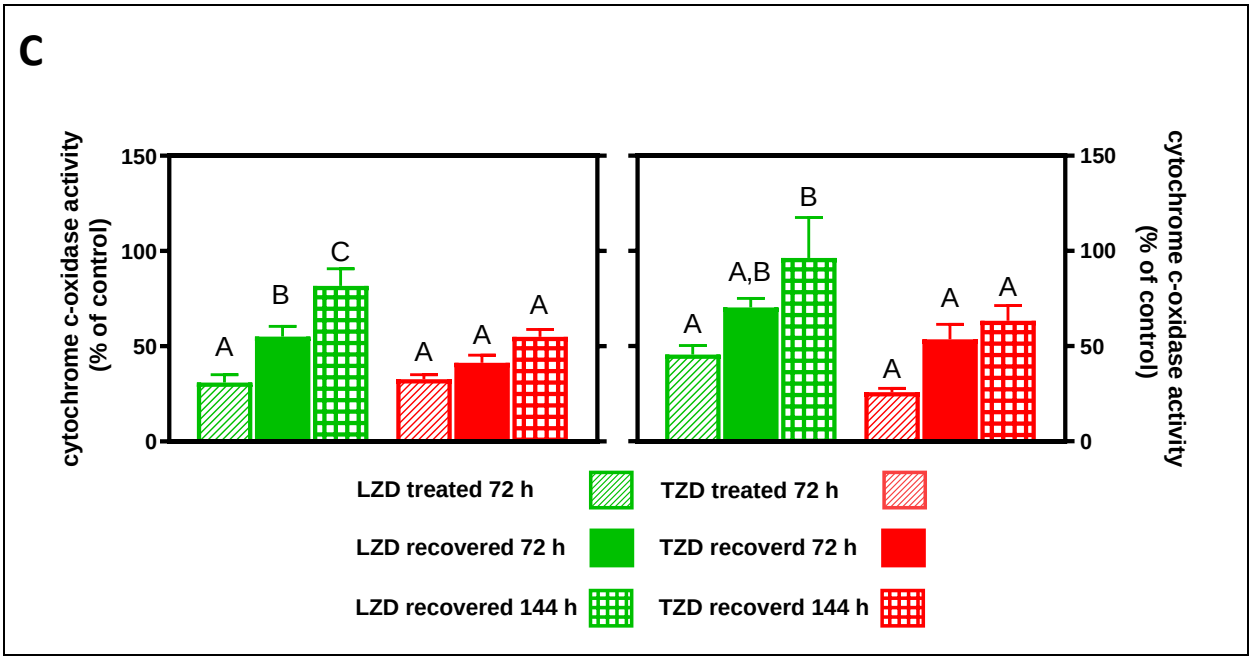


Figure 3: Effect of oxazolidinones on CYTox I expression and cytochrome *c*-oxidase activity after drug removal. UT-7 WT and UT-7 MPL cells were incubated during 72 hours with linezolid (LZD) and tedizolid (TZD), then transferred in to drug-free medium for the next 72 or 144 hours. **A**: Western blot analysis of the expression of CYTox I and Tom 20 (used for normalization). **B**: quantitative measurement (CYTox I/Tom 20 band density ratio). **C**: recovery of cytochrome *c*-oxidase activity (means ± SEM of triplicates in two independent experiments [when not visible, the bars are smaller than the symbols]) for one concentration (C_{max}) of both antibiotics (treated, diagonally-hatched bars; LZD 15 mg/L, TZD 3 mg/L), followed by either 72 h (72 h recovery, plain bars) or 144 h in drug free medium (144 h recovery, square-hatched bars). Statistical analysis (one-way ANOVA with Tukey-Kramer post-test for multiple comparison of all pairs of columns): values with different letters are significantly different from each other ($p<0.05$).