REVERSIBLE INHIBITION OF MITOCHONDRIAL PROTEIN SYNTHESIS DURING LINEZOLID-RELATED HYPERLACTATEMIA.

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The objective of the present study was to determine the mitochondrial toxicity mechanisms of linezolid-related hyperlactatemia. Five patients on a long-term schedule of linezolid treatment were studied during the acute phase of hyperlactatemia and after clinical recovery and lactate normalisation following linezolid withdrawal. Mitochondrial studies were performed in peripheral blood mononuclear cells (PBMCs) and consisted in measuring mitochondrial mass, mitochondrial protein synthesis homeostasis (cytochrome c oxidase -COX- activity, COX-II subunit expression, COX-II mRNA abundance and mtDNA content) and overall mitochondrial function (mitochondrial membrane potential and intact cell oxidative capacity).

During linezolid-induced hyperlactatemia we found extremely reduced protein expression (16% of remaining content compared to control values -100%-, p<0.001) of the mitochondrially coded, transcribed and translated COX-II subunit. Accordingly, COX activity was also found to be decreased (51% of remaining activity, p<0.05). These reductions were observed despite the amount of COX-II mtRNA transcripts being abnormally increased (297%, p=0.10=NS) and the mtDNA content remaining stable. These abnormalities persisted even after the correction by mitochondrial mass, which was mildly decreased during the hyperlactatemic phase. Most of the mitochondrial abnormalities returned to control ranges after linezolid withdrawal, lactate normalisation and clinical recovery.

Linezolid inhibits mitochondrial protein synthesis leading to decreased mitochondrial enzymatic activity responsible of linezolid-related hyperlactatemia that resolves upon discontinuation of linezolid.
INTRODUCTION

Linezolid belongs to a family of antibiotics (oxazolidinones) that inhibit bacterial protein synthesis by binding to 23S rRNA in the large ribosomal subunit and preventing the fusion of 30S and 50S ribosomal subunits and the formation of the translation initiation complex [1]. It has shown excellent efficacy against Gram-positive cocci including \textit{Staphylococcus aureus}, coagulase-negative staphylococci, enterococci and streptococci with a minimum inhibitory concentration (MIC) ranging from 0.5 to 4 mg/L [16]. Furthermore, linezolid has 100% oral bioavailability and reaches high concentrations in different tissues (skin, synovial fluid, bone, cerebrospinal fluid or lung), thus being a good alternative for the long-term treatment of orthopaedic implant infections, ventriculo-peritoneal shunts and other infections related to foreign bodies in which Gram-positive cocci are the main pathogens.

However, a major concern with this antibiotic is its safety profile, especially when it is administered for more than the 28-day period approved by the US Food and Drug Administration [6, 15]. Adverse events of linezolid include haematological disturbances (especially thrombocytopenia and anaemia), peripheral neuropathy, hyperlactatemia and metabolic acidosis [1, 2, 4, 6, 11, 12, 15, 23, 30]. Some of these events could be related to the capacity of linezolid to interfere with mitochondrial function [25, 29] due to similarities between bacterial and mitochondrial ribosomes. Experimental reports support this hypothesis. Nagiec et al. reported the inhibition of mitochondrial protein synthesis in human erythroleukaemia cells exposed to eperezolid, another oxazolidinone [24]. More recently other authors published decreased amount of mitochondrial respiratory chain subunits [10] or mitochondrial protein synthesis [19] in different tissue homogenates or isolated mitochondria, respectively, of rats treated with linezolid. All these studies also suggest that this effect is time and concentration-dependent, although their cross-sectional experimental design did not allow direct information to be obtained regarding the capacity of mitochondrial recovery after discontinuation of linezolid. Moreover, there are very few data from humans receiving treatment with linezolid that confirm experimental hypotheses. We have recently reported a decrease in mitochondrial enzyme activities in peripheral blood mononuclear cells (PBMCs) from 3 patients.
on linezolid therapy during the acute phase of symptomatic hyperlactatemia [29]. In addition, De Vriese et al. reported decreased enzyme activity of some mitochondrial respiratory chain proteins partially synthesised by mitochondrial ribosomes in the skeletal muscle, liver and kidney of a patient with severe hyperlactatemia [10]. In none of these four cases were data of mitochondrial outcome after hyperlactatemia resolution presented. We have conducted a prospective longitudinal study to assess the homeostasis of mitochondrial protein synthesis and function in selected patients developing linezolid-related hyperlactatemia both, during treatment and after discontinuing linezolid.
PATIENTS AND METHODS

Patients

In our hospital all the patients who receive linezolid for more than 1 month at standard oral dosage (600 mg/12h) are closely followed. Clinical symptoms of infection, linezolid-related or possibly-related adverse events (anaemia, thrombocytopenia, gastrointestinal discomfort…) were monitored and a haemogram was performed weekly. For the present study, blood lactate (as a surrogate marker of mitochondrial toxicity) was determined at least once a month or when hyperlactatemia was suspected based on clinical findings. When the patients developed hyperlactatemia, with or without clinical symptoms of metabolic acidosis, a blood sample was obtained for mitochondrial studies (see next section). In order to avoid confounders of mitochondrial toxicity, critically ill patients and those taking other potentially toxic drugs for mitochondria (i.e. aminoglycosides or antiretrovirals) were excluded from the study. Linezolid was withdrawn when a severe adverse event was observed (platelet count < 100,000 cells/mm3, haemoglobin < 9 g/L or severe gastrointestinal manifestations). An asymptomatic increase in serum lactate was not a criterion for stopping linezolid. In those patients who developed hyperlactatemia, a second sample was obtained for mitochondrial studies once linezolid was discontinued, patient became asymptomatic and lactate levels retuned to normality. For mitochondrial studies, 6 healthy volunteers matched by age and gender were included as controls. All individuals signed written consent to be included in the protocol.

Sample collection

For mitochondrial analysis we obtained 20 mL of venous blood during the hyperlactatemic episode during linezolid use and after linezolid discontinuation and hyperlactatemia resolution. PBMCs were isolated by Ficoll’s gradient [27] and protein content was measured according to the Bradford’s protein-dye binding-based method [5].

Determination of mitochondrial mass

The quantity of mitochondria was estimated by two different methodologies. One was spectrophotometric measurement of citrate synthase (CS) activity (EC: 4.1.3.7, nmols/min/mg protein), a mitochondrial matrix enzyme of the Krebs’ cycle, which remains highly constant in
mitochondria and is considered to be a reliable marker of mitochondrial content [3, 26, 28, 31].

The other method to measure mitochondrial mass was the western blot analysis of V-DAC amount (a nuclear encoded mitochondrial structural protein) corrected by β-Actin content (a nuclear encoded cytoplasmic protein) [22].

Studies on mitochondrial protein synthesis homeostasis.

Mitochondrial respiratory chain complex IV (COX, EC 1.9.3.1) enzyme activity: This was measured spectrophotometrically according to the Rustin et al. methodology [28], slightly modified for complex IV for minute amounts of biological samples [20]. COX activity was expressed in absolute values (nmol/min/mg protein), as well as in relative values compared to mitochondrial mass (by dividing absolute values by citrate synthase activity).

Mitochondrial protein synthesis: We assessed mitochondrial protein synthesis of the COX-II subunit (mitochondrially encoded, transcribed and translated) by western blot [22]. This expression was normalized by the content of β-actin signal to establish the relative COX-II abundance per overall cell protein, as well as per V-DAC to establish the relative COX-II abundance per mitochondria.

Mitochondrial RNA (mtRNA) quantification: Total RNA was obtained by an affinity column-based procedure (Rneasy, Qiagen). RNA (1 microgram/sample) was reverse-transcribed (reverse transcriptase Taqman, Applied Biosystems) using random primers. The real-time PCR reaction was performed using TaqMan universal mastermix (Applied Biosystems) in an ABI PRISM 7700 sequence detection system, in a total reaction volume of 25 µL. Quantification of COX-II mRNA was performed using the amplification conditions indicated by the supplier (Assay-by-design, Applied Biosystems) and the primers were: CAAACCACTTTCACCGCTACAC (forward) and GGACGATGGGCGATGAACTGT (reverse) and the FAM-labeled probe was AAATCTGTGGAGCAAACC. As a reference control the housekeeping nuclear-encoded 18S rRNA abundance was determined using a pre-made kit (Assay-on-Demand, Hs99999901_s1, Applied Biosystems). Absolute COX-II mtRNA content was expressed as the ratio of the mean COX-II mRNA value to the mean 18S rRNA.
value [13], while COX II mtRNA content relative to mitochondrial mass was calculated by dividing absolute values by citrate synthase activity.

Mitochondrial DNA (mtDNA) quantification: Total DNA was obtained by a standard phenol-chloroform extraction procedure. A fragment of the mitochondrial ND2 gene and the nuclear rRNA 18S gene were amplified in duplicate and separately by quantitative real-time PCR as previously reported [21]. The absolute content of mtDNA was expressed as the mtDNA to nDNA ratio (ND2mtDNA/18SnDNA), and mtDNA content relative to mitochondrial mass was obtained after dividing absolute value by citrate synthase activity.

Studies on overall cellular function.

Mitochondrial membrane potential: 750,000 fresh PBMCs were stained with 2.5 µg/mL of JC-1 probe (T-3168, Molecular Probes, Eugene, Oregon, USA) in 10%-Foetal Bovine Serum completed RPMI medium. The resulting cells were washed and analysed by Flow Cytometry (FACScan Cytometer, Becton Dickinson, San Jose, CA, USA) on FL1 and FL2 channels [9]. The results were expressed as percentage of PBMC with depolarised mitochondria with respect to the total amount of PBMC analysed.

Spontaneous mitochondrial oxidative activity: Oxygen usage was measured polarographically using a Clark electrode in a water-jacketed cell magnetically stirred at 37°C (Hansatech Instruments Limited, Norfolk, England) in 250 µL of respiration buffer using 100-200 µg of fresh cells. Intact-cell endogenous substrate oxidation was assessed as spontaneous PBMC oxygen uptake. The complete methodology is reported elsewhere [21, 28].

Statistical analysis.

Results were expressed as mean±SD and/or the percentage compared to control values (which were arbitrarily assigned 100%). The t-test for independent or paired measures (as needed) was used to search for differences.
From January to December 2005, 5 out of 13 patients receiving linezolid therapy for more than 1 month developed hyperlactatemia and were included in the present study. Mitochondrial enzymatic activity and spontaneous cell oxidation during the acute phase of hyperlactatemia of two patients have been previously reported [29]. In such two cases mitochondrial DNA, RNA and protein synthesis, as well as complete mitochondrial functionality studies have been added, during and after hyperlactatemia resolution. The clinical characteristics and serum lactate values determined during and after discontinuation of linezolid are shown in table I and figure 1. As can be seen, three patients were symptomatic and in two linezolid treatment was prematurely interrupted due to adverse events.

The amount of mitochondria was similar irrespective of the methodology employed, and was decreased during the hyperlactatemic phase (59% of control values for CS method, p<0·01; 60% of control values for VDAC method, p<0·05). After linezolid discontinuation and hyperlactatemia resolution, a trend to normalization of mitochondrial mass was observed (from 59% or 60% to 80%, respectively, p=0·17 for both methods).

We found a marked reduction in COX activity (a mitochondrial respiratory chain complex which is partially encoded by mtDNA) during the acute phase of hyperlactatemia of patients receiving linezolid compared to controls (51% respect to control values, p<0·05, figure 2), and an even greater reduction was observed for the expression of COX-II protein (one of the COX subunits encoded by mtDNA and translated by mitochondrial ribosomes), which was reduced to 16% of control value when assessed per cell (p<0·001, figure 2). These changes were observed despite an increase in COX-II mtRNA expression (297% of control values, p=0·1=NS) and no change in mtDNA levels (82% of control values, p=0·57) during the hyperlactatemic phase (figure 2). After linezolid discontinuation and normalization of lactate levels, mitochondrial abnormalities showed a significant trend toward normalization (from 51% to 130% for COX activity, p<0·01; from 16% to 72% for COX-II expression per cell, p<0·05, and from 297% to 210% for COX-II mtRNA abundance, p<0·05, figure 2 and 3). It is important to note that mitochondrial parameters conserved tendencies even after the correction by the
decrease in mitochondrial mass present in patients developing linezolid-related hyperlactatemia (figure 3).

No significant changes were found on the overall PBMC function irrespective of whether it was considered by spontaneous cell oxidation capacity or by mitochondrial membrane potential (figure 4).
DISCUSSION

In the present study, we evaluated the impact of standard dosages of 600 mg/12h of linezolid on mitochondrial activity using lactic acid serum levels as a surrogate marker. We studied 5 patients developing hyperlactatemia in whom we demonstrated a reduction in COX activity and COX-II protein synthesis in PBMCs, despite an increase in COX-II mtRNA production. All these abnormalities tended to disappear after discontinuing linezolid and serum lactate regained normal levels.

Our findings in humans offer a complete picture of what occurs in mitochondria during linezolid-related hyperlactatemia and corroborate previous experimental data suggesting the inhibitory action of linezolid on mitochondrial protein synthesis. Only few previous partial data had been reported to date regarding the in vivo effects of linezolid therapy. De Vriese et al. recently reported abnormally reduced enzymatic mitochondrial respiratory chain activity in liver, kidney and muscle of one patient under severe symptomatology after a 4-month course of linezolid [10]. All our hyperlactatemic patients were studied before three-month linezolid treatment and presented null or mild clinical manifestations. De Vriese et al. performed no further studies to determine the causes of such a reduction of COX activity. In this case, no mitotoxic effects were found in PBMC. Although some authors have argued against the validity of mitochondrial studies on PBMCs, mainly because these cells are not the typical target of mitochondrial disturbances, in our experience most mitochondrial defects can reliably be demonstrated in PBMCs [17, 21, 22]. In addition, very recently McKee et al. [19] demonstrated equal mitochondrial ribosomal structure and antibiotic effect in all studied tissues. We believe that for finding mitochondrial disturbances on PBMC it may be necessary to control crude results for mitochondrial content, which may increase or decrease in response to functional defects. Actually, we also found an important reduction in PBMC mitochondrial mass in all the hyperlactatemic patients analysed, irrespective of the methodology employed. This reduction in mitochondrial amount may not be the cause of the mitochondrial dysfunction observed in these patients. Conversely, we believe that it could be the consequence of linezolid mitochondrial protein synthesis inhibition, since mitochondrial parameters remained abnormal (or,
alternatively, showed a clear tendency to be lower than controls), when they were normalised by the amount of mitochondria. In this sense, reduction on mitochondrial mass could be suggesting that linezolid protein synthesis inhibition is limiting mitochondrial biogenesis. Finally, it is important to note that mitochondrial disturbances identified in the PBMCs of patients with linezolid-related hyperlactatemia could be even more pronounced in other tissues located in a more central place of lactate homeostasis, such as the liver or kidney which are, at the end, the main responsible of lactate raising in plasma.

Additionally, in these linezolid-induced hyperlactatemic patients, mtRNA abundance is increased, probably in a trend to compensate the linezolid inhibition of mitochondrial protein synthesis. Up-regulation of mtRNA levels in response to experimental inhibition of mitochondrial protein synthesis by thiamphenicol has been previously reported [8, 14]. Nevertheless, reduced enzymatic activity of the partially mitochondrial-coded COX was detected in our patients, suggesting that the up-regulatory mechanisms leading to increased mitochondrial DNA-encoded transcripts are not able to compensate linezolid-mediated mitochondrial translation inhibition.

Linezolid exerts a time-dependent antimicrobial action, but influence of linezolid concentration on adverse effects has been previously suggested by haematological alterations in patients with renal failure [18], provably associated with linezolid or metabolite accumulation [10]. Although serum linezolid concentration was not measured, no hepatic or renal impairment was present among the studied patients which could influence linezolid pharmacokinetics. Therefore individual susceptibility to mitochondrial toxicity could be related to mtDNA mutations on mt rRNA genes codifying for mitochondrial ribosomal subunits [25] or particular linezolid metabolism. McKee et al [19] reported that linezolid concentration that inhibits 50% of mitochondrial protein synthesis (IC$_{50}$) in rat and rabbit heart and liver mitochondria is between 3.37 to 5.26 mg/L. Considering that linezolid trough serum concentrations range from 1 to 6 mg/L, we hypothesise that patients with trough levels above IC$_{50}$ could maintain mitochondrial protein synthesis permanently inhibited and could be specially vulnerable to mitochondrial toxicity with respect to those patients with trough levels below IC$_{50}$. Since linezolid has a 100%
oral bioavailability and achieves trough serum levels over the MIC for most susceptible Gram-
positive microorganisms, it is necessary to further evaluate if guiding linezolid dosage using the
linezolid “trough level/MIC ratio” [7, 10] could prevent the development of the adverse events
related to mitochondrial protein synthesis inhibition while maintaining the clinical efficacy,
specially in patients with prolonged courses on linezolid.
ACKNOWLEDGMENTS

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REFERENCES


Table I. Clinical characteristics of individuals included in the study.

<table>
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<tr>
<th>Patient</th>
<th>Age/sex</th>
<th>Co-morbidity</th>
<th>Infection location</th>
<th>Aetiology of infection</th>
<th>Serum lactate&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Days on linezolid&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Adverse events</th>
<th>Stop linezolid for adverse event</th>
<th>Serum lactate&lt;sup&gt;3&lt;/sup&gt;</th>
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<td>Knee Osteosarcoma</td>
<td>Knee prosthesis</td>
<td>Vancomycin-resistant Enterococcus faecium</td>
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<td>15</td>
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<tr>
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<td>Methicillin-resistant Staphylococcus aureus</td>
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<td>Mild asthenia</td>
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<td>No</td>
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<td>Methicillin-resistant Staphylococcus epidermidis</td>
<td>43</td>
<td>30</td>
<td>Severe gastrointestinal discomfort</td>
<td>yes</td>
<td>17</td>
</tr>
</tbody>
</table>

<sup>1</sup> the highest value during linezolid therapy (mg/L).

<sup>2</sup> at the time of the first sample for mitochondrial studies.

<sup>3</sup> at least 15 days after discontinuing linezolid.
**Figure 1:** Lactate levels (lines) and sample collection for mitochondrial studies (downward arrows, first study; upward arrows, second study) for the patients developing linezolid-related hyperlactatemia. Dashed lines on patients lactate evolution correspond to the interval period between mitochondrial studies and shaded square area to lactate normality level.
Figure 2: Comparison of mitochondrial parameters between controls (100%, dashed line) and patients developing linezolid-related hyperlactatemia (columns, mean±SEM). P values correspond to comparison between values measured during and after linezolid treatment. Asterisks correspond to significant differences (*: p<0.05; **: p<0.01) between each column and control values.
**Figure 3:** Up: Individual values of mitochondrial protein synthesis homeostasis parameters measured in the study (up). Dashed lines correspond to the mean of controls, and shaded squares the normality interval (±2 SD). P values refer to statistical significance of the changes observed in patients developing linezolid-related hyperlactatemia after discontinuing linezolid (mt: mitochondrial; n: nuclear). Down: Western blot analysis of the mitochondrially coded cytochrome c oxidase subunit II (COX-II) expression with respect to both nuclear coded porin V-DAC (mitochondrial marker) and β-Actin (cellular marker) (L denotes patients receiving linezolid and C denotes control individuals; B corresponds to the second sample after hyperlactatemia resolution).
**Figure 4:** Overall mitochondrial function of PBMCs of patients developing linezolid-related hyperlactatemia. Dashed lines correspond to the mean of controls, and shaded squares the normality interval (±2 SD). P values refer to statistical significance of the changes observed in patients developing linezolid-related hyperlactatemia after discontinuing linezolid.
Spontaneous cellular oxidation

- Patient 1
- Patient 2
- Patient 3
- Patient 4
- Patient 5

Membrane potential

p=NS

% of depolarised mitochondria

Hyperlactatemia Normal lactate

Hyperlactatemia Normal lactate

p=NS