Experimental model of reversible myelosuppression caused by short-term, high-dose oxazolidinone administration

Emily J Hickey, CJ Gill, AS Misura, AF Flattery & GK Abruzzo

Aims: To develop a murine model of oxazolidinone-induced myelosuppression observed in man for identification of potentially less myelosuppressive compounds within this chemical therapeutic class. Methods: Female C3H mice were treated orally, once-daily, with linezolid for between 2 and 7 days. A total of 24 h after the last dose, mice were euthanized, blood was collected by cardiac puncture and analyzed for hematologic parameters against vehicle control animals. Results: Results from three independent experiments demonstrated that oral treatment with linezolid at 50 mg/kg produces mild, reversible anemia characterized by reticulocytopenia in greater than 4 days in mice. Conclusions: Use of this model to screen potential chemotherapeutic agents will facilitate discovery and guide structure–activity relationship chemistry for less myelosuppressive compounds.

Oxazolidinones represent a new class of antibacterials with potent antimicrobial activity against Gram-positive bacteria, such as staphylococci, streptococci and enterococci. This includes activity against methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant enterococci (VRE). Linezolid (Zyvox®) was the first oxazolidinone antibiotic to be approved in the USA and other countries worldwide that has shown in vitro activity and in vivo efficacy in animal models and clinical trials [1–3]. Linezolid is an inhibitor of the bacterial protein synthesis initiation complex formation, possibly by distorting the binding site for initiator-tRNA. [4,5] To date, linezolid has not demonstrated cross-resistance in bacterial strains resistant to other classes of antimicrobial agents, including other protein synthesis inhibitors [4–6]. Linezolid has demonstrated clinical efficacy in Gram-positive infections, including pneumonia, skin and soft tissue infections and infections caused by MRSA and VRE, with efficacy which is comparable to other antimicrobial agents with similar spectrums of activity but with the added advantage of efficacy against resistant pathogens [7–10]. However, the current label for linezolid carries a ‘black box’ warning related to clinical myelosuppression [11]. This is validated in the freedom of information (FOI) for this drug as well as in multiple clinical reports [12–19].

Materials & methods
Linezolid was dissolved in sterile distilled water as per manufacturer's instructions with subsequent dilutions in sterile distilled water. Chloramphenicol succinate was purchased from Sigma Chemical (MI, USA) and dissolved and diluted in sterile distilled water.

Female C3H mice (Harlan Laboratories) were purchased at 6–7 weeks of age and allowed to acclimatize in the facility for 1 week. Animals were housed in microisolator cages (Ancare Products, Inc.) and maintained with water and Purina Prolab RMH 3000 rodent chow. Animal protocols were approved by the Institutional Animal Care and Use Committee. All procedures were performed in accordance with the highest standards for the humane handling, care, and treatment of research animals and were approved by the Merck Institutional Animal Care and Use Committee. Procedures for the care and use of research animals at Merck meet or exceed all applicable local, national and international laws and regulations.

Keywords: animal models, infectious diseases, linezolid, myelosuppression, therapy

Oxazolidinones indicated varying degrees of sensitivity dependent upon strain of mouse used, with C3H being the most sensitive [20]. Studies were initiated to establish a short-course in vivo testing method in C3H mice that would require minimal treatment days and drug quantity needed to evaluate the potential myelosuppressive effects of novel oxazolidinones in early development. Such a model could then be used to screen novel oxazolidinone compounds and determine whether further evaluation is warranted.

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Three separate studies were performed. The objective of the initial study was to induce myelosuppression typical of the oxazolidinone class (using linezolid) compared with the documented suppression induced by chloramphenicol. C3H mice were treated with linezolid and chloramphenicol daily for 7 days by oral gavage in a 0.5-ml volume. Dosages used were linezolid at 300 mg/kg and chloramphenicol at 1500 mg/kg, with a vehicle-treated control group (n = 3–4).

The second experiment assessed the dose and time-dependency of myelosuppression with linezolid. Linezolid was administered at 150 or 300 mg/kg for a 2–5 day time-course with selected mice (n = 5/group/day) euthanized after each of those days to evaluate the hematological response over time with respect to dosage.

The final study assessed the dose effect with a down-titration of linezolid. Linezolid was administered from 300 to 50 mg/kg (n = 4 per group) in increments of 50, to find the lowest adverse effect level over time. In addition, a cohort group at 200 mg/kg was dosed for 4 days and sacrificed 7 days after the last treatment to assess the reversibility of this toxicity. Pharmacokinetic studies were completed for mice treated with 300, 150 and 50 mg/kg in this study.

Mice were sacrificed by CO2 inhalation 1 day after the last treatment, with the exception of the reversal arm of the last study, as designated per study. Blood was collected via cardiac puncture into K3-EDTA Microtainer (Becton Dickinson). Samples were analyzed on an Advia Hematology analyzer (Bayer Advia 120).

The data were analyzed via standard linear model and ANalysis Of VAriance (ANOVA). p-values less than 0.05 for all tests and comparisons were deemed significant unless otherwise indicated. The logarithmic scale was used since underlying assumptions of equal variance and normal distribution shape were better satisfied.

Results

In the initial study, C3H mice treated with linezolid (300 mg/kg per oral, once-daily) for 7 days demonstrated depression of red blood cell (RBC) counts, hematocrit, hemoglobin, reticulocytes, platelets and white blood cell (WBC) counts compared with vehicle-treated control mice. Chloramphenicol (1500 mg/kg) was administered as a positive control showing decreases in RBCs, hematocrit and hemoglobin compared with vehicle-treated control mice (Table 1). Reticulocyte and platelet counts are shown, but other parameters followed the same pattern of depletion (data not shown) (Table 1).

In the second study, reticulocytes showed the most sensitive response of parameters evaluated with a dose- and time-dependent decrease following oral administration of linezolid (Figure 1).

### Table 1. Reticulocyte and platelet count of treated groups.

<table>
<thead>
<tr>
<th></th>
<th>Reticulocyte count (x10e9 cells/l)</th>
<th>Platelet count (x10e3 cells/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle control</td>
<td>281 ± 16</td>
<td>1083 ± 139</td>
</tr>
<tr>
<td>Chloramphenicol 1500 mg/kg</td>
<td>166 ± 55*</td>
<td>781 ± 170</td>
</tr>
<tr>
<td>Linezolid 300 mg/kg</td>
<td>1.0 ± 0.03*</td>
<td>161 ± 47*</td>
</tr>
</tbody>
</table>

Counts recorded following 7 days of once a day, per oral administration of vehicle, chloramphenicol or linezolid in mice (n = 3; *p < 0.001).

Counts recorded following 2–5 days of per oral administration of vehicle or linezolid at 150 or 300 mg/kg in mice (n = 5; *p < 0.001) once-daily. Reticulocyte count on each day was significantly lower (p < 0.05) when linezolid was administered at 300 mg/kg compared with 150 mg/kg.
Hemoglobin, WBC, RBC and hematocrit responses were also dose- and time-dependent, with 4 days of dosing being the minimal period required to significantly inhibit these cell lines at 150 and 300 mg/kg (Figure 2). Platelet counts were decreased significantly following 5 days of dosing (Figure 3). Finally, 4-day administration of linezolid at all dosage levels significantly reduced reticulocyte counts. This effect exhibited dose-dependence with a more significant response at 300 mg/kg (p < 0.001) as compared with that at 50 mg/kg (p = 0.002) (Figure 4). WBC, RBC, hemoglobin and hematocrit also decreased over this 4-day study, but only at the highest of dosages: 200–300 mg/kg. There were no significant changes in platelet counts in this study. In the final experiment, a cohort were dosed at 200 mg/kg for 4 days followed by a 7-day washout period. All of these cohort mice euthanized 7 days after the last dose had normal reticulocyte and RBC values, demonstrating a complete reversal of these parameters.

**Discussion**

The need for new, safe and effective antibiotic agents is evident from the various surveillance studies documented to date [6,8]. Agents that display superior *in vitro* activity and *in vivo* efficacy must be evaluated for potential adverse effects to
ensure an acceptable therapeutic index. This can be very expensive and time consuming as clinical adverse effects are often evident only after long-term dosing. Green and colleagues reported on three clinical cases of myelosuppression. All three cases followed long-term linezolid therapy and documented pancytopenia similar to the reversibly chloramphenicol toxicity versus the previously documented thrombocytopenia found with linezolid therapy \[12\]. Our laboratory focused efforts on establishing a murine model predictive of clinical myelosuppression induced by the oxazolidinone class of compounds.

These results demonstrate the successful establishment of a murine model of linezolid-induced myelosuppression. The detection of myelosuppression in the C3H mouse was evident within 3 days of dosing. The objective of this initial study was to evaluate the hematological response in the mouse to high-dose chloramphenicol and linezolid. A positive finding would be a proof of concept that myelosuppression could be derived with administration of the compound for less than or equal to 7 days in the mouse. The C3H strain of mouse demonstrated changes in hematology values similar to those of treated rats in the linezolid FOI and in humans from the clinical literature. Knowing this response is time- and dose-dependent, the second study was performed to shorten the time-course and decrease the dramatic response seen at these initial dosages. Reticulocyte counts appeared to be the most sensitive measure within this model (Figure 1). Based on this and the other parameters evaluated, day 4 was determined to be an adequate end point for future studies.

The destruction of reticulocytes at the 150- and 300-mg/kg dosages, in addition to the significant decrease in hemoglobin, WBC, RBC and hematocrit counts at 150 mg/kg, suggested that lower dosages should be evaluated as a model standard. Examination of reticulocyte counts following 4 days of dosing from 300 to 50 mg/kg induced a significant reduction at all dosages (Figure 4). In this study, pharmacokinetics were evaluated for three of the six dose levels. The published FOI for linezolid denotes a no-effect level in the rat at a dosage of 20 mg/kg or an AUC of 49 µg*h/ml. The lowest dose tested that gave an effect in the 30-day rat toxicity study was 50 mg/kg or 140 µg*h/ml. Our lowest dose tested that yielded a significantly lower reticulocyte count was at 50 mg/kg or 78 µg*h/ml. This falls in line with the data reported in the FOI. Therefore, 50 mg/kg was chosen as the dosing standard for the screening studies.

**Expert commentary**

Linezolid, the first oxazolidinone to reach the market, has been associated with reversible, myelosuppression that is both time- and dose-dependent. Preclinical evaluation of linezolid in rats and dogs

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**Figure 3. Platelet count of treated groups.**

Counts taken following 2–5 days of per oral administration of vehicle or linezolid at 150 or 300 mg/kg in mice (n = 5; *p = 0.02; *p < 0.001) once a day.

**Figure 4. Reticulocyte count of treated groups following 4 days of therapy.**

Counts were recorded following 4 days of per oral administration of vehicle or linezolid at 50 to 300 mg/kg in mice (n = 4; †*p = 0.002; < 0.001) once-daily. AUC (µg*h/ml) is represented above the three doses tested.
at Pharmacia demonstrated mild, reversible anemia characterized by bone marrow hypocellularity, and decreased erythrocytes and reticulocytes with mild changes noted in white blood cells and platelets. In clinical trials in man, linezolid has been shown to have a greater likelihood of inducing thrombocytopenia. However, changes in neutrophil count and hemoglobin concentration were similar between the comparator groups and linezolid. The predominant thrombocytopenia seen in humans treated with linezolid differs from the initial reticulocytopenia found in this short-term mouse model. However, in the linezolid FOI, it appears that the erythroid line was also most sensitive when tested for 1 or 3 months in the rat and the dog, mimicking the effects seen in this short-term model.

Other antimicrobials, such as chloramphenicol, induce similar hematological responses to linezolid in animals and man. Chloramphenicol is known for two distinct responses. The more common is characterized by anemia with reticulocytopenia, slight leukopenia and thrombocytopenia, which is reversible, dose-dependent and similar to linezolid. However, this drug also causes an aplastic anemia with complicating and severe pancytopenia. Interestingly, this phenomenon occurs weeks to months after drug treatment and does not appear to be dose-dependent; however, it is often irreversible and fatal. The former anemia has been evaluated by Festing and colleagues, reporting on differences in the hematological response to chloramphenicol in four inbred and one outbred strain of mice. While chloramphenicol caused anemia and reticulocytopenia in all five strains, the four inbred strains tested showed a significant response at a lower dosage than the outbred strain of mouse used. Based on Festing’s findings, we chose the C3H inbred mouse for our studies as it showed similar changes in the hematological cells lines as those documented clinically with linezolid.

In summary, we have established a mouse model for determining myelosuppression caused by antimicrobial agents, particularly those in the oxazolidinone class. The myelosuppression can be induced with administration of linezolid at a dose as low as 50 mg/kg in the mouse for 4 days. Use of this model to screen potential chemotherapy agents will facilitate discovery and guide structure-activity relationship chemistry for the discovery and development of less myelosuppressive compounds.

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Bibliography

11. FOI-linezolid, Pharmacia.


