Activity of novel oxazolidinones against *Nocardia brasiliensis* growing within THP-1 macrophages

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Received 29 June 2009; returned 20 July 2009; revised 4 August 2009; accepted 4 August 2009

**Background:** *Nocardia* are organisms that can escape the effects of both immune response and antimicrobial agents, due to their potential capacity to grow intracellularly. In previous studies, we found that experimental oxazolidinones, DA-7157 and DA-7218, are active both *in vitro* and *in vivo*.

**Objectives:** In this study, we compare the ability of linezolid, DA-7157 and DA-7218 to inhibit intracellular growth of *Nocardia brasiliensis* within the human monocyte cell line THP-1.

**Methods and results:** The addition of oxazolidinones to the infected macrophage monolayer at concentrations 0.25, 1, 4 and 16 the MIC for *N. brasiliensis* resulted in an inhibitory effect on bacterial growth as follows DA-7157/C21 DA-7218 > linezolid.

**Conclusions:** The excellent intracellular antimicrobial activity detected suggests that these compounds could be effective in the treatment of actinomycetoma. However, more studies are needed both *in vitro* and *in vivo*, including clinical trials, to confirm this issue.

**Keywords:** actinomycetoma, linezolid, DA-7218, DA-7867

**Introduction**

Mycetoma in Mexico is mainly caused by actinomycetes, the predominant species being *Nocardia brasiliensis* (86.6% of cases).¹ Clinical lesions evolve very slowly, but sometimes can grow extensively, affecting a whole limb. The medical treatment of this disease requires that antibiotics be administered for several months, and this sometimes leads to the emergence of resistance.² Trimethoprim/sulfamethoxazole is the drug of choice, as it achieves adequate concentrations in the affected tissues, including bones. The high failure rate of treatments, however, prompts the use of drug combinations as well as the search for new active molecules.

Linezolid, a recently described oxazolidinone, has been used effectively in the therapy of patients with nocardiosis;³ however, it is expensive, and side effects such as peripheral neuropathy and myelosuppression have been reported during long-term use in some patients.⁴ New oxazolidinone compounds such as DA-7867 and DA-7218 (the DA-7157 pro-drug) have shown excellent activity both *in vitro* and *in vivo* against *N. brasiliensis*.⁵–⁸ In mice infected with *N. brasiliensis* and treated with DA-7218 (25 mg/kg), the number of lesions detected was lower than the number found in those treated with the same dose of linezolid,⁵ and DA-7867 was active at lower concentrations (10 mg/kg) than linezolid when applied once a day in a BALB/c experimental model.⁶ *Nocardia* can grow intracellularly in phagocytes and this makes treatment more difficult since antimicrobials need to penetrate deeply into fibrotic abscessed tissues. When drugs do not reach the bacteria, they can survive and produce new lesions. In order to predict which antimicrobial is useful to treat massive, chronic actinomycetomas, we consider it important to evaluate both its *in vitro* activity and the intracellular activity of the compound.

In the present study we standardized a model of *N. brasiliensis* infection using THP-1 macrophages to evaluate its intracellular replication in the presence of the oxazolidinones under test. Since this microorganism grows as clumps of filaments and the number of cfu does not represent the real quantity of bacteria (a nocardial clump can contain thousands of bacteria and produce only one colony), novel techniques have to be employed to determine nocardial growth.

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Materials and methods

Microorganisms

We utilized *N. brasiliensis* HUJEG-1, which was isolated from a thoracic case of mycetoma, and has been used extensively in immunological and susceptibility assays.\(^5,9\) It was kept in 10% skimmed milk at ~70°C until use.

Antimicrobial agents

Linezolid, DA-7157 and DA-7218 were provided by the Dong-A Pharmaceutical Company Research Laboratory (Yongin, South Korea). The MICs of linezolid, DA-7157 and DA-7218 for *N. brasiliensis* HUJEG-1 [measured by the broth microdilution method described by the CLSI (formerly the NCCLS)]\(^10\) were 0.12, 1 and 8 mg/L respectively.\(^6,7\)

Cell line

The human monocyte cell line THP-1 was maintained in RPMI 1640 medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS; Gibco-BRL) and 1 mM sodium pyruvate. The cell density was then determined in a haemocytometer and the cell suspension diluted as required in complete RPMI 1640 supplemented with 10% FCS and 6.25 ng/mL phorbol-12-myristate-13-acetate (Calbiochem Biosciences, Darmstadt, Germany) to obtain a density of 4×10⁵ cells/mL. A 1 mL aliquot of the cell suspension was seeded into each well of 24-well microplates (Costar Corning, Chamber Slide System, Nunc, Fisher Scientific, Pittsburgh, PA, USA) with the same macrophage densities and infection ratios were utilized for microscopic observation and, at the same time points, slides were stained with the Kinyoun method.

Quantification of nocardial growth by the Alamar Blue fluorometric method

A calibration curve was prepared by placing 0.6, 0.8, 1, 1.2, 1.4, 1.6, 1.8 and 2×10⁶ cfu of the unicellular suspension of *N. brasiliensis* HUJEG-1 in 1.5 mL Eppendorf tubes (Fisher Scientific), centrifuging at 10000 rpm for 10 min and discarding the supernatant. A 1 mL aliquot of Alamar Blue (Trek Diagnostics Systems, Cleveland, OH, USA), diluted 1:9 in Luria broth medium (Sigma), was added and the tubes incubated at 37°C for 24 h with agitation. The tubes were centrifuged again and 200 μL of supernatant was collected into microplate wells and the fluorescence determined in a Multi-Detection Microplate Reader (Synergy HT, Biotek Instruments, Winooski, VT, USA) with Gen5 software at an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

Determination of the intracellular activity of the oxazolidinones

A 3:1 multiplicility of infection (MOI) was chosen to determine the effect of antimicrobials on the nocardial intracellular growth. Two hours after infecting the monolayer, the medium was discarded and the monolayer washed twice with warm PBS, pH 7.4. The antimicrobials were added at 0.25×, 1×, 4× and 16× the MIC of each compound in RPMI 1640 with 10% FCS and incubated for a further 6 h at 37°C in 5% CO₂. After this time, most of the macrophages were infected with nocardial cells. The culture medium was discarded and 1 mL of cold distilled water was added and incubated for 15 min. In order to release the intracellular bacteria the monolayer was destroyed by pipetting back and forth several times and the suspension was collected in 1.5 mL Eppendorf tubes. Nocardial growth was quantified by the Alamar Blue method described above.

Statistical analysis

Potential differences among the groups against a control without oxazolidinones was established by Kruskal–Wallis analysis of variance (ANOVA), Dunn’s two-sided multiple comparison test with control and Dunnett’s simultaneous confidence intervals for treatment versus control.

Results

Preparation of the unicellular suspension

*Nocardia* normally grow as a mass of filaments. By grinding the bacterial mass and centrifuging at 100 g we obtained a suspension composed of short filaments and individual bacteria (Figure 1). When staining with propidium iodide we observed a viability of >99% (not shown).

Growth of *N. brasiliensis* HUJEG-1 within the THP-1 monolayer

The analysis of bacterial growth on the monolayer cells in relation to the post-infection time showed that, after 1 h, only one-tenth of the inoculum remained associated with the macrophages, either attached to or engulfed by the phagocytes.
Intracellular activity of oxazolidinones against *N. brasiliensis*

Figure 1. Bacterial suspension of *N. brasiliensis* HUJEG-1 used to infect the monolayers. Clumps of bacteria (left) growing on BHI were mechanically disaggregated with an Evelham–Potter device obtaining a suspension composed of individual and short filaments of *Nocardia* (right).

Figure 2. Infection of the THP-1 macrophage monolayer with a unicellular suspension of *N. brasiliensis*. (a) At 1 h after infection a few nocardial cells were observed inside the macrophages, ×100. (b) At 6 h after infection the monolayer was still intact with an increased number of nocardial cells inside the phagocytes, ×100. (c) At 12 h after infection we observed the production of nocardial clumps with peripheral destruction of the monolayer, ×10. (d) At 24 h after infection most of the monolayer was destroyed, ×10. (e) At 48 h after infection intensely stained filament clumps were observed with scanty macrophages, ×10. (f) At 12 h after infection the nocardial infection induced macrophage fusion and production of multinuclear cells, ×60. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*. 
When we observed the Kinyoun-stained slides of the infected macrophage monolayers, at 1 h there were many infected phagocytes with scarce short or unicellular fragments of bacteria in their cytoplasm (Figure 2a). Six hours after infection, the phagocytes were full of *Nocardia* cells with a few clusters of filaments growing outside them (Figure 2b). Twelve hours after infection, there were abundant giant microcolonies of *Nocardia* surrounded by clear areas of devastation of the macrophage monolayer. Macrophages were rounded and accumulated around the necardial growth. They fused to produce binuclear or multinuclear cells (Figure 2c and f). Destruction of the monolayer was more evident after 24 h, with abundant *Nocardia* microcolonies intensely stained with fuchsin (Figure 2d). At 48 h, the monolayer was almost completely destroyed, with scanty macrophages found among the necardial microcolonies (Figure 2e).

As the amount of *Nocardia* observed under the microscope did not correlate with the viable count, we measured the bacterial load by determining the reduction of Alamar Blue. In Figure 3 (left-hand graph) we show a standard curve made with several known amounts of bacteria from a cellular suspension. Using this method we were able to measure between $6 \times 10^5$ and $1.8 \times 10^6$ cfu. Beyond these ranges resolution was poor. When monitoring necardial growth in the macrophage monolayer we observed a slight increase in the first 6 h; after this point, bacteria started to grow exponentially and, after 12 h, reached $\geq 1.8 \times 10^6$—approximately the upper limit of the technique (Figure 3, right-hand graph). In the supernatant, we always had high concentrations of bacteria produced by the rupture of macrophages, and extracellular growth was evident (Figure 3, right-hand graph).

For determination of the intracellular effect of the drugs, we selected 6 h of incubation with the compounds since at this time there was a large number of intracellular bacteria and the monolayer was still undamaged.

**Effect of tested oxazolidinones on the necardial growth**

As seen in Figure 4, linezolid shows a dose-dependent activity with statistical significance for all concentrations. The experimental oxazolidinones, DA-7157 and DA-7218, also showed a remarkable activity, which was statistically significant for all concentrations compared with both control and linezolid ($P=0.003$).

**Discussion**

There are few studies on the growth of *Nocardia* inside macrophages, most of them performed with *Nocardia asteroides* type 6 (now *Nocardia cyriacigeorgica*). Beaman and Smathers\(^{11,12}\) used rabbit alveolar macrophages to study infection with strains of *N. asteroides* and described how the macrophage response was related to the virulence of the organism, the highly virulent strain being able to induce a dramatic macrophagic response that sequentially resulted in the formation of cellular aggregates, the appearance of round cells and multinucleated giant cells, and the subsequent destruction of the monolayer with the production of necardial clumps floating in the medium—similar findings to those in the present study with *N. brasiliensis* using an MOI of 3:1. Traditionally, it has been considered that the species belonging to the formerly *N. asteroides* complex are less virulent than *N. brasiliensis* since they mainly cause diseases in immunosuppressed patients; however, it seems that the innate defence mechanisms have a similar behaviour against both species.

Beaman and Smathers\(^{11}\) also noticed the limitations of quantification using viable counting since the floating clumps gave rise to one colony only. As we wanted to quantify the effect of the oxazolidinones on necardial growth, a respirometric assay was developed based on a previously published study for *Staphylococcus aureus*.\(^{13}\) Alamar Blue has the property to change the wavelength of fluorescence once it is reduced. When we quantified necardial growth using known quantities of a unicellular suspension the method could determine up to $1.8 \times 10^6$ cfu.

Actinomycetoma is a tumour-like lesion with multiple abscesses and fistulae. The aetiological agents are found as microcolonies, although smaller amounts are found as intracellular living microorganisms. There are few successful therapeutic...
Intracellular activity of oxazolidinones against \textit{N. brasiliensis}

![Graph showing Nocardia growth control, Linezolid, DA-7218, and DA-7157 concentrations](image)

**Figure 4.** Antimicrobial activities of linezolid, DA-7157 and DA-7218 against \textit{Nocardia brasiliensis} HUJEG-1 after 8 h of replication inside THP-1 macrophages. The measurements were made in triplicate; each point represents the mean of the assays and error bars represent the standard deviations. There were significant differences at all concentrations \((P = 0.003)\).

schemes to treat this condition. This may be due to the fact that, \textit{in vitro}, few antibiotics can achieve optimal tissue concentrations or lack the ability to kill both intracellular and extracellular bacteria.

Oxazolidinones have been effective \textit{in vitro}; DA-7867 seemed very promising since MIC values were lower than those of linezolid (0.03 mg/L compared with 2 mg/L for the isolate used in the animal experiments); yet, however, this potency was not observed in the murine model. In contrast, DA-7157 showed a better effect, inhibiting the production of extensive lesions, and most of the animals were completely cured. With linezolid we observed that DA-7157 is 4- to 8-fold more potent against \textit{S. aureus} \cite{15} it should be possible to use lower doses (200 mg) every 12 h in order to avoid or minimize side effects, whilst still maintaining its antibacterial activity. The excellent intracellular antimicrobial activity detected suggests that these compounds could be very effective in the treatment of actinomycetoma. However, more studies are needed both \textit{in vitro} and \textit{in vivo}, including clinical trials, to confirm this issue.

**Funding**

This research was performed with the support of the Department of Dermatology of the ‘Dr. José E. González’ University Hospital, Universidad Autónoma de Nuevo León. DA-7157 and DA-7218 were provided by S. H. Choi, of the Research Laboratory, Dong-A Pharmaceutical Co., Ltd, Yongin, and the College of Pharmacy, Sungkyunkwan University, Suwon, Korea.

**Transparency declarations**

None to declare.

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