Antimicrobial activities of benzoxazinorifamycin (KRM-1648) and clarithromycin against *Mycobacterium avium–intracellulare* complex within murine peritoneal macrophages, human macrophage-like cells and human alveolar epithelial cells

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Profiles of the in-vitro antimicrobial effects of KRM-1648 and clarithromycin against *Mycobacterium avium–intracellulare* complex (MAC) within murine peritoneal macrophages, the THP-1 human macrophage cell line and the A-549 type II alveolar epithelial cell line were determined. MAC organisms grew more rapidly in A-549 and THP-1 cells than in murine peritoneal macrophages. Peritoneal macrophages produced significant amounts of reactive nitrogen intermediates in response to MAC infection, but A-549 and THP-1 cells did not. KRM-1648 progressively killed *M. intracellulare* residing in macrophages, but did not completely eliminate *M. intracellulare* from A-549 and THP-1 cells. Moreover, in the case of *M. intracellulare*-infected A-549 and THP-1 cells, bacterial regrowth was observed during the middle to late phase of cultivation. Clarithromycin exhibited moderate levels of microbicidal activity against *M. intracellulare* residing in peritoneal macrophages, THP-1 cells and A-549 cells. The profiles of clarithromycin-mediated killing or inhibition of the intracellular organisms in A-549 and THP-1 cells were similar to those observed for organisms within peritoneal macrophages.

Introduction

Disseminated *Mycobacterium avium–intracellulare* complex (MAC) infections are frequently encountered in patients in the advanced stage of AIDS, and are usually intractable, partly because of the severely depressed host defence mechanisms in AIDS patients. Development of new drugs with strong anti-MAC activity is therefore urgently desired. We and others have found that both benzoxazinorifamycin (KRM-1648) and clarithromycin have strong in-vitro and in-vivo anti-MAC activities. KRM-1648 had much lower MICs against MAC than did clarithromycin, and the microbicidal activity of KRM-1648 against both MAC organisms growing extracellularly in a liquid medium and those growing intracellularly in murine peritoneal macrophages was much greater than that of clarithromycin. Nevertheless, the therapeutic efficacy of KRM-1648 against MAC infection in mice was not as good as expected based on its in-vitro anti-MAC activity. Therefore, the in-vitro activity of KRM-1648, as measured by its MICs and bactericidal effects against extracellular or intracellular MAC, is not satisfactorily predictive of the bacteriological response of KRM-1648-treated mice.

It has been reported that *Mycobacterium tuberculosis* and *M. avium* invade and replicate within A-549 cells (a human type II alveolar epithelial cell line), that the degree of invasiveness is in the order of virulence of the organisms and that intracellular multiplication of *M. tuberculosis* is much more vigorous in A-549 cells than in human monocytes. McDonough & Kress also suggested the possibility that *M. tuberculosis* gains access to the host lymphatic system and circulatory system by directly penetrating the alveolar epithelial lining of infected lung, and that non-professional phagocytes play important roles as sites of infection and multiplication of these mycobacteria. In this context, we have attempted to compare the behaviour of MAC in lung epithelial cells with that in macrophages, and have determined the efficacies with which KRM-1648 and clarithromycin kill the organisms within these cells. In this study, we examined the profiles of the growth of MAC within A-549 cells, the THP-1 human macrophage cell line, which has immature macrophage...
function, and murine peritoneal macrophages when cultured in medium in the presence or absence of either KRM-1648 or clarithromycin.

Materials and methods

Organisms

*M. avium* strain N-444 (which shows low virulence in mice) and *Mycobacterium intracellulare* strain N-260 (highly virulent in mice), both of which we isolated from patients with MAC infection, were cultured in 7H9 broth (Difco Laboratories, Detroit, MI, USA). Bacterial suspensions were prepared in phosphate-buffered saline (PBS) containing 0.1% (w/v) bovine serum albumin (BSA) and frozen at –80°C until use.

Mice

Female BALB/c mice were purchased from Japan Clea Co., Osaka, Japan.

Antimicrobial agents

KRM-1648 (Kaneka Corporation, Hyogo, Japan) and clarithromycin (Taisho Pharmaceutical Co., Tokyo, Japan), which were initially dissolved in dimethyl sulfoxide, were diluted in prescribed media before use. The maximum concentration of compound detected in lungs (C_{\text{max(lung)}}) in mice administered the drugs at oral doses equivalent to the clinical dosage was as follows: 0.8 mg/L for KRM-1648 when given at 2 mg/kg (personal communication; Dr T. Hidaka, Kaneka Corporation), and 7.0 mg/L for clarithromycin when given at 10 mg/kg.

MIC and MBC determination

MICs of test drugs were determined as reported previously, by either an agar dilution method using Middlebrook 7H11 medium or a broth dilution method using 7HSF medium (a broth medium with the same composition as 7H11 agar but without malachite green), as described by Yajuku et al. MBCs were determined as described previously. Briefly, after MIC determinations using 7HSF medium, MBCs were determined by inoculating 10 μL samples from wells in which test agents allowed no visible growth of the organisms, on to a 7H11 agar plate, followed by 14 day cultivation. MBCs were read as minimum concentrations of drugs causing >99.9% killing of the inoculated organisms.

Intracellular growth of organisms

Macrophage monolayer cultures were prepared by seeding 1 × 10^6 of Zymosan A-induced peritoneal exudate cells of 8- to 12-week-old BALB/c mice on 16 mm culture wells (Becton Dickinson & Co., Lincoln Park, NJ, USA). Monolayer cultures of A-549 cells (American Type Culture Collection, Rockville, MD, USA) and THP-1 cells (American Type Culture Collection) were prepared by seeding 1 × 10^5 or 2 × 10^5 and 3 × 10^5 or 4 × 10^5 of cultured cells, respectively, on 16 mm culture wells and the latter cells were pretreated with 20 ng/mL phorbol myristate acetate for 18 h before use to arrest their growth. These cells were then infected with 4 × 10^5 or 5 × 10^5 cfu/mL of test organisms in a 0.5 mL portion of Ham’s F-12K medium supplemented with 5% fetal bovine serum (FBS) (Bio Whittaker Co., Walkersville, MD, USA) at 37°C in a CO₂-95% humidified air for 2 h. After washing with 2% (v/v) FBS–Hanks’ balanced salt solution (HBSS) to remove extracellular organisms, the MAC-infected cells were cultured in 1.0 mL of 1% (v/v) FBS–Ham’s F-12K medium (5% FBS for peritoneal macrophages) in the presence or absence of test antimicrobials for up to 7 days. At intervals, the cells were lysed with 0.07% (w/v) sodium dodecyl sulphate followed by subsequent neutralization with 6% (w/v) BSA-PBS, and were then washed with distilled water by centrifugation (2000g for 30 min). The recovered organisms were resuspended in 1.0 mL of distilled water, and 10 μL portions of serial 10-fold dilutions were spotted on to 7H11 agar plates. The number of residual bacterial cfu was estimated by counting microcolonies, formed after cultivation for 5–7 days at 37°C in a CO₂ incubator, microscopically at × 15 magnification.

Reactive nitrogen intermediates production by MAC-infected cells

Culture fluids from MAC-infected cells were harvested and the NO₃ concentration in them was measured using Griess reagent, as an indicator of the extent of production of reactive nitrogen intermediates (RNIs) during cultivation, as described previously.

Statistical calculation

Statistical analysis was performed using Student’s t-test.

Results

Intracellular growth of MAC in macrophages, THP-1 cells and A-549 cells

Figure 1 shows the rate of bacterial growth of *M. avium* N-444 (low virulence) and *M. intracellulare* N-260 (high virulence) in peritoneal macrophages, THP-1 cells and A-549 cells. The growth rate of *M. intracellulare* N-260 was consistently higher than that of *M. avium* N-444 regardless of the type of cell in which the organisms were treated with 20 ng/mL phorbol myristate acetate (P < 0.01). The growth of both MAC organisms was much more vigorous in THP-1 and A-549 cells than in peritoneal macrophages.
KRM-1648 activity against MAC in lung epithelial cells

As shown in Figure 2a, peritoneal macrophages produced significant amounts of RNIs during cultivation after MAC infection. In this case, the peritoneal macrophages infected with *M. intracellulare* N-260 displayed higher levels of RNI release than did those infected with *M. avium* N-444. Both THP-1 and A-549 cells failed to produce appreciable amounts of RNIs in response to MAC infection (Figure 2, panels b and c). The cytotoxicity of the MAC organisms against peritoneal macrophages, THP-1 cells, and A-549 cells was examined in terms of lactate dehydrogenase release from infected cells; neither *M. avium* N-444 nor *M. intracellulare* N-260 had any cytotoxic effects on these cells (data not shown).

**Figure 2.** Production of reactive nitrogen intermediates by murine peritoneal macrophages (a), THP-1 cells (b) and A-549 cells (c) infected with *M. avium* N-444 (○) or *M. intracellulare* N-260 (●). The ordinate indicates the MAC infection-induced NO₂⁻ production by test cells, calculated as [NO₂⁻ accumulation by MAC-infected cells] – [NO₂⁻ accumulation by uninfected cells]. Each plot shows means ± S.E.M. (n = 3; S.E.M. bars were omitted when values were <0.2).

As shown in Figure 2a, peritoneal macrophages produced significant amounts of RNIs during cultivation after MAC infection. In this case, the peritoneal macrophages infected with *M. intracellulare* N-260 displayed higher levels of RNI release than did those infected with *M. avium* N-444. Both THP-1 and A-549 cells failed to produce appreciable amounts of RNIs in response to MAC infection (Figure 2, panels b and c). The cytotoxicity of the MAC organisms against peritoneal macrophages, THP-1 cells, and A-549 cells was examined in terms of lactate dehydrogenase release from infected cells; neither *M. avium* N-444 nor *M. intracellulare* N-260 had any cytotoxic effects on these cells (data not shown).

**Antimicrobial activity of KRM-1648 and clarithromycin against MAC within peritoneal macrophages, THP-1 cells and A-549 cells**

Figure 3 shows the effects of KRM-1648 0.8 mg/L against the intracellular growth of *M. intracellulare* N-260 residing in peritoneal macrophages, THP-1 cells and A-549 cells. The organisms in peritoneal macrophages were progressively killed by KRM-1648 during a 7 day cultivation (Figure 3a), but KRM-1648-mediated bacterial elimination was incomplete for *M. intracellulare* N-260 within THP-1 and A-549 cells (Figure 3b and c). In THP-1 and A-549 cells, rapid bacterial killing was observed during the first
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Figure 3. Microbicidal activity of KRM-1648 against *M. intracellulare* N-260 within peritoneal macrophages (a), THP-1 cells (b) and A-549 cells (c). *M. intracellulare*-infected cells were cultured in the presence (●) or absence (○) of KRM-1648 at 0.8 mg/L. Each plot shows means ± S.E.M. (n = 3). Asterisks indicate significant differences between the numbers of bacterial cfu recovered from KRM-1648-treated cells and untreated cells (⁎P < 0.05; ⁎⁎P < 0.01).

24 h of incubation, and progressive regrowth of the organisms occurred thereafter. This closely resembles the mode of behaviour of MAC organisms in the visceral organs (lungs and spleen) of infected mice treated with KRM-1648.8,19

We next examined changes in KRM-1648-susceptibility of *M. intracellulare* organisms before and after cultivation of infected THP-1 and A-549 cells in medium containing KRM-1648. In this experiment, MICs of KRM-1648 were measured by the agar dilution method using 7H11 medium.10 The MIC for the parent *M. intracellulare* N-260 strain was 0.1 mg/L. Ten colonies of each of these organisms were isolated from *M. intracellulare*-infected THP-1 and A-549 cells after 7 days' cultivation in medium containing KRM-1648 at the *C*max (0.8 mg/L); the MICs of KRM-1648 were found to be between 0.05 and 0.1 mg/L. The MIC for nine colonies recovered from THP-1 cells was 0.1 mg/L and that for one other colony was 0.05 mg/L. The MIC for eight colonies recovered from A-549 cells was 0.1 mg/L and that for the remaining two colonies was 0.05 mg/L. This indicates that *M. intracellulare*-resistant mutants were not generated from the bacterial populations residing in THP-1 or A-549 cells during cultivation periods of the infected cells in the medium containing KRM-1648 at 0.8 mg/L for at least 7 days.

Figure 4 shows the effects of clarithromycin at the *C*max (7 mg/L) against *M. intracellulare* in peritoneal macrophages, THP-1 cells or A-549 cells. Less dramatic effects were seen than those observed for the case of peritoneal macrophages with KRM-1648, and differences between the three cell lines were less marked. In this experiment, we did not observe the generation of clarithromycin-resistant mutants from *M. intracellulare* populations in THP-1 or A-549 cells during cultivation of these cells in the presence of clarithromycin at 7 mg/L. That is, when ten colonies of the organism were isolated from *M. intracellulare*-infected THP-1 cells or A-549 cells after 7 days' cultivation, the MIC of clarithromycin for all of these colonies was 6.25 mg/L. This value was identical to the MIC for the parent *M. intracellulare* N-260 strain.

Figure 5 shows the effects of KRM-1648 and clarithromycin added at either the MIC or the MBC, which were determined by the broth dilution method using 7HFS medium, against intracellular *M. intracellulare* N-260 in peritoneal macrophages or A-549 cells. KRM-1648 at the MBC (1 mg/L) progressively killed organisms residing in macrophages (Figure 5a), whereas progressive regrowth was observed from day 1 to day 7 in A549 cells (Figure 5b). KRM-1648 added at the MIC (0.06 mg/L) inhibited the growth of organisms in macrophages, while in A-549 cells it transiently eliminated organisms during the first 3 days of cultivation but bacterial regrowth occurred subsequently (Figure 5b). Clarithromycin added at the MIC (4 mg/L) or MBC (16 mg/L) caused progressive killing of *M. intracellulare* N-260 in macrophages (Figure 5c) as well as in A-549 cells (Figure 5d). Killing efficacy was saturated even at the MBC.

Discussion

Concerning the vigorous growth of MAC organisms in A-549 lung epithelial cells and THP-1 cells with immature macrophage function,14 the following can be stated. First, these cells lacked RNI-producing activity in response to stimulation by MAC infection, unlike mouse peritoneal macrophages, which produced significant amounts of RNIs.
KRM-1648 activity against MAC in lung epithelial cells

Thus, the unrestricted growth of MAC residing in A-549 and THP-1 cells could be in response to the failure of these cells to produce RNIs, antimicrobial effectors for expression of macrophage antimycobacterial activity. Second, peritoneal macrophages infected with highly virulent *M. intracellulare* N-260 released larger amounts of RNIs than did macrophages infected with the less virulent *M. avium* N-444. This suggests that RNIs do not play crucial roles in manifestation of the macrophage antimycobacterial activity against MAC and require collaboration with other kinds of microbicidal effector molecules, including free fatty acids and cationic antimicrobial peptides.

Another interesting finding of the present study is that *M. intracellulare* organisms within A-549 and THP-1 cells were more resistant to KRM-1648-mediated bactericidal effects than were those in peritoneal macrophages (Figure 3). This is consistent with the finding by Mehta *et al.* of amikacin-mediated killing of *M. tuberculosis* in A-549 cells and in macrophages. Notably, *M. intracellulare* in A-549 and THP-1 cells was not completely inhibited even by extracellular concentrations (0.8–1 mg/L) of KRM-1648 that were more than ten-fold the MIC value (0.06 mg/L). This profile is very different from that of KRM-1648-mediated killing of *M. intracellulare* within peritoneal macrophages.

(Figure 2). Thus, the unrestricted growth of MAC residing in A-549 and THP-1 cells could be in response to the failure of these cells to produce RNIs, antimicrobial effectors for expression of macrophage antimycobacterial activity. Second, peritoneal macrophages infected with highly virulent *M. intracellulare* N-260 released larger amounts of RNIs than did macrophages infected with the less virulent *M. avium* N-444. This suggests that RNIs do not play crucial roles in manifestation of the macrophage antimycobacterial activity against MAC and require collaboration with other kinds of microbicidal effector molecules, including free fatty acids and cationic antimicrobial peptides.

(Figure 4). Microbicidal activity of clarithromycin against *M. intracellulare* N-260 within peritoneal macrophages (a), THP-1 cells (b) and A-549 cells (c). *M. intracellulare*-infected cells were cultured in the presence (●) or absence (○) of clarithromycin at 7.0 mg/L. Asterisks indicate significant differences between the numbers of bacterial cfu recovered from clarithromycin-treated cells and untreated cells (*P < 0.05; **P < 0.01). Other details are the same as for Figure 3.

(Figure 5). Microbicidal activity of KRM-1648 (a, b) and clarithromycin (c, d) against *M. intracellulare* N-260 within peritoneal macrophages (a, c) or A-549 cells (b, d). *M. intracellulare*-infected cells were cultured in the presence (●, △) or absence (○) of the indicated antimicrobial agents at the MIC (●) (KRM-1648, 0.06 mg/L; clarithromycin, 4 mg/L) or MBC (△) (KRM-1648, 1 mg/L; clarithromycin, 16 mg/L). MICs and MBCs were determined by the broth dilution method using 7HSF medium. Asterisks indicate significant differences between the numbers of bacterial cfu recovered from drug-treated cells and untreated cells (*P < 0.05; **P < 0.01). Other details are the same as for Figure 3.
macrophages. As proposed by Frehel et al.,23 the ineffec-
tiveness of KRM-1648 against \textit{M. intracellulare} in A-549 and
THP-1 cells may have been partly caused by limited
access of the drug to organisms as immature phagosomes in
which virulent MAC pathogens reside and lysosomes in
which KRM-1648 may accumulate, may not fuse with each
other. Although the results obtained in this study pertain to
a particular \textit{M. intracellulare} N-260 strain, they indicate
that it is important to consider the roles of alveolar epithe-
lial cells as sites of bacterial invasion and multiplication in
the lungs of MAC patients who are receiving chemothera-
peutic regimens that include KRM-1648 treatment.

On the other hand, clarithromycin displayed moderate
levels of antimicrobial activity against \textit{M. intracellulare}
residing in peritoneal macrophages, THP-1 cells or A-549
cells. Profiles of clarithromycin-mediated killing and
inhibition of \textit{M. intracellulare} within these three types of
cells did not differ from each other markedly (Figure 4).
Since clarithromycin was effective against \textit{M. intracellulare}
in A-549 cells as well as the organisms in peritoneal macro-
phages, it appears that the efficacy of clarithromycin
delivery to \textit{M. intracellulare} growing in A-549 cells are
nearly the same as those in macrophages.

Recently, Bermudez et al.24 have reported that MAC
organisms which are adapted to the intracellular environ-
ment of macrophages invade macrophages by complement
receptor- or mannose receptor-independent pathways,
unlike MAC growing extracellularly, and that such organ-
isms resist tumour necrosis factor-\(\alpha\)-mediated microbicidal
activity of host macrophages. Further studies of the effica-
cies of these drugs against MAC in macrophages and A-549
cells are currently under way using organisms growing
intracellularly in macrophages as an inoculum for infection.

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