Antimicrobial activity of picolinic acid against extracellular and intracellular *Mycobacterium avium* complex and its combined activity with clarithromycin, rifampicin and fluoroquinolones

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**Objectives:** A natural metal ion chelator, picolinic acid (PA), is known to potentiate macrophage antimycobacterial activity. Here, we studied the antimicrobial activity of PA against extracellular and intramacrophage *Mycobacterium avium* complex (MAC) organisms.

**Methods:** MAC organisms, MAC-infected macrophages or MAC-infected type II pneumocytes were cultured in the presence or absence of PA with or without antimycobacterial drugs, and residual bacterial cfu of extracellular or intracellular MAC were counted on 7H11 agar plates.

**Results:** First, PA exhibited antimicrobial activity against extracellular and intramacrophage MAC. The effect of PA was mimicked by other metal ion-chelating agents, such as ethylenediaminetetraacetic acid and \(O_2O'_0\)-bis (2-aminophenyl) ethyleneglycol-\(N_2N_2N_2N_0\)-tetraacetic acid. Second, PA potentiated antimicrobial effects of a two-drug combination of clarithromycin/rifampicin and some fluoroquinolones (levofloxacin, sitafloxacin and gatifloxacin) against extracellular and intramacrophage MAC. Similar combined effects of PA with clarithromycin/rifampicin were also seen in the case of MAC residing within type II alveolar epithelial cells.

**Conclusions:** PA exerted an appreciable anti-MAC activity, when used singly or in combination with some antimycobacterial drugs (clarithromycin/rifampicin and fluoroquinolones), suggesting the usefulness of PA as an adjunct for clinical antimicrobial chemotherapy of MAC infections.

Keywords: macrophages, chelating agents, immunoadjuvant therapy, immunomodulators

**Introduction**

Clinical management of *Mycobacterium avium* complex (MAC) infection is difficult, since it is frequently encountered in immunocompromised hosts, particularly AIDS patients, and MAC organisms are moderately to highly resistant to common antituberculosis drugs such as isoniazid, ethambutol, pyrazinamide and rifampicin.¹ Some macrolides, such as clarithromycin and azithromycin, in combination with rifamycin derivatives (rifampicin and rifabutin) or other drugs are appreciably efficacious in treating disseminated MAC infections in AIDS patients. Despite this, MAC pulmonary infections are generally intractable owing to a lack of anti-MAC therapeutics (and their companion drugs) with excellent in vivo activity.¹⁻³ The urgent problems are that therapeutic efficacies of present multidrug regimens of patients with MAC infection, in terms of sputum conversion and clinical improvement of patients and the relapse rate after combined chemotherapy, are at unsatisfactory levels.⁴⁻⁶ Since the development of new classes of anti-MAC drugs is unpromising at present, the most practical strategy is to develop regimens to treat patients with refractory MAC diseases using ordinary antimycobacterial drugs in combination with adjunctive immunomodulators (called adjunctive immunotherapy).⁷

For this purpose, development of new classes of immunomodulators other than immunopotentiating cytokines [interferon-\(\gamma\) (IFN-\(\gamma\)), interleukin-12, etc.], particularly those with no severe adverse effects and low cost, is desired. Such types of immune response modifiers, which appear to be useful in potentiating host resistance to mycobacterial infections, include ATP and its...
analogues, imidazoquinoline, diethyldithiocarbamate, poloxamer, dibenzopyran, 10a,25-dihydroxvitamin D3, glucosaminyl muramyldipeptide, and heat-killed Mycobacterium vaccae.7

Picolinic acid (PA), a naturally occurring product of degradation of tryptophan, is a cheap and safe chelating agent for metal ions, such as Zn2+ and Fe2+ ions, and chromium picolinate is generally used by overweight or obese persons as a dietary supplement that may promote weight loss.8,9 PA is also known to up-regulate host immune responses, especially macrophage cell functions.10,11 It has recently been demonstrated by Pais and Appelberg12 that PA reduced intramacrophage growth of MAC organisms. Notably, PA in combination with IFN-γ completely inhibits mycobacterial growth in macrophages, and this effect of PA is dependent on host macrophage apoptosis but independent of macrophage activity in producing reactive nitrogen intermediates (RNI), reactive oxygen intermediates (ROI) and tumour necrosis factor-α (TNF-α).12 In this context, it has also been reported that PA is endowed with a regulatory effect on the cell cycle and inhibitory effects on bacterial growth and tumour cell growth.13-15 Therefore, it is possible that PA-mediated intracellular bacterial killing in MAC-infected macrophages12 may be in part owing to antimicrobial activity of PA against MAC organisms. In this study, we thus examined the antimicrobial activity of PA against extracellular MAC and found that it actually exerted significant levels of growth inhibitory activity against MAC. We also studied profiles of the antimicrobial activity of PA against intracellular MAC residing within macrophages (professional phagocytes) and type II pneumocytes (non-professional phagocytes), in particular, in terms of combined effects of PA with antimycobacterial drugs, including clarithromycin, rifampicin and fluoroquinolones.

Materials and methods

Microorganisms

MAC N-444 (serovar 8) and MAC N-260 (serovar 16), which were isolated from patients with MAC infection in Japan and identified as M. avium and Mycobacterium intracellulare, respectively, by DNA probe testing, were used. For preparation of a bacterial suspension as an inoculum, test organisms were grown in 7H9 broth (Difco Laboratories, Detroit, MI, USA) at 37°C. Bacterial suspensions prepared with PBS containing 1% BSA were gently sonicated using a sonicator (Model UR-20P; Tomy Seiko Co., Tokyo, Japan) for 20 min and the recovered microorganisms were then washed with distilled water by centrifugation. The resulting bacterial pellet was suspended in 0.25 mL of distilled water and the number of cfu of recovered organisms was counted on 7H11 agar plates.

Intracellular growth of MAC

The intracellular growth of MAC organisms inside mouse peritoneal macrophages, THP-1 human macrophages (ATCC, Rockville, MD, USA), and A-549 human type II alveolar epithelial cells (A-549 cells) (ATCC) was measured by the following experimental systems.16,17

Mouse macrophage system. Zymosan A-induced murine peritoneal exudate cells (3 × 107), suspended in 0.2 mL of 5% FBS-RPMI 1640 medium (RPMI medium), were seeded in a microculture well (96-well flat-bottom plate; Becton Dickinson & Company, Lincoln Park, NJ, USA) and incubated at 37°C in a CO2 incubator (5% CO2/95% humidified air) for 2 h. After washing with 2% FBS-Hanks’ balanced salt solution (HBSS), the resultant macrophage monolayer was infected with test MAC organisms by incubating it in the medium (100 μL) containing 6 × 106 cfu of bacteria in a CO2 incubator for 1.5 h. Infected macrophages were then washed with 2% FBS-HBSS and then cultivated in 0.2 mL of 5% FBS-RPMI medium in the presence or the absence of test drugs in a CO2 incubator for up to 7 days. At intervals cultured macrophages were lysed with 0.07% SDS for 10 min. After neutralization of SDS with 6% BSA, the resultant macrophage cell lysate was centrifuged at 2000 g for 20 min to collect bacterial cells and the number of cfu of recovered organisms was counted on 7H11 agar plates.

THP-1 macrophage system. THP-1 mononcytic cells (5 × 104 cells) were cultured in 5% FBS-RPMI medium containing 50 ng/mL PMA in a microculture well at 37°C in a CO2 incubator for 18–22 h, in order to differentiate the mononcytic cells to mature macrophages. After washing with 2% FBS-HBSS, the resultant macrophage monolayer was infected with test MAC organisms by incubating it in the medium (0.1 mL) containing 1 × 106 cfu of bacteria in a CO2 incubator for 1.5 h. After rinsing with 2% FBS-HBSS, infected macrophages were cultured in 0.2 mL of 5% FBS-RPMI medium in the presence or the absence of test drugs in a CO2 incubator for up to 7 days. At intervals the number of residual bacterial cfu in cultured macrophages was counted as mentioned for the mouse macrophage system.

A-549 cell system. A-549 cells (4 × 104 cells) suspended in 5% FBS-RPMI medium were seeded in 96-well tissue culture plates and incubated for 18 h in order to allow attachment to the wells.
Significant bactericidal activity (counting was done on 7H11 agar plates. Each bar indicates the mean mentioned above.

After rinsing with 2% FBS-HBSS, the infected cells were cultured in MAC N-444 (high-virulence MAC). As shown in Figure 1(b), the antimicrobial activity of PA against extracellular MAC N-444 and HIDA (a Fe²⁺-specific iron chelator) exerted a weak anti-MAC action. Notably both EDTA and BAPTA exerted bactericidal activity against MAC. A chelator for Fe³⁺, Fe⁴⁺ and Ca²⁺ ions, BAPTA, exhibited the most potent inhibitory action. This is consistent with previous finding by Gomes et al. that some iron chelating agents, such as desferrioxamine and N,N’bis(2-hydroxybenzyl) ethylenediamine-N₂N’-diacetic acid were active in inhibiting extracellular and intramacrophage growth of MAC. Moreover, the addition of 2 mM FeSO₄ to 7H1SF medium caused partial but significant restoration of PA-mediated inhibition of extracellular MAC N-444 (Figure 2b). Therefore, it appears that the activity of PA in inhibiting killing extracellular MAC is principally attributable to its iron-chelating function, which causes the depletion of nutrient iron essential for bacterial growth. These findings indicate that the potentiation of anti-MAC antimicrobial function of macrophages by treatment with PA previously observed by Pais and Appelberg is at least partly owing to the direct antimicrobial action of PA against MAC organisms through depriving the bacteria of available nutrient iron.

As shown in Figure 3, PA in combination with clarithromycin/rifampicin exhibited significant combined effects against extracellular MAC N-444 and N-260 strains. However, moderate combined effects were achieved by using PA in combination with levofloxacin in both the cases of MAC N-444 and N-260 strains. Figure 4 shows the antimicrobial effects of PA and other Fe-chelating agents (EDTA, BAPTA and HIDA) in combination with antimycobacterial drugs (clarithromycin/rifampicin, levofloxacin) against extracellular MAC N-444. All the metal but significant growth inhibition of MAC N-260. Table 1 shows MICs (in terms of μM and mg/L concentrations) of PA, clarithromycin, rifampicin and fluoroquinolones for MAC N-444 and N-260 strains measured by the broth dilution method using 7H1SF medium. The MIC of PA for MAC N-444 was smaller than that for MAC N-260, confirming that the low-virulence MAC N-444 strain is more susceptible to PA compared with the high-virulence MAC N-260 strain. Although the MICs of PA for the MAC strains were high (25–50 mM), these concentrations of PA are known not to cause severe cytotoxic effects, as described in the Discussion section.

Since PA chelates metal ions, such as Zn²⁺ and Fe²⁺, it is likely that its antimicrobial activity against MAC organisms is owing to its ability to chelate metal ions, especially Fe ions which are an essential nutrient for bacteria. As shown in Figure 2(a), not only PA but also other kinds of metal ion chelators, including EDTA (a chelator for Ca²⁺, Mg²⁺, Fe²⁺, etc.) and BAPTA (a chelator for Fe²⁺, Fe³⁺ and Ca²⁺), had antimicrobial activity against extracellular MAC N-444 and HIDA (a Fe²⁺-specific iron chelator) exerted a weak anti-MAC action. Notably both EDTA and BAPTA exerted bactericidal activity against MAC. A chelator for Fe³⁺, Fe⁴⁺ and Ca²⁺ ions, BAPTA, exhibited the most potent inhibitory action. This is consistent with previous finding by Gomes et al. that some iron chelating agents, such as desferrioxamine and N,N’bis(2-hydroxybenzyl) ethylenediamine-N₂N’-diacetic acid were active in inhibiting extracellular and intramacrophage growth of MAC. Moreover, the addition of 2 mM FeSO₄ to 7H1SF medium caused partial but significant restoration of PA-mediated inhibition of extracellular MAC N-444 (Figure 2b). Therefore, it appears that the activity of PA in inhibiting killing extracellular MAC is principally attributable to its iron-chelating function, which causes the depletion of nutrient iron essential for bacterial growth. These findings indicate that the potentiation of anti-MAC antimicrobial function of macrophages by treatment with PA previously observed by Pais and Appelberg is at least partly owing to the direct antimicrobial action of PA against MAC organisms through depriving the bacteria of available nutrient iron.

Results

Antimicrobial activity of PA against extracellular MAC

PA was examined for its antimicrobial activity against extracellular MAC N-444 (low-virulence MAC) and N-260 strain (high-virulence MAC). As shown in Figure 1, PA caused growth inhibition of both MAC strains in a dose-dependent manner. PA at 20 mM completely inhibited bacterial growth of MAC N-444 and, moreover, exerted a weak bactericidal effect (Figure 1a). However, as indicated in Figure 1(b), the antimicrobial activity of PA against MAC N-260 was significantly weaker than its activity against MAC N-444 (P < 0.01). PA even at 20 mM caused only partial 87

### Table 1. MICs of test antimicrobials and PA for MAC N-444 and MAC N-260 strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>CLR</th>
<th>RIF</th>
<th>LVX</th>
<th>STX</th>
<th>GAT</th>
<th>PA</th>
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<tr>
<td></td>
<td>MIC (μM)</td>
<td></td>
<td></td>
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<tr>
<td>N-444</td>
<td>5.4 (4)</td>
<td>9.7 (8)</td>
<td>22 (16)</td>
<td>4.9 (2)</td>
<td>11 (4)</td>
<td>25 × 10⁴ (3 × 10⁴)</td>
</tr>
<tr>
<td>N-260</td>
<td>5.4 (4)</td>
<td>9.7 (8)</td>
<td>22 (16)</td>
<td>9.8 (4)</td>
<td>21 (8)</td>
<td>50 × 10⁴ (6 × 10⁴)</td>
</tr>
</tbody>
</table>

CLR, clarithromycin; RIF, rifampicin; LVX, levofloxacin; STX, sitafloxacin; GAT, gatifloxacin.

In parentheses, MICs are indicated in terms of mg/L.
ion-chelating agents exhibited significant combined effects with clarithromycin/rifampicin (Figure 4a). Notably, the most obvious combined effect was seen between BAPTA and clarithromycin/rifampicin, followed by EDTA and clarithromycin/rifampicin. This finding indicates that all the test agents with Fe-chelating ability (PA, EDTA, BAPTA and HIDA) are capable of exerting potently increased antimicrobial activity against extracellular MAC organisms when used in combination with clarithromycin/rifampicin. However, somewhat weak but significant levels of combined effects were seen for combinations of levofloxacin with the test chelating agents (Figure 4b). In this experiment, the most obvious combined effect was seen between EDTA and levofloxacin, but BAPTA showed no combined effect when used with levofloxacin. Notably, in most cases, test chelating agents having Fe-chelating ability potentiated the anti-MAC antimicrobial activities of clarithromycin/rifampicin and levofloxacin when used in combination with the antitymocobacterial drugs.

Antimicrobial activity of PA, used singly or in combination with some antitymocobacterial drugs, against extracellular MAC

We next examined the antimicrobial activity of PA and its effects on the expression of antimicrobial activities of some antitymocobacterial drugs against intramacrophage MAC. As shown in Figure 5(a), PA caused growth inhibition of MAC N-444 residing in THP-1 human macrophages in a dose-dependent manner. Notably, as shown in Figure 5(b), PA activity against intramacrophage MAC N-444 was partly blocked by the addition of holo-transferrin (iron-saturated transferrin) at 1 mg/mL concentration. In separate experiments, apo-transferrin at 1 mg/mL inhibited intramacrophage growth of MAC N-444 by 58 \pm 1\% (n = 4) and exhibited significant combined effects with PA against the intramacrophage MAC organisms (P < 0.01): PA alone, 76 \pm 1\% inhibition; PA + apo-transferrin, 86 \pm 2\% (H. Tomioka, S. Cai, K. Sato and T. Shimizu, unpublished results). It thus appears that holo-transferrin, added in culture medium, is efficiently delivered to and accumulated in macrophage phagosomal vesicles engulfing mycobacterial organisms, as reported by Clemens and Horwitz, and subsequently supplies sufficient amounts of essential nutrient iron to MAC organisms replicating inside macrophage phagosomal vesicles. In contrast, it is thought that apo-transferrin molecules delivered to macrophage phagosomal vesicles cause moderate levels of iron deprivation for intraphagosomal MAC organisms and that PA further potentiates this effect.

Next, we examined the antimicrobial activity of PA against intramacrophage MAC, when used in combination with some antitymocobacterial drugs at concentrations achievable by
oral administration of them at clinical dosages. As illustrated in Figure 6, PA potentiated the bactericidal activity of clarithromycin/rifampicin at clinical dosages against intramacrophage MAC. Next, we examined the effects of PA on the expression of antimicrobial activities of some fluoroquinolones, added at clinical concentrations, against intramacrophage MAC. As shown in Figure 7, PA significantly increased the growth inhibitory activity of levofloxacin and sitafloxacin against intracellular MAC replicating within THP-1 macrophages, although fluoroquinolones are generally known to be weakly efficacious in chemotherapy of MAC infections in humans and in animals.21–23 In this case, these quinolones in combination with PA failed to exhibit microbicidal effects on MAC organisms replicating within THP-1 macrophages. Notably, these quinolones were more or less endowed with bactericidal activity against intramacrophage MAC by combination with PA.

Figure 4. Antimicrobial activity of PA and other metal ion-chelating agents, used singly or in combination with clarithromycin/rifampicin (a) or levofloxacin (b), against extracellular MAC. MAC N-444 strain was cultured in 7H9F medium with or without addition of 20 mM PA or 10 mM of other metal ion-chelating agents (EDTA, BAPTA, HIDA), clarithromycin (CLR)/rifampicin (RIF) (1/4 C_{max} each: CLR, 0.58 mg/L; RIF, 1.6 mg/L) or levofloxacin (LVX) (1/2 C_{max}, 1.0 mg/L) alone or in combination for 4 days. *Significant combined effect (P < 0.01). The other details are the same as in Figure 1.
Antimicrobial activity of PA, used singly or in combination with clarithromycin/rifampicin, against intracellular MAC residing inside type II pneumocytes

It is known that type II alveolar epithelial cells play roles as sites of bacterial entry and growth in lung infections owing to mycobacterial pathogens. Thus, we examined the antimicrobial activity of PA and its combined effects with clarithromycin/rifampicin against intracellular MAC residing within A-549 cells. As shown in Figure 9(a), PA at 20 mM concentration exhibited bactericidal activity against MAC residing inside A-549 cells. Moreover, PA significantly potentiated the antimicrobial activity of C\text{max} concentrations of clarithromycin/rifampicin against intracellular MAC replicating within A-549 cells (Figure 9b). Since A-549 cells are lacking in effective antimicrobial functions against MAC organisms, it appears that the observed activity of PA against intracellular MAC inside A-549 cells is mainly owing to the direct antimicrobial activity of PA itself.

Discussion

In the present study, it was found that PA exhibited direct antimicrobial activity against extracellular and intramacrophage MAC organisms presumably by depriving intraphagosomal microenvironment of free nutrient iron essential for the intraphagosomal growth of the parasites (Figures 2a, b and 5b). This is consistent with the finding by Pais and Appelberg that the mechanisms of PA-mediated augmentation of macrophage anti-MAC activity do not involve ROIs and RNIs that are known as the major antimicrobial effector molecules produced by macrophages. It thus appears that the activity of PA in potentiating macrophage anti-MAC activity is largely attributable to its direct antimicrobial activity against MAC organisms. In this context, it has been reported that an excess of iron hampers the Nramp-1-encoded function of host macrophages in exerting antimicrobial activity against intraphagosomal MAC organisms, indicating the function...
of \textit{Nramp-1}-encoded protein in the transport of iron out of the parasite-harbouring macrophage phagosomes.29

As indicated in Figure 8(a), PA exhibited significant combined activity against intramacrophage MAC when used in combination with levofloxacin (LVX) (a), sitafloxacin (STX) (b) or gatifloxacin (GAT) (c) alone or in combination for 4 days. *Significantly smaller than the control value ($P < 0.05$). The other details are the same as in Figure 5.

In this context, recent studies have suggested a role for tryptophan catabolite PA as an important regulator of macrophage activities. It has been reported that PA is a selective inducer of macrophage inflammatory protein-1\textalpha{} and -1\textbeta{}.30 Moreover, it has been indicated that PA exhibited protective effects on mice infected with \textit{Candida albicans}, by increasing the expression of TNF-$\alpha{}$ and interleukin-1 at sites of infection, presumably owing to activation of host macrophages.31 It thus appears that PA may potentiate macrophage antimicrobial activity by up-regulating macrophage production of antimicrobial effector molecules other than ROIs and RNIs. In such macrophage antimicrobial functions, participation of free fatty acids (such as arachidonic acid) and cationic antimicrobial peptides (such as \alpha{},\beta{}-defensins and protegrin-like peptides)32–35 seems to be critical.

The mechanisms, by which PA potentiates the antimicrobial activities of clarithromycin/rifampicin against intramacrophage MAC, may be essentially different from each other. That is, the antimicrobial action of PA owing to its Fe-chelating activity appears to play central roles in the potentiation of the antimicrobial activity of clarithromycin/rifampicin against intramacrophage MAC. On the contrary, the macrophage-activating effects of PA seem to play the major role in the augmentation of the antimicrobial activity of levofloxacin against MAC residing within macrophages.

In this context, recent studies have suggested a role for tryptophan catabolite PA as an important regulator of macrophage activities. It has been reported that PA is a selective inducer of macrophage inflammatory protein-1\textalpha{} and -1\textbeta{}.30 Moreover, it has been indicated that PA exhibited protective effects on mice infected with \textit{Candida albicans}, by increasing the expression of TNF-$\alpha{}$ and interleukin-1 at sites of infection, presumably owing to activation of host macrophages.31 It thus appears that PA may potentiate macrophage antimicrobial activity by up-regulating macrophage production of antimicrobial effector molecules other than ROIs and RNIs. In such macrophage antimicrobial functions, participation of free fatty acids (such as arachidonic acid) and cationic antimicrobial peptides (such as \alpha{},\beta{}-defensins and protegrin-like peptides)32–35 seems to be critical.

Since the development of new classes of anti-MAC drugs is unpromising at present, the most practical strategy is to develop adjunctive drugs for current chemotherapeutic regimens.7 In this context, PA is a cheap and safe metal ion-chelating agent and facilitates absorption of Zn$^{2+}$ and chromium ions from the intestine when orally administered, and chromium picolinate is generally
used by overweight or obese persons as a dietary supplement for weight loss and muscle building.\(^8,9\) It has been reported that intraperitoneal administration of PA to rats at a dose of 30 mmol (presumably yielding \(\sim 200-400\) mM concentrations in the body fluids) caused no marked histological and ultrastructural changes in the brain.\(^30\) This may mean that the doses of PA (~20 mM) used here for macrophage studies do not cause severe toxic effects in vivo.

In the present study, it was found that PA significantly potentiated the antimicrobial activities of clarithromycin/rifampicin and fluoroquinolones (levofloxacin, sitafloxacin and gatifloxacin) against intramacrophage MAC when used in combination with these antimicrobials. This finding suggests that PA may be useful as an adjunct of antimicrobial chemotherapy of MAC patients with antitubercular drugs. It is of interest to note that PA exerted significant levels of combined activity against intracellular MAC residing inside macrophages and lung epithelial cells when added to the medium in combination with fluoroquinolones. This finding suggests possible usefulness of fluoroquinolones for chemotherapy of MAC infections when administered in combination with PA as an adjunctive agent. This is meaningful from the viewpoint of the clinical efficacy when used as monotherapy, and are generally only weak activity against MAC organisms, have no significant antimycobacterial activity. This is meaningful from the viewpoint of the combined activity against intracellular MAC as an adjunct of antimicrobial chemotherapy of MAC patients with fluoroquinolones (levofloxacin, sitafloxacin and gatifloxacin) that has recently been reported that moxifloxacin is efficacious in treating MAC-infected mice.\(^38\)

In any case, the present study gave us the following important and promising findings: PA potentiates antimicrobial activities of clarithromycin/rifampicin against both intracellular MAC and intracellular MAC residing within macrophages and lung epithelial cells. Further studies are currently under way using in vivo experimental systems.

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Transparency declarations

No declarations were made by the authors of this paper.

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