Azithromycin blocks autophagy and may predispose cystic fibrosis patients to mycobacterial infection

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Azithromycin is a potent macrolide antibiotic with poorly understood antiinflammatory properties. Long-term use of azithromycin in patients with chronic inflammatory lung diseases, such as cystic fibrosis (CF), results in improved outcomes. Paradoxically, a recent study reported that azithromycin use in patients with CF is associated with increased infection with nontuberculous mycobacteria (NTM). Here, we confirm that long-term azithromycin use by adults with CF is associated with the development of infection with NTM, particularly the multi-drug-resistant species Mycobacterium abscessus, and identify an underlying mechanism.

We found that in primary human macrophages, concentrations of azithromycin achieved during therapeutic dosing blocked autophagosome clearance by preventing lysosomal acidification, thereby impairing autophagic and phagosomal degradation. As a consequence, azithromycin treatment inhibited intracellular killing of mycobacteria within macrophages and resulted in chronic infection with NTM in mice. Our findings emphasize the essential role for autophagy in the host response to infection with NTM, reveal why chronic use of azithromycin may predispose to mycobacterial disease, and highlight the dangers of inadvertent pharmacological blockade of autophagy in patients at risk of infection with drug-resistant pathogens.

Introduction
Azithromycin is a potent antibiotic and is frequently used in prophylaxis and treatment regimens for mycobacterial infections (1). Long-term use of azithromycin and other macrolide antibiotics has increased as a result of reports of improved clinical outcomes in patients with cystic fibrosis (CF) (2), chronic obstructive pulmonary disease (3), panbronchiolitis (4), asthma (5), and chronic transplant rejection (6). These benefits are believed to result from pharmacological properties and may predispose cystic fibrosis patients to mycobacterial infection (1).

Worryingly, several studies have identified a synchronous increase in mycobacterial infection of CF patients, predominantly with the multi-drug-resistant, highly pathogenic nontuberculous mycobacteria (NTM) Mycobacterium abscessus (8, 9), for which there is no reliable treatment (1). We therefore wondered whether azithromycin paradoxically impairs host immunity against mycobacteria. Macroautophagy (referred to herein as autophagy) appears crucial for an effective cellular response against mycobacteria (10). Since the macrolide bafilomycin disrupts autophagy in vitro (11), we examined whether azithromycin blocks autophagy and thereby impairs intracellular killing of NTM. Here we present clinical data suggestive of an association between long-term azithromycin treatment and development of NTM infection in CF patients. At concentrations achieved during therapeutic dosing, azithromycin impaired lysosomal degradation of both autophagosomes and phagosomes and led to failure of intracellular killing of mycobacteria and development of chronic infection with M. abscessus in mouse models.

Results
Long-term azithromycin use may predispose CF patients to NTM infection. Escalating azithromycin use over the last 5 years has mirrored an increase in patients colonized or infected with NTM in our adult CF center (Figure 1A), not explicable by changes in sputum sampling (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI46095DS1) or microbial culture methods. Long-term azithromycin use was associated with developing infection with NTM, particularly M. abscessus (P = 0.0009, 2-tailed uncorrected χ2; Figure 1B). When adjusted for age—which was identified, as in previous studies (9), as a significant covariate (P = 0.01; Supplemental Table 2) —azithromycin use remained significantly associated with NTM disease (P = 0.00046; odds ratio 9.8, 95% CI 2.09–45.87). Similar associations of chronic azithromycin use with NTM infection have been reported for an Israeli CF cohort (12), which suggests that azithromycin might impair host immunity against mycobacteria.

Azithromycin blocks autophagosome degradation. Since autophagy appears crucial for an effective cellular response against mycobacteria (10), we investigated whether azithromycin blocks autophagosome degradation by using primary human THP-1 macrophages transiently transfected with a FLAG-tagged fusion protein of LC3 and GFP-labeled autophagosomes, as well as with a marker of autophagosome-lysosome fusion. Here we show that therapeutic concentrations of azithromycin inhibited degradation of lysosomal cargos in macrophages infected with M. abscessus.
Autophagy is routinely assayed by quantifying autophagosomes, achieved either by measuring levels of LC3-II, a protein that specifically associates with autophagosomes, or by detection of LC3-II+ vesicles (13). In cell lines, azithromycin caused a dose-dependent increase in both the number of LC3-II+ vesicles and the amount of LC3-II protein (EC$_{50}$, 4.5 μg/ml; Figure 1, C and D). This suggests an accumulation of autophagosomes, which could result from either their increased synthesis or their decreased degradation. We therefore examined the effect of azithromycin in the presence of the lysosomal inhibitor bafilomycin A$_1$ (BafA$_1$), which blocks LC3-II destruction (11). Cotreatment of azithromycin and BafA$_1$ resulted in no significant increase in LC3-II levels compared with BafA$_1$ alone (Figure 1E), which indicates that azithromycin does not induce autophagosome synthesis, but rather blocks LC3-II degradation. Moreover, the azithromycin-induced rise in LC3-II was not accompanied by decreases in mTOR-dependent signaling (monitored by phosphorylation of S6 and p70S6 kinase); however, it did increase p62 levels. (G) Azithromycin dose-dependently increased LC3-II and p62 levels in primary macrophages. (H) Similar effect of azithromycin, assessed by LC3-II and p62 levels, in primary macrophages derived from clinically stable CF patients and healthy controls (n = 3 per group). *P < 0.05; **P < 0.005.
elevations in the endogenous autophagy substrate p62 (ref. 15 and Figure 1, F and G). Azithromycin treatment in vitro resulted in accumulation of LC3-II and p62 in primary macrophages from CF patients similar to that in cells from healthy controls (Figure 1H and Supplemental Figure 1).

To confirm that azithromycin blocks autophagy-mediated degradation at, or below, concentrations achieved in clinical practice (between 12 μg/ml and 53 μg/ml in sputum, but several hundred-fold higher in phagocytic cells; refs. 7, 16), we examined the clearance of 2 known autophagy substrates: mutant (Q74) huntingtin (17) and A53T α-synuclein (18). Azithromycin treatment retarded the basal and rapamycin-enhanced degradation of A53T α-synuclein and led to enhanced accumulation of intracellular aggregates of Q74 huntingtin (Figure 2, A and B).

We next examined whether inhibition of LC3-II clearance by azithromycin was caused by failure of autophagosome-lysosome fusion, as seen with long-term treatment with BafA1; treatment (11), or failure of lysosomal function, a mechanism we recently reported (19). Confocal microscopy of HeLa cells coexpressing mCherry-LC3 and the lysosomal marker lgp120 revealed that although the number of LC3+ vesicles per cell increased with rapamycin, BafA1, or azithromycin treatment, the degree of LC3 colocalization with lgp120 was not significantly affected by azithromycin, but was, as expected, enhanced by rapamycin and inhibited by BafA1 (Figure 3A). However, azithromycin prevented acidification of autophagosomes. In HeLa cells stably expressing the mCherry-GFP-LC3 reporter, azithromycin treatment significantly increased nonacidified autophagosomes (i.e., mCherry-GFP+) and reduced acidified autophagosomes (mCherry-GFP–; Figure 3B).

Azithromycin blocks autophagic clearance and autophagosome acidification. (A) Azithromycin reduced basal (black) and rapamycin-induced (gray) clearance of mutant (A53T) α-synuclein in stable inducible PC12 cells. The A53T α-synuclein transgene was induced with doxycycline for 48 hours and then switched off (by antibiotic removal) before cells were treated with vehicle alone, rapamycin (200 nM), and/or azithromycin for a further 24 hours. (B) Azithromycin reduced clearance of mutant (Q74) huntingtin. Aggregates of GFP-Q74 huntingtin were reduced by rapamycin treatment, but increased when autophagy was blocked by either BafA1 or azithromycin (4–16 μg/ml). Con, control. *P < 0.05; **P < 0.005. Scale bar: 10 μm.
stimuli (Supplemental Figure 5), which suggests an inhibition of phagosome-triggered cytokine release. These data suggest that azithromycin treatment might compromise the host response to mycobacterial infection both by preventing degradation of mycobacteria within autophagosomes and phagosomes and by disrupting release of cytokines, such as TNF-α, that promote autophagy-dependent intracellular killing of mycobacteria.

Intracellular killing of mycobacteria is impaired by azithromycin. We therefore examined the effect of azithromycin on the survival of mycobacteria within primary human macrophages. Autophagy is critical for intracellular killing of M. tuberculosis and M. bovis BCG (10, 20), but also pathogenic NTM, including M. abscessus (Supplemental Figure 6). Over a wide range of concentrations, azithromycin blocked basal intracellular killing of M. bovis BCG and M. abscessus, as well as preventing the ability of the autophagy inducer rapamycin to enhance killing (Figure 6, A and B). The control of M. tuberculosis and NTM infection in vivo is crucially dependent on the inflammatory cytokines IFN-γ and TNF-α, which enhance intracellular killing of mycobacteria through the induction of autophagy (10). As expected, azithromycin treatment

Figure 3
Azithromycin blocks acidification of autophagosomes without impairing lysosomal function. (A) Azithromycin did not impair autophagosome-lysosome fusion. Confocal microscopy of HeLa cells coexpressing LC3-mCherry with the lysosomal marker lgp120-GFP revealed that although rapamycin, BafA₁, and azithromycin (16 μg/ml) treatment increased the total number of LC3⁺ vesicles per cell, the colocalization of LC3⁺ vesicles with lgp120 (LC3⁺/lgp120⁺) was not significantly different from controls after azithromycin treatment, but increased with rapamycin and decreased with BafA₁ treatment. (B) Azithromycin impaired autophagosome acidification. HeLa cells stably expressing the mCherry-GFP-LC3 construct were treated with either 8–16 μg/ml azithromycin or BafA₁ for 24 hours and analyzed by confocal microscopy. Compared with the control, azithromycin caused a significant reduction in the number of acidified LC3⁺ vesicles, but increased total LC3⁺ vesicles per cell. Number of vesicles was normalized to control cells. **P < 0.005. Scale bars: 10 μm.
Azithromycin blocks autophagosomal and phagosome acidification by impairing lysosomal function. (A) Prevention of IFN-γ–induced autophagic flux. RAW 264.7 cells stably expressing mFRET-GFP-LC3 were treated with vehicle alone (as control), BafA1 (200 nM), azithromycin (40 μg/ml), IFN-γ (200 ng/ml), or azithromycin plus IFN-γ. Representative images are shown. Quantification of acidified (mCherry+GFP+; green) vesicles revealed a significant reduction in basal and IFN-γ–induced autophagosomal acidification by azithromycin treatment. (B) Reduced acidification of lysosomes in primary human macrophages. Double-labeled (FITC and TMR) dextran was loaded into lysosomes before cells were treated for 4 hours with azithromycin (80 μg/ml) or BafA1 (400 nM). As determined by confocal fluorescence analysis, azithromycin significantly reduced lysosomal acidification compared with controls, as did BafA1. Intracellular pH calibrations were performed as described in Supplemental Methods. (C) Azithromycin decreased acidification of phagosomes containing M. abscessus. Patient-derived M. abscessus strains were heat-killed, double-labeled with FAM and Alexa Fluor 633, and incubated for 24 hours with primary human macrophages untreated or treated with azithromycin (20 μg/ml) or BafA1 (100 nM). Representative confocal/DIC images of macrophages with intracellular labeled M. abscessus show increased mycobacterial FAM fluorescence (indicating reduced acidification) after treatment with azithromycin or BafA1. Also shown is quantification by flow cytometry of FAM fluorescence of macrophages that have internalized M. abscessus (i.e., Alexa Fluor 633+). Corresponding phagosomal pH values (see Supplemental Methods) demonstrated significant alkalinization of phagosomes with azithromycin or BafA1 treatment. Scale bars: 10 μm.

**Figure 5**
Azithromycin blocks phagosomal degradation and phagosome-lysosome fusion. (A) OVA-coated beads were incubated with primary human macrophages (1-hour pulse, 23-hour chase). Internalized beads were released, and the amount of OVA coating was quantified by flow cytometry after incubation with fluorescent anti-OVA antibody. Treatment with azithromycin (20 μg/ml) significantly reduced OVA degradation compared with untreated cells, to levels close to those achieved by BafA1 (100 nM) or leupeptin/pepsstatin. (B) Human primary macrophages were incubated (1-hour pulse, 4-hour chase) with TMN- and biotin-conjugated dextran to load lysosomes and then fed with IgG-coated streptavidin-conjugated fluorescent latex beads (30-minute pulse, 2-hour chase) with no treatment or in the presence of azithromycin (80 μg/ml) or BafA1 (400 nM). Beads were recovered by cell disruption, and the degree of bound dextran fluorescence was quantified by flow cytometry. Shown are a representative histogram and average geometric mean fluorescence of triplicate samples.

Discussion
We found that azithromycin blocked lysosomal degradation of autophagosomes and phagosomes, which, while potentially con-
Azithromycin blocks intracellular killing of mycobacteria. (A and B) Human macrophages were infected with luminescent mycobacteria — M. bovis BCG (A) and M. abscessus (B) — for 2 hours, washed, and exposed to the indicated concentrations of azithromycin (μg/ml) and/or 200 nM rapamycin for 24 hours. Viable intracellular mycobacteria were then assessed by measuring cell-associated luminescence after cell lysis. (C) IFN-γ and TNF-α enhanced intracellular killing of M. abscessus in human macrophages, and this was blocked by azithromycin pretreatment. Results were normalized to levels obtained without cytokine addition. (D) Patient-derived M. abscessus strains were incubated with peripheral blood from healthy subjects pretreated for 24 hours with vehicle alone or increasing concentrations of azithromycin. After 72 hours of incubation at 37°C, viable mycobacteria were quantified after sample lysis by counting CFUs. Addition of azithromycin, while reducing mycobacterial growth (white), prevented any additional autophagy-dependent killing achieved by coincubation with IFN-γ (200 U/ml) or rapamycin (100 nM). (E) Induction of macrolide resistance in M. abscessus. M. abscessus-lux was grown for 6 days in liquid culture (i.e., Middlebrook 7H9 plus ADC enrichment) in the presence of 0.1 μg/ml azithromycin or vehicle alone, washed, and resuspended in RPMI with 10% FCS with the indicated concentrations of azithromycin. Viable mycobacteria were assessed by measuring luminescence after 24 hours. *P < 0.05; **P < 0.005.

Figure 6

The net effect of azithromycin during clinical NTM infection is a growing clinical problem, with the potential to block intracellular killing of mycobacteria and promote chronic infection with NTM in vivo. Infection of CF patients with the multi-drug-resistant NTM M. abscessus is a growing clinical problem, leading to accelerated deterioration in lung function despite aggressive antibiotic therapy (8, 9). Previous epidemiological studies (12) and our current analysis suggest an association between long-term azithromycin use and development of M. abscessus infection, although definitive proof will require a prospective multicenter study with standardized sputum sampling and culture methods. Our findings provide a plausible mechanism for how chronic azithromycin use might predispose to NTM infection in CF patients and raise potential concerns about long-term macrolide treatment for other inflammatory lung diseases, use of macrolides in population-based eradication programs for trachoma and other infectious diseases (27), and clinical introduction of nonantibiotic macrolides as antiinflammatory agents.

The net effect of azithromycin during clinical NTM infection will depend on the balance between its direct antibiotic effect and its ability to block intracellular killing of mycobacteria. For slow-growing NTM species, such as the M. avium-intracellulare complex (MAC), which do not possess inducible macrolide resistance genes, azithromycin therapy (as part of multi-drug regimens) is usually highly effective. Whether it remains so during long-term, low-dose, intermittent treatment is unclear, particularly when concentrations of macrolides within MAC-containing phagosomes are likely to be reduced in vivo to below bacteriostatic levels (28). For rapidly growing NTM species, such as M. abscessus, that can acquire macrolide resistance (Figure 6E) through rapid induction of erm(41), single amino acid ribosomal mutation (1), and biofilm formation (23), the likely effect of long-term azithromycin therapy will be to promote infection, as seen in our mouse infection model. Azithromycin treatment also influenced cytokine production in vitro and in vivo, leading to reduced levels of macrophage- and dendritic cell–derived IL-12 and TNF-α and skewing T cell responses away from IFN-γ and toward IL-4. Although the mechanism underlying these effects remains to be elucidated, the net result will likely be further inhibition of autophagic killing (25, 26).

We demonstrated that azithromycin blocked autophagy in primary macrophages from stable CF patients as effectively as in those from healthy controls. Although basal levels of the autophagy substrate p62 were lower in CF macrophages, potentially as a result of transcriptional regulation (11), its levels were increased in both control and CF cells by azithromycin, indicative of blocked autophagic clearance.

Although the ability of azithromycin to block autophagy could be clinically exploitable in anticancer therapy, where tumor susceptibility to chemotherapy may be enhanced (29), our data suggest
that its impairment of autophagic degradation in macrophages will compromise host immunity to mycobacteria and potentially block the efficacy of adjuvant IFN-γ therapy often used in patients with difficult NTM or M. tuberculosis infection. Our findings have profound implications for the treatment of multi-drug-resistant mycobacteria, including M. tuberculosis, in which the ability of azithromycin to block intracellular bacterial killing is not accompanied by a direct antibiotic effect.

Methods
Further information can be found in Supplemental Methods.

Culture of cell lines and generation of human macrophages
HeLa and COS-7 cells were grown as previously described. Human monocyte-derived macrophages were isolated from peripheral blood as previously described (30), obtained from healthy consented subjects (approved by Regional NHS Research Ethics Committee), or from patients with CF (clinically stable and not taking azithromycin).

Lysosomal and phagosomal pH measurements
Human primary macrophages were loaded with TMR-FITC dextran (1-hour pulse, 4-hour chase). Cells were then treated with compounds and visualized by live cell fluorescence/differential interference contrast (fluorescence/DIC) confocal imaging. To determine the phagosomal pH, primary macrophages were incubated (1-hour pulse, 23-hour chase) with albumin-coated, FAM-conjugated 645-nm (Far Red) latex beads, patient-derived heat-killed M. abscessus strains double-labeled with FAM and Alexa Fluor 633, or PFA-killed M. tuberculosis H37Rv double-labeled with FITC and Alexa Fluor 633. Macrophages were pretreated for 24 hours with compounds. Analysis was carried out by either live cell fluorescence/DIC confocal imaging or flow cytometry, in which the fluorescence of cells with internalized particles (FarRed or Alexa Fluor 633 for beads and mycobacteria, respectively) was measured. Intracellular calibration was achieved as detailed in Supplemental Methods.

Phagosomal degradation assays
Human macrophages were incubated (30-minute pulse, 2-hour chase) with OVA-conjugated fluorescent latex beads at 4°C, 37°C, or 37°C with 15-minute protease inhibitor pretreatment. Macrophages were pretreated with BafA1 or azithromycin for 24 hours prior to phagocytosis; treatment continued throughout the experiment. Beads were then recovered by cell disruption in the presence of protease inhibitors, then incubated with OVA-specific biotinylated antibody and fluorescent streptavidin, which was quantified by flow cytometry.

Figure 7
Azithromycin promotes M. abscessus infection in vivo. (A) C57BL/6 mice were infected by aerosol challenge with azithromycin-resistant M. abscessus. Treatment with azithromycin (100 mg/kg) by gavage for 5 days per week resulted in failure of M. abscessus clearance from lungs. (B) Representative lung histology demonstrating that azithromycin treatment led to persistent lung infection associated with extensive granulomas (arrows) and peribroncholar inflammation at day 30. Ziehl-Neelsen staining confirmed the presence of large numbers of intracellular mycobacteria in azithromycin-treated, but not control, animals. Scale bars: 200 μm (histology); 10 μm (Ziehl-Neelsen). (C) Extent and severity of lung lesions in M. abscessus–infected mice at days 15 and 30 of treatment with vehicle alone or azithromycin. (D) Assessment of intracellular cytokine profiles for lung macrophages, dendritic cells, and CD4+ and CD8+ effector T cells during infection in the presence or absence of chronic azithromycin treatment.
Phagosome-lysosome fusion assays

Human macrophages were inoculated with mycobacteria (MOI 10:1) for 2 hours at 37°C, repeatedly washed, and then incubated for 24 hours at 37°C in the presence or absence of azithromycin (80 μg/ml) or BaF4. Beads were removed by cell disruption, and the degree of bound fluorescent dextran was quantified by flow cytometry, and average geometric mean fluorescence was determined.

In vitro mycobacterial infection assays

Primary human macrophages. Macrophages were inoculated with mycobacteria (MOI 10:1) for 2 hours at 37°C, repeatedly washed, and then incubated for 24 hours at 37°C in the presence or absence of azithromycin or rapamycin, as indicated. Cells were finally lysed, and luminescence was measured.

Whole blood mycobacterial infection assays. Patient-derived M. abscessus strains were grown to log phase in Middlebrook 7H9 media. Heparinized peripheral blood from healthy consented subjects was inoculated with M. abscessus after pretreatment for 24 hours with vehicle alone or azithromycin and incubated for 72 hours at 37°C. Viable mycobacteria were quantified (CFU) on microplates. Where indicated, IFN-γ or rapamycin were added to blood at the time of infection with mycobacteria.

Induction of macrophage resistance in vitro. M. abscessus-bac was grown in liquid culture in the presence of 0.1 μg/ml azithromycin or vehicle alone, washed, and resuspended in RPMI 10% with FCS with the indicated concentrations of azithromycin. Viable mycobacteria were assessed by measuring luminescence after 24 hours.

In vivo mycobacterial infection

Mice were challenged with M. abscessus as previously described (24). Control (saline) and azithromycin groups (n = 5 per group) were treated 5 days per week at a concentration of 100 mg/kg by gavage. On days 15 and 30 after infection, bacterial loads in the lungs and spleen were determined by histological and flow cytometry analysis (see Supplemental Methods). Bacterial counts were determined by plating serial dilutions of homogenates of lungs after 3–7 days.

Statistics

Densitometric analysis on the immunoblots was done by Image J software. P-values for the Cellomics array scan counting and for other experiments requiring multiple comparisons were determined by factorial ANOVA using STATVIEW software (version 4.53). P-values for all other assays were determined using 2-tailed Student’s t test. A P-value of 0.05 or less was considered significant. Experiments were performed on at least 3 separate occasions with at least triplicate samples for each condition and represented as mean ± SD.

To test for an association between NTM status and azithromycin, we used a logistic regression model adjusted for covariates. Forward logistic regression was used to select covariates associated with NTM status (P < 0.05) from age, forced expiratory volume in 1 second, BMI, sex, colonization with Pseudomonas aeruginosa, CF-related liver disease, CF-related diabetes, CF-related asthma, allergic bronchopulmonary aspergillosis, and gastroesophageal reflux disease. Analyses were performed in R (http://www.r-project.org).

Study approval

All experimental protocols were approved by the Animal Care and Usage Committee of Colorado State University and complied with NIH guidelines.

Acknowledgments

This work was supported by grants from the Wellcome Trust (Senior Clinical Fellowships to R.A. Floto and D.C. Rubinsztein), the NIH Biomedical Research Centre at Addenbrooke’s Hospital, Papworth Hospital NHS Trust, and the Jodi and Lucinda Dunmore Foundation. The authors thank Juliet Fowler and Christian Laughton for help with microbiology and Jane Elliott and Judy Ryan for help with clinical data collection.

Received for publication December 14, 2010, and accepted in revised form June 8, 2011.

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