β-Lactamase inhibition by avibactam in Mycobacterium abscessus

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Objectives: Two β-lactams, cefoxitin and imipenem, are part of the reference treatment for pulmonary infections with Mycobacterium abscessus. M. abscessus has recently been shown to produce a broad-spectrum β-lactamase, BlaMab, indicating that the combination of β-lactams with a BlaMab inhibitor may improve treatment efficacy. The objectives of this study were to evaluate the impact of BlaMab production on the efficacy of β-lactams in vitro and to assess the benefit of BlaMab inhibition on the activity of β-lactams intracellularly and in an animal model.

Methods: We analysed the mechanism and kinetics of BlaMab inactivation by avibactam, a non-β-lactam β-lactamase inhibitor currently in Phase III of development, in combination with ceftazidime for the treatment of serious infections due to Gram-negative bacteria. We then deleted the gene encoding BlaMab to assess the extent of BlaMab inhibition based on a comparison of the impact of chemical and genetic inactivation. Finally, the efficacy of amoxicillin in combination with avibactam was evaluated in cultured human macrophages and in a zebrafish model of M. abscessus infection.

Results: We showed that avibactam efficiently inactivated BlaMab via the reversible formation of a covalent adduct. An inhibition of BlaMab by avibactam was observed in both infected macrophages and zebrafish.

Conclusions: Our data identify avibactam as the first efficient inhibitor of BlaMab and strongly suggest that β-lactamase inhibition should be evaluated to provide improved therapeutic options for M. abscessus infections.

Keywords: non-tuberculous mycobacteria, M. abscessus, therapy, β-lactams, cystic fibrosis

Introduction

Since the individualization of Mycobacterium abscessus as a distinct species in 1992,1 this fast-growing Mycobacterium has been recognized as an important pathogen.2 M. abscessus produces numerous virulence factors3 and causes a wide spectrum of diseases. Skin and soft tissue infections following tattooing4 and accidental or iatrogenic inoculation5 generally resolve with antibiotic therapy.6 Pulmonary diseases have a poorer prognosis, especially in the context of cystic fibrosis or severe gastrointestinal reflux.7,8 The treatment of these infections is difficult owing to the limited number of active antibiotics that are available. M. abscessus is intrinsically resistant to antituberculous agents.6 Clarithromycin cannot be uniformly recommended as a first-line drug since erm RNA methylase genes conferring macrolide resistance have been detected in members of the M. abscessus complex.9 The gene is functional in the subspecies abscessus, whereas an internal deletion is present in members of the bolletii subspecies that had previously been classified in the massiliense species.10 The treatment of M. abscessus infections therefore relies on a few antibiotics including amikacin, linezolid, tigecycline and the parenteral β-lactams imipenem and cefoxitin. The latter
drugs have moderate in vitro activity, with MICs of 4 and 32 mg/L, respectively. Most other β-lactams have no appreciable in vitro activity. Resistance to β-lactams in mycobacteria results from the combination of several mechanisms, which have not been well characterized in M. abscessus. Impermeability of the mycomembrane has been shown to hamper the activity of cephalosporins in Mycobacterium chelonae. Substitution of the classical β-lactam targets, i.e., penicillin-binding proteins, by β-amino-transpeptidases may further decrease the activity of penicillins and cephalosporins. As shown in Mycobacterium tuberculosis, the production of a chromosome-encoded Ambler class A β-lactamase (BlaMab) may participate in β-lactam resistance.

In M. tuberculosis, irreversible β-lactamase inhibition by clavulane improves the activity of β-lactams, and the combination of clavulanate with meropenem is sporadically used for XDR tuberculosis. However, M. abscessus BlaMab is not inhibited by clavulanate, sulbactam or tazobactam and, strikingly, these drugs are in fact hydrolysed by the β-lactamase. We show here that avibactam, a non-β-lactam β-lactamase inhibitor currently being evaluated in Phase III trials in combination with ceftazidime for the treatment of infections due to Gram-negative bacteria (http://clinicaltrials.gov/ct2/results?term=avibactam&Search=Search), is a potent inhibitor of BlaMab in vitro. A BlaMab-deficient mutant was constructed to assess the extent of β-lactamase inhibition in vitro, in cultured macrophages and in a recently developed zebrafish model of M. abscessus infection. These approaches show that BlaMab is the major determinant of β-lactam resistance in M. abscessus and that β-lactamase inhibition should be considered to improve the treatment of M. abscessus infections.

Materials and methods

Strains, growth conditions and in vitro susceptibility testing

Derivatives of reference strain M. abscessus CIP104536 (ATCC 19977) with a rough (R) and a smooth (S) morphotype were grown in Middlebrook 7H9 broth supplemented with 10% (v/v) oleic acid, albumin, dextrose, catalase (OADC; BD-Difco) and 0.05% (v/v) Tween 80 (Sigma) (7H9sB) at 30°C with shaking (150 rpm). The R morphotype was used in the zebrafish model because the S form is avirulent in this model. The S morphotype was used for the macrophage model since this form is easier to manipulate and to enumerate. To construct the ΔblaMab mutants, the blaMab gene (MAB_2875) of the M. abscessus CIP104536 R and S morphotypes was replaced by a zeocin resistance gene using one-step homologous recombination (see Figure S1 and Supplementary methods section, available as Supplementary data at JAC Online). M. abscessus CIP104536 R and S mutants were constructed using the microdilution method in 96-well round-bottom microplates as described in the Supplementary data.

Biochemical analysis of BlaMab inactivation by avibactam

The cloning, production in Escherichia coli BL21 (DE3) and purification of a recombinant form of BlaMab have been reported elsewhere. The kinetic constants for BlaMab inhibition were determined for a two-step reaction (Figure 1a) as previously described (further details are available as Supplementary data).

Activity of combinations of β-lactams and avibactam in THP-1 macrophages

Macrophages were infected with M. abscessus CIP104536 R expressing tdTomato (300 cfu) were injected into the caudal vein of 30 h post-fertilization embryos of zebrafish (Danio rerio, ‘golden’ mutant) (further details are available as Supplementary data). Infected larvae were transferred into 96-well plates and exposed to various concentrations of amoxicillin (512–12800 mg/L) with or without 50 mg/L avibactam in water for 5 days. The drug-containing water was renewed daily for 5 days. The viability of the infected embryos was evaluated daily by an assessment of cardiac activity, and the dissemination of M. abscessus was evaluated by fluorescence microscopy. All zebrafish experiments were carried out at the University Montpellier 2, according to European Union guidelines for the handling of laboratory animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm) and approved by the Direction Sanitaire et Vétérinaire de l’Hérault and Comité d’Ethique for the Expérimentation Animale de la Région Languedoc Roussillon (CEEA-LR) under the reference CEEA-LR-13007.

Statistical analysis

For the biochemical analysis of BlaMab inhibition, standard errors correspond to the 95% CI of the fits performed with SigmaPlot software. The results of the MIC determinations are the medians of three independent replicates. The results of the macrophage experiments are the means ± SD of at least three independent experiments. The Mann–Whitney U-test and Kruskall–Wallis test were used to compare the intracellular activity of the antibiotics. For the zebrafish infection model, the experiments were performed at least in triplicate. The data for the replicates were pooled for the construction and comparison of survival curves. The efficacy of the different antibiotics was compared using the log-rank test for survival and Fisher’s exact test for the frequency of abscesses. All statistical analyses were performed with Epi Info™ software version 7.1.3 (CDC, Atlanta, GA, USA).

Results

Deletion of the blaMab β-lactamase gene

To explore the role of β-lactamase BlaMab in β-lactam resistance, the corresponding gene (MAB_2875) was deleted from the chromosome of M. abscessus CIP104536 (Figure S1). Extracts and whole-cell suspensions of the WT strain rapidly hydrolysed the chromogenic cephalosporin nitrocefin (Figure S2). β-Lactamase activity was not detected in mutant ΔblaMab, indicating that BlaMab is the only β-lactamase produced by M. abscessus CIP104536.

Since the susceptibility of M. abscessus to β-lactams varies according to the culture medium, we determined MICs both in the reference medium for susceptibility testing, CAMHB, and in the medium routinely used in research laboratories for the optimal growth of mycobacteria, Middlebrook 7H9 broth supplemented with 10% OADC and 0.05% Tween 80 (7H9sB). In both media, M. abscessus CIP104536 was highly resistant to all β-lactams except cefoxitin, imipenem and meropenem, as previously
The deletion of blaMab dramatically reduced the MICs of penicillins and first-, second- and third-generation cephalosporins (except ceftazidime), in both CAMHB and 7H9sB medium (Table 1). These results indicate that Bla Mab is the major determinant of high-level resistance to penicillins and most cephalosporins in *M. abscessus*. The absence of activity of ceftazidime and aztreonam against mutant D blaMab suggests that the transpeptidases of *M. abscessus* are not inhibited by these antibiotics.

Activity of β-lactams combined with avibactam against *M. abscessus*

To explore BlaMab inhibition in whole bacterial cells, we compared the MICs of β-lactams against *M. abscessus* CIP104536 in the presence or absence of avibactam (Table 1). Similar results were obtained for the S and R morphotypes. In 7H9sB medium, this β-lactamase inhibitor decreased the MICs of amoxicillin, cefalotin, cefuroxime, cefamandole and ceftriaxone (Table 1). The addition of avibactam to the culture medium and the deletion of blaMab had strikingly similar impacts on the MICs, indicating that Bla Mab was completely inhibited in the 7H9sB medium. Avibactam had no intrinsic antibacterial activity since the drug did not modify the MICs of β-lactams against mutant D blaMab and did not inhibit the growth of the *M. abscessus* strains (MICs >256 mg/L).

The MICs of cefoxitin and imipenem are generally higher in the reference medium, CAMHB, than in 7H9sB. A comparison of MICs of additional β-lactams against the ΔblaMab mutant in the two media (Table 1) indicated that all β-lactams were intrinsically less active in CAMHB (2- to 8-fold). A complete inactivation of BlaMab by avibactam was not achieved in CAMHB medium since the addition of the inhibitor to the growth medium did not reduce the MICs of β-lactams to the same extent as the deletion of blaMab. Thus, both β-lactams and avibactam were less active in CAMHB than in 7H9sB medium.

![Figure 1](image_url)

**Figure 1.** Kinetics of BlaMab inhibition by avibactam. (a) Reaction scheme. E, BlaMab; I, avibactam; EI, non-covalent inhibitor-enzyme complex; EI*, carbamylated enzyme; $K_i$ was defined as the ratio of $k_{-1}$ over $k_1$. (b) Time-dependent inhibition of BlaMab by avibactam. BlaMab (0.25 nM) was incubated with nitrocefin (100 μM) and avibactam (0.1, 0.25, 0.5, 1, 2.5 and 4 μM). Progress curves (solid lines) were fitted (black dotted lines) to Equation 1 (available as Supplementary data) to obtain the pseudo-first-order rate constant $k_{obs}$. (c) Determination of carbamylation rate constant $k_2/K_i$. $k_{obs}$ was plotted as a function of avibactam concentration and $k_2/K_i$ was deduced from the slope of the resulting line according to Equation 2 (available as Supplementary data). (d) Kinetics of BlaMab decarbamylation. BlaMab (1 μM) was incubated with avibactam (5 μM) for 20 min. The mixture was diluted 10000-fold and recovery of enzyme activity was measured using nitrocefin (100 μM) as the substrate (grey solid curve). Under the assay conditions the concentrations of BlaMab and avibactam were 100 and 500 pM, respectively. The progress curve was fitted (black dotted line) to Equation 3 (available as Supplementary data) to obtain $k_{off}$. The black solid curve corresponds to the hydrolysis of nitrocefin (100 μM) by uninhibited BlaMab (100 pM). The dashed line represents spontaneous nitrocefin hydrolysis.
In vitro inhibition of BlaMab

Avibactam is unique among β-lactamase inhibitors since carbamylation of the enzyme active-site serine is fully reversible according to the reaction scheme depicted in Figure 1(a). The efficacy of this mode of inhibition depends upon both the carbamylation and decarbamylation rates. Since the inhibition of *M. abscessus* β-lactamase has not been previously investigated, we have purified a soluble form of BlaMab and determined its inhibition kinetics using nitrocefin as the substrate (Figure 1). Avibactam inhibited BlaMab in a time-dependent manner with a carbamylation rate constant ($k_2/K_i$) of $4.9 + 1.2 \times 10^5$ M$^{-1}$ s$^{-1}$ (Figure 1b and c), close to that observed for the prototypic Ambler class A β-lactamase TEM-1 ($1.6 + 1.2 \times 10^5$ M$^{-1}$ s$^{-1}$). Decarbamylation occurred with a rate constant of $0.047 \pm 0.0001$ min$^{-1}$ (Figure 1d), implying that half the enzyme recovered its activity within 15 min in the absence of avibactam. This value is also similar to the decarbamylation rate constant reported for TEM-1 ($0.045 \pm 0.022$ min$^{-1}$). The hydrolysis of avibactam has been reported for a single β-lactamase, KPC-2, from *Klebsiella pneumoniae*. In our study, the hydrolysis of avibactam by BlaMab was not detected by mass spectrometry (data not shown). Together, these results indicate that avibactam inhibits β-lactamases from *M. abscessus* and from the Enterobacteriaceae with similar efficiencies.

Intramacrophagic activity of amoxicillin combined with avibactam

Macrophages were infected with *M. abscessus* CIP104536 S and its ΔblaMab derivative and exposed to various concentrations of amoxicillin and avibactam; the surviving bacteria were enumerated after 2 days of incubation by plating serial dilutions of macrophage lysates. In the absence of antibiotic, *M. abscessus* CIP104536 S and its ΔblaMab Derivative grew in THP-1-derived macrophages, leading to a 10-fold increase in the cfu numbers in 2 days (Figure 2).

### Table 1. MICs (mg/L) of β-lactams in CAMHB and 7H9sB media with or without 4 mg/L avibactam

<table>
<thead>
<tr>
<th>β-Lactam</th>
<th>CAMHB medium CIP104536</th>
<th>ΔblaMab</th>
<th>7H9sB medium CIP104536</th>
<th>ΔblaMab</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>−avibactam +avibactam</td>
<td>−avibactam +avibactam</td>
<td>−avibactam +avibactam</td>
<td>−avibactam +avibactam</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>&gt;256 256</td>
<td>8 16</td>
<td>&gt;256 8</td>
<td>4 4</td>
</tr>
<tr>
<td>Cefalotin</td>
<td>&gt;256 256</td>
<td>16 16</td>
<td>&gt;256 8</td>
<td>4 4</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>256 32</td>
<td>16 16</td>
<td>32 8</td>
<td>4 8</td>
</tr>
<tr>
<td>Cefamandole</td>
<td>&gt;256 64</td>
<td>16 16</td>
<td>128 8</td>
<td>4 4</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>&gt;256 256</td>
<td>64 32</td>
<td>64 8</td>
<td>8 8</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>&gt;256 &gt;256</td>
<td>&gt;256 &gt;256</td>
<td>&gt;256 &gt;256</td>
<td>&gt;256 &gt;256</td>
</tr>
<tr>
<td>Ceftaroline</td>
<td>32 32</td>
<td>32 32</td>
<td>16 8</td>
<td>8 16</td>
</tr>
<tr>
<td>Imipenem</td>
<td>8 8</td>
<td>4 4</td>
<td>4 2</td>
<td>2 2</td>
</tr>
<tr>
<td>Meropenem</td>
<td>16 8</td>
<td>8 8</td>
<td>4 4</td>
<td>4 4</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>&gt;256 &gt;256</td>
<td>&gt;256 &gt;256</td>
<td>&gt;256 &gt;256</td>
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Results are the medians of three independent experiments performed with the S morphotype of *M. abscessus* CIP104536 and its ΔblaMab derivative. MICs observed for the R variant of *M. abscessus* CIP104536 were not different (data not shown).

**Figure 2.** Intracellular activity of amoxicillin (AMC) combined with avibactam. Fold change in cfu between 0 and 2 days post-infection. Results are the means ± SEM of at least three independent experiments. *P < 0.05 versus untreated control using the Mann–Whitney U-test.
Amoxicillin at 8, 32 and 128 mg/L prevented the intracellular growth of the \( \text{D} \text{bla} \text{Mab} \) mutant (Figure 2a). Amoxicillin alone had no effect against the WT CIP104536 strain, whereas this drug in combination with avibactam (16 mg/L) exhibited dose-dependent activity (Figure 2b). However, comparison with the \( \text{D} \text{bla} \text{Mab} \) mutant indicated that a complete inactivation of Bla Mab by avibactam was not achieved inside the macrophages.

Efficacy of avibactam in a zebrafish model of \( M. \text{abscessus} \) infection

A recently developed zebrafish model\(^{22} \) of \( M. \text{abscessus} \) infection was used to assess the in vivo inhibition of Bla Mab by avibactam. In this model, zebrafish embryos infected by \( M. \text{abscessus} \) CIP104536 R developed abscesses mainly located in the CNS within 4 days after infection (Figure 3a and b), leading to a rapid decrease in survival that began at day 5 post-infection (Figure 3c). Amoxicillin at the highest non-toxic concentration (Figure S3) significantly decreased the mortality of infected larvae (Figure 3c, \( P<10^{-4} \)). The addition of avibactam (50 mg/L) further increased the survival (\( P<10^{-6} \)). The efficacy of amoxicillin and of the amoxicillin/avibactam combination was also evaluated by enumerating infected larvae with abscesses by live fluorescence microscopy (Figure 3d). Amoxicillin alone was active in reducing the proportion of embryos with abscesses (62% of embryos infected by the WT strain and left untreated developed abscesses versus 40% for embryos infected by the WT strain and treated with amoxicillin, \( P=0.0006 \)). Avibactam improved the efficacy of amoxicillin (17%, \( P=0.00003 \)). Overall, these results indicate that avibactam inhibited Bla Mab in the conditions that prevail in the abscesses since the drug improved the efficacy of amoxicillin. As expected, amoxicillin was active in the absence of avibactam against mutant \( \text{D} \text{bla} \text{Mab} \) (Figure 3c and d).

Activity of the amoxicillin/avibactam combination against a collection of \( M. \text{abscessus} \) isolates

The variability in the in vitro activity of \( \beta \)-lactams and avibactam was evaluated in a collection of 16 \( M. \text{abscessus} \) clinical isolates from a previous study (Table S1).\(^{11} \) Amoxicillin alone did not inhibit the growth of any of the 16 isolates up to a concentration of 256 mg/L. Avibactam at 4 mg/L improved the activity of amoxicillin. The MICs of amoxicillin in the presence of avibactam were in the same range as the MICs of cefoxitin (4 to 32 mg/L versus...
8 to 32 mg/L, respectively). These results indicate that amoxicillin displayed antibacterial activity against all isolates following β-lactamase inhibition by avibactam.

Discussion

β-lactamases BlaMab from M. abscessus and BlaC from M. tuberculosis have similar broad hydrolysis spectra. In contrast, the β-lactamases have opposite behaviours with respect to their interaction with β-lactamase inhibitors since BlaMab efficiently hydrolyses clavulanate, sulbactam and tazobactam, whereas BlaC is inhibited by these drugs, including irreversible inactivation in the case of clavulanate. In this study, we show that avibactam is a potent inhibitor of purified BlaMab (Figure 1), whereas the inhibition of BlaC by this non-β-lactam inhibitor was detected only at high concentrations. The identification of avibactam as an efficient inhibitor of BlaMab prompted us to evaluate the benefit of β-lactamase inhibition on the activity of β-lactams against M. abscessus in vitro, intracellularly and in an animal model.

In order to evaluate the efficacy of avibactam for the in vitro inhibition of BlaMab, we constructed a β-lactamase-deficient mutant of M. abscessus. In 7H9SB medium, the MICs of β-lactams against this mutant were very close to those observed for the parental strain in the presence of 4 mg/L avibactam, indicating that a low concentration of inhibitor is sufficient to fully inhibit BlaMab (Table 1). An inhibition of BlaMab was also observed in a set of 16 clinical isolates (Table S1). In the absence of functional BlaMab, several representatives of the three main classes of β-lactams (penams, cephalosporins and carbapenems) were found to be active against M. abscessus. This observation indicates that the cross-linking step of peptidoglycan synthesis, which involves two pathways in M. abscessus, is inhibited by a large spectrum of β-lactams, although most of them are devoid of antibacterial activity due to their hydrolysis by BlaMab. In 7H9SB medium, the MICs of the two drugs used for the treatment of M. abscessus infections, imipenem and ceftazidime, were 4 and 16 mg/L. In the absence of functional BlaMab, the MICs of several additional β-lactams belonged to the same range (4–8 mg/L). These results indicate that avibactam may potentially extend the therapeutic options among β-lactams for the treatment of M. abscessus infections. Of note, the MICs of all the β-lactams were relatively high even in the absence of BlaMab. Thus, low permeability may also contribute to intrinsic β-lactam resistance in M. abscessus, as previously proposed for M. chelonae.

In order to determine the intracellular activity of avibactam, we assessed the efficacy of the amoxicillin/avibactam combination in macrophages (Figure 2). Because amoxicillin is one of the best substrates of BlaMab, this β-lactam provides a stringent assay for the intracellular inhibition of BlaMab by avibactam. Amoxicillin inhibited the intracellular growth of mutant ΔblaMab at concentrations close to the MIC in 7H9SB medium, indicating that the drug penetrates into the macrophages and is intracellularly active (Figure 2a). An assay of the amoxicillin/avibactam combination against the parental strain revealed the intracellular inhibition of BlaMab by avibactam (Figure 2b). However, the inhibition of the β-lactamase was partial since the number of intracellular bacteria was higher for chemical (Figure 2b) than for genetic (Figure 2a) enzyme inactivation. This may reflect a limited penetration of avibactam into the macrophages. Of note, avibactam has been reported to be active in THP-1 monocytes infected by Pseudomonas aeruginosa.

The in vivo evaluation of β-lactam efficacy in rodent models of M. abscessus infection suffers from several limitations, including the spontaneous clearance of infection and difficulties in achieving sufficient blood levels, especially with carbapenems, which are rapidly hydrolysed by renal dehydropeptidase. A zebrafish model of M. abscessus infection has recently been developed and used for the in vivo evaluation of drug efficacy. We used this model, which offers several advantages, including speed, low cost and ethical acceptability, to assess the inhibition of BlaMab in infectious forms of M. abscessus. Avibactam efficiently increased survival and decreased abscess formation in zebrafish larvae treated with amoxicillin (Figure 3). Thus, the inhibition of BlaMab increased the in vivo activity of amoxicillin.

Conclusions

M. abscessus lung infections have a poor prognosis, especially in the context of underlying pulmonary diseases such as cystic fibrosis. Active β-lactams currently include only ceftoxin and carbapenems, which are parenteral drugs. Here we show that the spectrum of active β-lactams can be extended by the inhibition of M. abscessus β-lactamase BlaMab. This could potentially provide alternatives to imipenem and ceftoxin with better PK/PD parameters. Since the approved inhibitors, clavulanate, sulbactam and tazobactam, are hydrolysed by BlaMab, avibactam is the only inhibitor that can be currently considered. We have shown that this drug efficiently inhibits BlaMab by the reversible formation of a covalent adduct and is active intracellularly and in the zebrafish model. In humans, avibactam has shown an excellent tolerance profile in Phase I and II studies. The drug penetrates into epithelial lining fluid and its activity is not impaired by pulmonary surfactant. The combination of avibactam and ceftazidime is currently in Phase III trials for treatment of nosocomial pulmonary infections and complicated urinary tract and intra-abdominal infections. Unfortunately, this combination is not relevant to the treatment of M. abscessus infections due to the lack of activity of ceftazidime. In addition, avibactam is being developed as a parenteral drug. The development of an orally bioavailable combination of a β-lactam with a BlaMab inhibitor is an attractive approach to improve therapeutic options for M. abscessus infections. The excellent in vitro activity of avibactam and the unique mode of action of this inhibitor suggest that derivatives of this drug should be considered for the development of this type of combination.

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β-lactamase inhibition in Mycobacterium abscessus

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Supplementary data
Supplementary methods, Figures S1 to S3 and Table S1 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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