Extended-spectrum antibodies protective against carbapenemase-producing Enterobacteriaceae

David Skurnik, Damien Roux, Stephanie Pons, Thomas Guillard, Xi Lu, Colette Cywes-Bentley and Gerald B. Pier*

Division of Infectious Diseases, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA 02115, USA

*Corresponding author. Tel: +1-617-525-2269; Fax: +1-617-525-2510; E-mail: gpier@rics.bwh.harvard.edu

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Background: Carbapenem-resistant Enterobacteriaceae (CRE) are responsible for worldwide outbreaks and antibiotic treatments are problematic. The polysaccharide poly-((β-1,6)-N-acetyl glucosamine (PNAG) is a vaccine target detected on the surface of numerous pathogenic bacteria, including Escherichia coli. Genes encoding PNAG biosynthetic proteins have been identified in two other main pathogenic Enterobacteriaceae, Enterobacter cloacae and Klebsiella pneumoniae. We hypothesized that antibodies to PNAG might be a new therapeutic option for the different pan-resistant pathogenic species of CRE.

Methods: PNAG production was detected by confocal microscopy and its role in the formation of the biofilm (for E. cloacae) and as a virulence factor (for K. pneumoniae) was analysed. The in vitro (opsonophagocytosis killing assay) and in vivo (mouse models of peritonitis) activity of antibodies to PNAG were studied using antibiotic-susceptible and -resistant E. coli, E. cloacae and K. pneumoniae. A PNAG-producing strain of Pseudomonas aeruginosa, an organism that does not naturally produce this antigen, was constructed by adding the pga locus to a strain with inactive alg genes responsible for the production of P. aeruginosa alginate. Antibodies to PNAG were tested in vitro and in vivo as above.

Results: PNAG is a major component of the E. cloacae biofilm and a virulence factor for K. pneumoniae. Antibodies to PNAG mediated in vitro killing (>50%) and significantly protected mice against the New Delhi metallo-β-lactamase-producing E. coli (P = 0.02), E. cloacae (P = 0.0196) and K. pneumoniae (P = 0.006), against K. pneumoniae carbapenemase (KPC)-producing K. pneumoniae (P = 0.02) and against PNAG-producing P. aeruginosa (P = 0.0013). Thus, regardless of the Gram-negative bacterial species, PNAG expression is the sole determinant of the protective efficacy of antibodies to this antigen.

Conclusions: Our findings suggest antibodies to PNAG may provide extended-spectrum antibacterial protective activity.

Introduction

Initially described in Staphylococcus epidermidis, then in Staphylococcus aureus and Escherichia coli, as a product of the proteins encoded in the ica (Staphylococcus spp.)1 or pga (E. coli)2 loci, the surface polysaccharide poly-((β-1,6)-N-acetyl-glucosamine (PNAG) has now been detected in numerous Gram-positive and Gram-negative bacteria.3 Previous studies have shown that monoclonal antibodies (MAbs) and polyclonal antibodies to PNAG can kill MDR and even pan-resistant strains of the Burkholderia cepacia complex (BCC) in vitro and protect mice in a setting of peritonitis.4 PNAG antibodies were also protective in similar pre-clinical studies against potentially MDR bacterial species such as E. coli5 and Acinetobacter baumannii6. In addition, genome analysis found that Klebsiella pneumoniae, Enterobacter cloacae and Stenotrophomonas maltophilia all contain an intact pga locus and may thus be able to produce PNAG.7 Therefore, antibodies to PNAG have the potential to prevent or treat a broad variety of infections caused by MDR Gram-negative bacteria.3,6 In the present work, we tested this hypothesis by analysing the ability of antibodies to PNAG to kill and protect against infections caused by carbapenemase-producing, carbapenem-resistant Enterobacteriaceae (CRE). The study focused on the New Delhi metallo-β-lactamase-1 (NDM-1)- and K. pneumoniae carbapenemase (KPC)-producing strains, which represent major threats to patients in both the community and the hospital setting. We focused on three major Enterobacteriaceae species of clinical importance: E. coli, K. pneumoniae and E. cloacae.8,9 A confirmatory study was conducted using Pseudomonas aeruginosa, the only major MDR pathogen that does not naturally produce PNAG, to determine whether expression of PNAG using a cloned pga locus led to a significant protective effect of antibodies in this non-natural setting.
Materials and methods

A full description of the methods is available as Supplementary data at JAC Online. Bacterial strains, plasmids and primers are listed in Tables S1 and S2 (available as Supplementary data at JAC Online).

Bacterial strains

E. cloacae strains were provided by Astrid Rey, Sanofi, Toulouse, France. K. pneumoniae K2 was provided by Alan S. Cross, University of Maryland, Baltimore, USA. The E. coli, E. cloacae and K. pneumoniae NDM-1 strains were obtained from the CDC (USA) and the KPC strains were provided by Barry Kreiswirth, Rutgers New Jersey Medical School, Newark (USA). P. aeruginosa PA14 was available in the laboratory. The NDM-producing strains used in this study were resistant to all β-lactams tested (including carbapenems and aztreonam), ciprofloxacin, amikacin and gentamicin, and demonstrated MICs of colistin and polymyxin B ≤1 mg/L.10 The KPC-bearing K. pneumoniae strains all carried KPC-3 and belong to the epidemic ST258 clone, are endemic in New York and New Jersey11 and were resistant to all β-lactams tested, had intermediate resistance to amikacin (MIC 32 mg/L) and gentamicin (MIC 8 mg/L) and were susceptible to tetracyclines (doxycycline and minocycline), colistin (MIC 1 mg/L), tigecycline (MIC 1 mg/L) and polymyxin B (1 mg/L).

Genetic manipulations

Deletion of pgaC in K. pneumoniae was done following the method of Datsenko and Wanner.12 P. aeruginosa PA14 transposon (Tn) mutants were obtained from the PA14 Tn insertion library.13 Introduction of either a single pgaA gene or the entire pga locus was done by conjugation between P. aeruginosa PA14 and an E. coli Sm10 carrying pUCP18::pgaA or pUCP18::pga followed by selection on lysogeny broth (LB) agar supplemented with tetracycline (75 mg/L) and Irgasan (25 mg/L).

Confocal microscopy

Experiments followed previously described protocols with minor modifications.3

Flow cytometry

Bacteria were grown in tryptic soy broth (TSB) medium overnight at 37°C and then left at room temperature for 24 h before fixing with

Figure 1. E. cloacae and PNAG production. Detection of PNAG on the surface of E. cloacae 2a using immunofluorescence confocal microscopy (a) and flow cytometry (b). Binding of MAb F598 to PNAG conjugated to AF488 [green fluorescence in (a) and green curve in (b)]. No binding with the control MAb conjugated to AF488 was detected [lack of green fluorescence in (a) and blue curve in (b)]. In (a), SYTO 83 was used to visualize DNA (red fluorescence) and an overlay of red and green channels is presented. (c) Formation of biofilms after 2 days of growth at room temperature comparing E. cloacae 2a, E. cloacae 2a Δpga and E. cloacae 2a Δpga (pUCP18::pga). (d) Oposonaphagocytic killing of E. cloacae 2a mediated by polyclonal antibodies and MAb to PNAG. Bars represent mean percentage of killing relative to the control containing NGS or the control MAB F429. All standard deviations (not shown) were <15%. Assays were done in duplicate. C’, complement; absence of killing arbitrarily assigned as 1% killing. Dilutions of polyclonal antibodies and amounts (in μg) of MAb to PNAG tested are provided at the top of each bar.
paraformaldehyde (PFA). Samples were then pelleted and washed with PBS and incubated with either MAB F429 to *P. aeruginosa* alginate directly conjugated to AF488 (2.5 μg/mL) or MAB F598 to PNAG directly conjugated to AF488 (2.5 μg/mL), then placed in PBS containing 0.5% BSA overnight at 4°C. Samples were then washed with PBS and resuspended in 500 μL of PBS and placed into flow cytometry tubes for FACS analysis.

**Biofilm assays**

Biofilm production was assessed as previously described by measuring the incorporation of crystal violet after growth of bacterial cultures in glass tubes at 37°C for 24 h containing TSB medium.

**Opsonophagocytic activity of PNAG-specific antibodies against the major species of pathogenic Enterobacteriaceae**

The opsonophagocytic assays followed published protocols except that the differentiated HL60 promyelocytic cell line (ATCC) was used as a source of phagocytes.

**Protection studies**

Mice were housed under specific pathogen-free conditions and all animal experiments were conducted under protocols approved by the Harvard Medical Area Institutional Animal Care and Use Committee.

To evaluate the in vivo protective efficacy of antibody to PNAG, we used either an intraperitoneal or intravenous (via retro-orbital injection) infection model in mice, as described previously. Briefly, mice (C3H/HeN, female, 6–8 weeks of age) were injected intraperitoneally with PBS, 0.2 mL of normal goat serum (NGS), or PNAG-specific goat antiserum raised to a vaccine containing 9GlcNH₂-TT 24 and 4 h before infection. Bacteria were grown overnight in LB and then resuspended in sterile PBS to ~5 × 10⁸ to 5 × 10⁹ cfu/mL.

**Statistical analysis**

Non-parametric data were evaluated by the Mann–Whitney U-test for two-group comparisons and by the Kruskal–Wallis test for multi-group comparisons, with Dunn’s multiple comparison test for pairwise comparisons. Parametric data were analysed by t-tests (for two-group comparisons) and ANOVA (for multi-group comparisons).

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**Figure 2.** *K. pneumoniae* and PNAG production. Detection of PNAG on the surface of *K. pneumoniae* K2. (a) Immunofluorescence confocal microscopy. (b) Flow cytometry analyses. Binding of MAB F598 to PNAG conjugated to AF488 [green fluorescence in (a) and green curve in (b)]. No binding of the control MAB [lack of green fluorescence in (a) and blue curve in (b)]. In (a), SYTO 83 was used to visualize DNA (red fluorescence). Bacteria tested in (a): *K. pneumoniae* K2, *K. pneumoniae* K2 ΔpgaC and *K. pneumoniae* K2 ΔpgaC (pUCP18::pgaC) (c). Impact of loss of PNAG on the virulence of *K. pneumoniae* K2: C3H/H3N mice (8/group) were challenged intraperitoneally with *K. pneumoniae* K2 (KpK2) ΔpgaC (KpK2ΔpgaC). (d) Opsonophagocytic killing of *K. pneumoniae* K2 mediated by polyclonal antibodies or MAbs to PNAG. Bars indicate mean percentage of killing relative to controls containing NGS or the control MAB F429. All standard deviations (not shown) were <13%. Assays were done in duplicate. C’, complement; absence of killing arbitrarily assigned as 1% killing. Dilutions of polyclonal antibodies and amounts (in μg) of MAbs to PNAG tested are provided at the top of each bar.
or ANOVA with Tukey’s multiple comparison test for pair-wise comparisons. Survival analysis utilized the Kaplan–Meier method. All analyses used GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA).

Results

PNAG production in E. cloacae and K. pneumoniae

An initial bioinformatic analysis indicated an intact pga locus was present in the genomes of most available sequences of strains of E. cloacae and K. pneumoniae, predictive of PNAG expression. Using both immunofluorescence–confocal microscopy and FACS, PNAG was detected on the surface of E. cloacae, as evidenced by the binding of the human MAb F598 to PNAG (Figure 1). MAb F598 reactivity was retained after treatment of cells with chitinase, but lost after treatment with the PNAG-specific hydrolytic enzyme dispersin B18,19 (Figure S1), as well as after treatment with sodium metaperiodate, which can only degrade glucosamine residues linked β-(1→6) (Figure S1). PNAG produced by E. cloacae contributed to biofilm formation (Figure 1c), with significantly less production in the PNAG negative strain E. cloacae 2A ΔpgaC (P < 0.001). Biofilm production was restored in the E. cloacae ΔpgaC (pUCP18::pgaC) strain, in which the pgaC gene was provided by trans complementation (Figure 1c). Opsonic killing of E. cloacae 2A could be mediated by both polyclonal antibodies and the MAb to PNAG (Figure 1d). Identical results were obtained with a second strain, E. cloacae T2 (Figure S2a and b).

As with E. cloacae, the presence of the pga locus in K. pneumoniae K2 was associated with production of surface PNAG that was lost once the pgaC gene was deleted and restored when complemented back.

Figure 3. PNAG production by NDM-1-producing Enterobacteriaceae and in vitro killing by PNAG antibodies. Detection of PNAG on the surface of NDM-1-producing Enterobacteriaceae strains using immunofluorescence confocal microscopy (left panels). Binding of MAb F598 to PNAG conjugated to AF488 results in green fluorescence. SYTO 83 was used to visualize DNA (red fluorescence). Bacteria tested: NDM-1-producing E. coli, K. pneumoniae and E. cloacae (a, b, c left panels, respectively). Digestion with the PNAG-degrading enzyme dispersin B, but not a control enzyme, chitinase, resulted in loss of binding of MAb F598. Opsonophagocytic killing of NDM-1-producing E. coli, K. pneumoniae and E. cloacae (a, b, c, right panels, respectively) mediated by polyclonal antibodies or MAbs to PNAG. Bars represent mean percentage of killing relative to control containing NGS or the control MAb F429. All standard deviations (not shown) were <15%. Assays were done in duplicate. C′, complement; absence of killing arbitrarily assigned as 1% killing. Dilutions of polyclonal antibodies and concentrations (in μg) of MAbs to PNAG tested are provided at the top of each bar.
In *K. pneumoniae*, PNAG was also found to be a virulence factor with significantly fewer mice becoming moribund or dying \((P, 0.01)\) in a mouse model of peritonitis when comparing animals infected with the *K. pneumoniae* K2 pgaC strain with those infected with the WT strain. Antibodies to PNAG were also able to mediate opsonophagocytic killing of *K. pneumoniae* K2 (Figure 2(d)).

In vitro and in vivo activity of PNAG antibodies against NDM-1-producing *E. coli*, *K. pneumoniae* and *E. cloacae*

PNAG was expressed in all strains of *E. coli*, *K. pneumoniae* and *E. cloacae* carrying the carbapenemase-producing NDM-1 plasmid (Figure 3a–c, left panels). PNAG production allowed the in vitro killing of these bacteria by antibodies to this antigen (Figure 3a–c, right panels). In a mouse model of peritonitis, administration of antibodies to PNAG were also able to mediate opsonophagocytic killing of *K. pneumoniae* K2 (Figure 2(d)).

**PNAG expression and protection against KPC-producing *K. pneumoniae***

In addition to NDM-1, KPC is the other major carbapenemase associated with pan-resistant Gram-negative bacterial infections. KPC are primarily produced by *K. pneumoniae* and KPC strains are responsible for both sporadic individual infections and severe outbreaks.\(^{20,21}\) Production of the plasmid-borne KPC did not interfere with PNAG production (Figure S3) and antibodies to PNAG were able to kill KPC-producing strains of *K. pneumoniae* in vitro.

For in vivo experiments, control and immune sera were injected intraperitoneally or intravenously followed by intraperitoneal challenge with 10^8 cfu/mouse. Significantly fewer bacteria \((P, 0.001)\) were recovered from the livers of the mice injected with PNAG-immune serum compared with the control serum (Figure 5a). The human MAb F598 to PNAG was next evaluated for protective efficacy. Various doses were injected intraperitoneally into mice followed by intravenous injection of 10^9 cfu of *K. pneumoniae* KPC (Figure 5b). Significantly fewer mice became moribund or died after receiving 100 μg of MAb F598 compared with mice immunized with 100 μg of the control MAb F429 \((P = 0.01)\). Injecting lower doses of MAb F598 led to progressive reductions in protection. Polyclonal antibodies to the 9GlcNH2-TT vaccine were also significantly protective against intravenous challenge with *K. pneumoniae* KPC \((P = 0.02, \text{Figure 5b})\).

**PNAG production in *P. aeruginosa***

To further validate that antibodies to PNAG could be protective against any Gram-negative pathogenic bacterial species producing...
PNAG, the pga locus from E. coli was introduced into WT and alginate-negative P. aeruginosa, a species that does not carry a native pga locus or produce PNAG. This antigen was not detected on the surface of WT P. aeruginosa (Figure S4), although alginate was, as previously reported. Interestingly, no PNAG was detected at the surface of WT PA14 after introduction of the E. coli pga locus (Figure 6a). We hypothesized that this could be due to production of alginate, a cell-surface polymer with a charge opposite to that of PNAG, and that elimination of alginate would allow PNAG production in the strain carrying pUCP18::pga. P. aeruginosa with a Tn insertion in the algD gene needed for alginate synthesis and transformed with pUCP18::pga had detectable PNAG on the surface (Figure 6b, right panel). No alginate was detected on this strain (Figure 6b, left panel). The same result was obtained with P. aeruginosa PA14 with Tn insertions in the algF or algJ gene: no PNAG was detected at the surface of alginate-deficient P. aeruginosa carrying only the pgaA gene on pUCP18. Identical results were obtained with another P. aeruginosa strain, FRD1. As with strain PA14, once the production of alginate was removed, PNAG was detected at the surface of the strains carrying pUCP18::pga.

In mice challenged intraperitoneally with 4 × 10^7 cfu of the PNAG-producing strain PA14 Tn::algD (pUCP18::pga), significantly fewer animals became moribund following passive transfer of antibody to PNAG compared with the group immunized with NGS or PBS (Figure 6b, P = 0.0013, log-rank test). Antibodies to PNAG did not have an effect on infection with the PNAG-negative parental strain PA14 Tn::algD (Figure 6c). A >1 log higher challenge dose of this strain, 5 × 10^8, was required to achieve similar levels of lethality compared with the strain producing PNAG, presumably due to the decreased virulence associated with loss of surface polysaccharides. Of note, the PNAG-producing P. aeruginosa PA14 is comparably virulent in mice to the alginate-producing WT PA14 strain.

**Discussion**

Class A and B carbapenemases can hydrolyse nearly all β-lactams, including carbapenems, which are among the last drugs remaining for the treatment of MDR Gram-negative infections. K. pneumoniae producing these carbapenemases have spread globally and are now endemic in the USA, Israel, Greece and Italy. More recently, NDM-producing Enterobacteriaceae appeared and are disseminating from South Asia and Northern Africa. Mortality from invasive CRE infections reaches 40%. This situation demands new treatments for these infections, particularly using approaches such as immunotherapy, which likely has the advantage of being independent of the issues associated with the emergence of being independent of the issues associated with the emergence of antibiotic resistance.
and dissemination of antibiotic resistance. However, most immunotherapies are limited because antibodies usually target an antigen specific to one bacterial species or strain within a species. Thus, no immunotherapy has ever been developed with the potential to have a broad spectrum in the same manner as mainstream antibiotics such as β-lactams, aminoglycosides or fluoroquinolones. Indeed,

![Image](https://example.com/image.png)

**Figure 6.** Impact of PNAG production in *P. aeruginosa* on protective efficacy of antibody to PNAG. (a) Detection of PNAG on the surface of *P. aeruginosa*. Binding of MAb F598 to PNAG conjugated to AF488 results in green fluorescence. SYTO 83 was used to visualize DNA (red fluorescence). The overlap of the green and red colour is presented in the bottom of each panel. WT *P. aeruginosa* PA14 carrying a cloned *pga* locus [PA14 WT (pUCP18::*pga*)] was positive for alginate production, but negative for PNAG. *P. aeruginosa* PA14 with a Tn insertion in the *algD* gene to inactivate alginate production (PA14 Tn::*algD*) with a cloned *pga* locus [PA14 Tn::*algD* (pUCP18::*pga*)] was negative for alginate, but positive for PNAG production. (b) C3H/H3N mice were passively immunized with PBS (4 mice), NGS (6 mice) or PNAG-specific goat immune serum (6 mice) intraperitoneally 24 and 4 h before intraperitoneal challenge with PNAG-producing *P. aeruginosa* PA14 Tn::*algD* (pUCP18::*pga*) (4 × 10⁷ cfu/mouse). (c) C3H/H3N mice (n = 8/group) were passively immunized with either NGS or PNAG-specific goat immune serum intraperitoneally 24 and 4 h before intraperitoneal challenge with PNAG- and alginate-negative *P. aeruginosa* PA14 Tn::*algD* (5 × 10⁷ cfu/mouse). P values by log-rank test comparing NGS with antibodies with PNAG.

**Table 1.** Antibodies to PNAG: in vivo activity against MDR and pan-resistant Gram-negative bacteria

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<tr>
<th>Bacterial species</th>
<th>Method of immunization</th>
<th>Challenge route</th>
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<tr>
<td><em>E. coli</em> NDM-1</td>
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<td><em>K. pneumoniae</em> NDM-1</td>
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<td>PNAG-producing <em>P. aeruginosa</em></td>
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<td><em>B. cepacia</em> complex</td>
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<td>lethality&lt;sup&gt;+&lt;/sup&gt; this study</td>
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and dissemination of antibiotic resistance. However, most immunotherapies are limited because antibodies usually target an antigen specific to one bacterial species or strain within a species. Thus, no immunotherapy has ever been developed with the potential to have a broad spectrum in the same manner as mainstream antibiotics such as β-lactams, aminoglycosides or fluoroquinolones. Indeed,
for Gram-negative bacteria, the most effective immunological approaches target highly variable antigens such as capsules and LPS O polysaccharides, making it quite challenging to produce comprehensive immunotherapies for these organisms. This situation can now be addressed as it has been demonstrated that a chemically and structurally conserved surface polysaccharide, PNAG, is present on many microbial pathogens, and antibodies raised to specific glycoforms of this antigen can provide effective immunotherapy in laboratory animal settings.1–5,7,27 This approach was extended and further validated in this study by showing that PNAG-expressing MDR Gram-negative bacteria can be killed by antibodies to PNAG and protection achieved in mouse models, thus making immune effectors to this antigen an extended-spectrum immunotherapeutic targeting MDR pathogens.

This conclusion is supported by the in vitro and in vivo efficacy of PNAG antibodies against NDM-1 and KPC-producing Enterobacteriaceae species commonly responsible for human infections. A further proof of principle used P. aeruginosa producing PNAG to confirm that antibodies to this antigen were protective even in a non-natural setting of PNAG production. PNAG also contributed to biofilm production by E. cloacaе and was a virulence factor in a murine model of K. pneumoniae infection. Antibodies to PNAG mediated opsonophagocytic killing in vitro and protection in vivo against all WT and MDR strains tested using different challenge routes. While OXA-48-type carbapenem-hydrolysing class D β-lactamase-producing strains are increasingly reported in enterobacterial species,28 they were not tested in this study. Nonetheless, the constellation of data presented indicate the potential to use immunotherapy against PNAG as a broad-spectrum antibody against multiple pathogens and that its efficacy will be unaffected by the mechanism of antibiotic resistance (Table 1).

Another advantage of this approach is that in this study, and in previous reports,5,6,15,29–31 PNAG is frequently found to be a virulence factor when the virulence of PNAG-negative strains is compared with parental WT strains in animal models of infection. This reduces the impact of the loss of PNAG production to avoid immune effectors, inasmuch as virulence will likely be frequently compromised. This outcome is in contrast to our recent demonstration22,23 that acquisition of antibiotic resistance was associated with an increase in virulence in animal models of infection, a worrisome finding in that the emergence of spread of resistant strains could also promote the spread of more virulent bacterial pathogens.

A Phase I clinical trial evaluating the safety and pharmacokinetics of MAb F598 to PNAG has been successfully completed,33 and the design of a Phase II trial is currently being finalized. In addition, the production of a vaccine to generate functional antibodies to PNAG in humans will be completed and tested in 2016. Therefore, the potential for a vaccine against infectious agents, a way easier to possibly treat many of the major Gram-negative pathogenic organisms, including those with MDR or pandrug-resistant (PDR) phenotypes, may be realized.

**Funding**

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**Supplementary data**

A full description of the methods, Tables S1 and S2 and Figures S1 to S4 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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