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1 **Temocillin: a new candidate antibiotic for local antimicrobial delivery in orthopaedic surgery?**

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9
10 Running title: Assessing temocillin performance in bone cement

11
12 Keywords: Bone cement, elution, impact strength

13 **Objectives**

14 To assess the performance of the Gram negative-specific antibiotic temocillin in
15 polymethylmethacrylate (PMMA) bone cement pre-loaded with gentamicin, as a strategy for local
16 antibiotic delivery.

17 **Methods**

18 Temocillin was added at varying concentrations to commercial gentamicin-loaded bone cement. The
19 elution of the antibiotic from cement samples over a two week period was quantified by HPLC-MS.
20 The eluted temocillin was purified by fast protein liquid chromatography and minimum inhibitory
21 concentration (MIC) for a number of antibiotic-resistant *Escherichia coli* determined. The impact
22 strength of antibiotic-loaded samples was determined using a Charpy-type impact testing apparatus.

23 **Results**

24 HPLC-MS data showed temocillin eluted to clinically significant concentrations within 1 h in this
25 laboratory system and the eluted temocillin retained antimicrobial activity against all organisms
26 tested. Impact strength analysis showed no significant difference between cement samples with or
27 without temocillin.

28 **Conclusions**

29 Temocillin can be added to bone cement and retains its antimicrobial activity after elution. The
30 addition of up to 10% temocillin did not affect the impact strength of the cement. The results show
31 that temocillin is a promising candidate for use in antibiotic loaded bone cement.

32 **Keywords:** temocillin, bone cement, antibiotic elution, antimicrobial activity

33

34 **Introduction**

35 In the UK during 2012, bone cement was used in 54% of total primary hip replacements (including
36 21% hybrid cemented/cementless procedures) and 86% of total primary knee replacements (including
37 <1% hybrid procedures), equating to approximately 150,000 arthroplastic operations. The use of
38 antibiotic-loaded cement in primary hip replacement procedures has increased from 73% in 2004 to
39 89% in 2012. Similarly the use of antibiotic-loaded cement in primary knee replacement procedures
40 has increased from 87% in 2003 to 98% in 2012.¹ Adding one or more prophylactic antibiotics to
41 cement has been shown to reduce postoperative infection rates.² Due to the wide range of organisms
42 that can contribute to prosthetic joint infections and problems with antibiotic-resistant bacteria, an
43 increasing range of antibiotics need to be available for addition to bone cement.

44 Temocillin is a β -lactam antibiotic resistant to hydrolysis by most β -lactamases, due to the presence of
45 6- α -methoxy group, which stabilizes the molecule against hydrolysis by many such enzymes.^{3,4} A
46 substantial minority of prosthetic joint infections are caused by Gram negative bacteria, most notably
47 Enterobacteriaceae such as *Escherichia coli*, *Klebsiella* spp. and *Proteus* spp.⁵⁻⁷ Temocillin is
48 effective against organisms expressing a range of extended spectrum β -lactamases (ESBL), including
49 some carbapenem resistant species.⁸⁻¹⁰ We envisage that if temocillin could be used as a locally
50 delivered antimicrobial for orthopaedic surgery, the most likely context in which it would be
51 employed would be in combination with gentamicin. Gentamicin is a well-established additive to
52 bone cement that gives protection against Gram positives such as Staphylococci which are the most
53 common cause of prosthetic joint infection.^{11,12} In such a situation, temocillin (which does not show
54 antagonistic interaction with gentamicin⁴) would give protection against Gram negatives, including

55 gentamicin resistant organisms such as ESBL-producers, possibly in revision surgery in patients with
56 a history of infection of the prosthesis with an ESBL-producing Gram-negative organism.

57

58 **Methods**

59 Temocillin was added at varying concentrations to gentamicin-containing Refobacin Bone Cement R
60 (Biomet). Bone cement was mixed in a HiVac mixing bowl according to manufacturer's instructions
61 and set in 5 mm × 9 mm diameter plastic moulds. Bone cement was allowed to cure for 1 h then
62 stored at -20°C. The bone cement samples were submerged in 0.1 M ammonium acetate solution and
63 aliquots taken at 0, 1, 2, 6, 24, 48, 72, 168 and 336 h (14 days). Eluted temocillin and gentamicin
64 concentrations were quantified by LC-MS using a Phenomenex Luna C18 (2) column coupled to a
65 Finnigan LCQ ion trap Mass spectrometer. The isocratic mobile phase for detection of temocillin
66 consisted of 60 % (v/v) acetonitrile 0.1 % (v/v) trifluoroacetic acid at a flow rate of 0.05 mL/min. For
67 detection of gentamicin an isocratic mobile phase of 40 % (v/v) methanol 0.1 % (v/v) trifluoroacetic
68 acid at a flow rate of 0.05 mL/min was used. The use of volatile ammonium acetate solution as a
69 buffer (rather than a standard buffer such as phosphate buffered saline) allowed direct analysis of the
70 eluate by HPLC using electrospray ionisation MS, without the need for a desalting step. The mass
71 spectrometer was operated with an ESI source in positive ion mode with a source voltage of 4.5 kV,
72 sheath gas flow 80 (arbitrary units), and capillary temperature 250 °C. Detection of antibiotic was
73 carried out using selected ion monitoring at 437 m/z corresponding to the temocillin sodium adduct
74 $[M + Na]^+$ or 478 m/z corresponding to the protonated gentamicin C1 component $[M + H]^+$.
75 Antibiotic concentration was determined by linear regression to a standard calibration curve with a
76 correlation coefficient (R^2) of >0.99 for each antibiotic. Method validation was carried out by
77 analysing standard solutions of each antibiotic (n=3) at 10 mg/L 100 mg/L and 400 mg/L over 5
78 hours and on 3 separate days to determine interday and intraday variation respectively. Temocillin
79 analysis showed an interday coefficient of variation (%CV) ranging from 0.98 - 5.33 and an intraday
80 %CV ranging from 5.47 - 13.00. Gentamicin analysis showed an interday %CV ranging from 4.25 -

81 11.61 and an intraday %CV ranging from 12.05 - 19.98. Temocillin samples eluted during the first 24
82 hours of bone cement elution assays were pooled, separated from gentamicin by fast protein liquid
83 chromatography using a HiTrap SP 5mL ion exchange column (GE Healthcare) and MICs for a
84 number of *Escherichia coli* strains determined by broth micro-dilution method.¹³ The MIC values for
85 eluted temocillin were compared to the MIC values for a standard temocillin solution determined
86 using the same method. Impact analysis of bone cement samples was carried out using a Charpy-type
87 Hounsfield plastic impact testing apparatus^{14,15} and statistical analysis was carried out using Analysis
88 of Variance function in Microsoft Excel software.

89 **Results**

90 *Kinetics of antibiotic elution*

91 When bone cement samples containing temocillin at various concentrations and gentamicin at 1.25 %
92 (w/w) were placed in buffer solution to allow elution of the antibiotic, the highest concentration of
93 eluted temocillin was 3051 ± 264 mg/L after 336 h (14 days) in eluate from the cement samples
94 containing temocillin at 10% (w/w) (Fig. 1b). Similar samples containing 5% and 1.25% (w/w)
95 temocillin produced lower concentrations of temocillin, at 1337 ± 427 mg/L and 327 ± 91 mg/L,
96 respectively, after 336 h (Fig. 1b). In contrast gentamicin concentration was highest from the cement
97 sample containing 1.25% temocillin (1380 ± 290 mg/L of gentamicin after 336 h) (Fig 1d). The
98 samples containing 5% and 10% temocillin gave 400 ± 170 mg/L and 490 ± 180 mg/L of eluted
99 gentamicin, respectively, after the same period of elution.

100

101 *Activity of the eluted temocillin*

102 MICs of the eluted temocillin were measured against several strains of *E. coli* (Table 1) after
103 chromatographic separation from the eluted gentamicin, in order to confirm that the temocillin
104 retained its antimicrobial activity after incorporation into the bone cement and subsequent elution.
105 Results were compared against the MICs determined a standard solution of temocillin that had not

106 been in contact with bone cement. The MICs determined using the eluted and standard temocillin
107 solutions were comparable for all strains tested and in line with published data for temocillin-
108 susceptible strains.¹⁶ Hence temocillin retained its antimicrobial activity after elution from the bone
109 cement. The range of MICs observed for the different strains can be attributed to varying (low) levels
110 of resistance that would be expected between different isolates of these types.

111 *Impact analysis*

112 The results of the impact analysis showed no significant difference in the impact strength of bone
113 cement containing 1.25 % (w/w) gentamicin with or without temocillin at ($P > 0.05$) (see
114 supplementary Figure S1

115 **Discussion**

116 The data presented here show that temocillin is a promising candidate for antibiotic-loaded bone
117 cement delivery strategies. The temocillin is not degraded by the elevated temperatures during the
118 cement curing process and retains its antimicrobial activity, which is still detectable in the eluate up to
119 2 weeks later. Antimicrobial activity of the eluted temocillin was confirmed with a range of *E. coli*
120 strains, including a laboratory strain (DH5 α) and recent clinical isolates expressing ESBLs, of the type
121 that might require the use of temocillin in the bone cement during a joint revision operation in a
122 patient with a history of periprosthetic infection with such an organism. The concentrations of eluted
123 temocillin and gentamicin exceeded MIC values for susceptible strains within the first hour of elution
124 in this laboratory system. This result may be important in a clinical setting since it indicates that the
125 antibiotic-loaded bone cement could provide effective antimicrobial prophylaxis during the
126 perioperative period (hip and knee replacement operations typically take 1-2 h) and it may be
127 beneficial that active antibiotic continues to elute during the postoperative period. Increasing the
128 percentage of temocillin within the bone cement produced larger concentrations of eluted temocillin.
129 An increase in the amount of temocillin from 1.25% (w/w) to 5 or 10 % (w/w) also led to an
130 unexpected decrease in gentamicin elution of approximately 3-fold by 336 h. The antibiotic elution
131 experiments measured cumulative elution into a single buffer which was not replaced. When higher

132 concentrations of antibiotic were present in the buffer, this may have affected subsequent elution of
133 antibiotic. This could explain the reduced elution of gentamicin from the bone cement containing
134 higher concentrations of temocillin. If there was a large initial release of temocillin into the buffer as
135 was seen at 5 and 10% temocillin concentrations, this may have inhibited the release of gentamicin
136 into the same buffer. Although substantial, we believe that this difference in gentamicin elution is
137 unlikely to be clinically significant because no reduction in gentamicin elution was observed during
138 the critical perioperative period (typically 1-2 h),¹⁷ and the eluted concentration of gentamicin in this
139 laboratory system exceed the in the MICs of susceptible organisms such as staphylococci (typically <
140 1 mg/L) by at least 140-fold by the time of the first sample (<1 min).^{13,18} In addition to its favourable
141 elution and antimicrobial properties, up to 10% (w/w) of temocillin can be added to commercial
142 gentamicin-containing bone cement without detrimental effect on the impact strength of the cement.
143 However a number of other groups have carried out tests looking at different mechanical properties of
144 bone cement and shown a detrimental effect of high loading of antibiotic on these mechanical
145 properties. These data should be taken into account before deciding on a concentration of temocillin
146 within bone cement. Lautenschlager *et al.* showed that increasing amounts of gentamicin caused a
147 gradual, proportional decrease in the compressive and diametral tensile strength of the bone cement. It
148 was noted that above 11.25% gentamicin (w/w), compressive strength dropped below levels
149 recommended in ASTM guidelines.¹⁹ Studies looking at increasing amounts of daptomycin and
150 gentamicin showed that fatigue limits decreased with increasing antibiotic concentrations and gave
151 optimum loading concentrations of 3.4% and 4.78-6.5% respectively.^{20,21} The data shown here
152 indicate that temocillin is a promising candidate for inclusion in antibiotic-loaded bone cement that
153 may be a useful tool in combating Gram negative prosthetic joint infection.

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159 **Transparency Declarations**

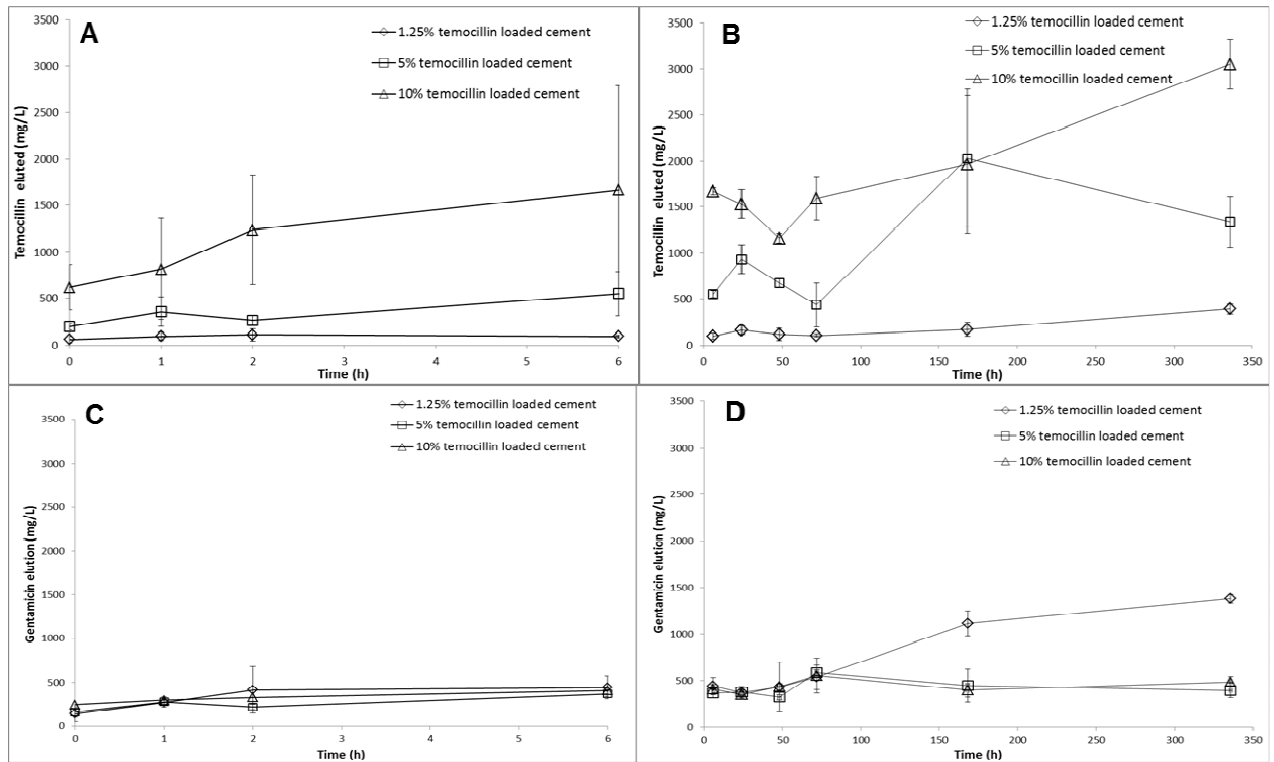
160 None.

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215 **Fig 1** A) Elution of temocillin up to 6 hours, B) elution of temocillin from 6 - 336 hours, C) elution
 216 of gentamicin up to 6 hours and D) elution of gentamicin from 6 - 336 hours. Bone cement
 217 samples initially contained 1.25% (w/w) gentamicin and varying amount of temocillin as
 218 indicated. Bone cement samples were immersed without buffer change and aliquots of eluate
 219 analysed over 336 hours. Results are shown as the mean of 3 separate experiments \pm standard
 220 deviation
 221
 222
 223
 224
 225

<i>E. coli</i> strain	Eluted temocillin ^A	Standard temocillin
	MIC (mg/L)	MIC (mg/L)
DH5- α	3.8 - 7.6	3.1
AmpC expressing strain	1.9 - 3.8	3.1 - 6.3
SHV-1 expressing strain	3.8 - 7.6	6.3
orthopaedic isolate from infected prosthesis	15.3	6.3
temocillin susceptible ESBL producing strain -1	15.3	12.5
temocillin susceptible ESBL producing strain -2	15.3	6.3 - 12.5

226

227 **Table 1.** MICs for a range of *E.coli* strains determined by broth microdilution method using eluted temocillin and standard antibiotic solutions.

^A Temocillin samples eluted during the first 24 hours of bone cement elution assays were pooled and separated from gentamicin using ion exchange chromatography. The concentration of temocillin was quantified by HPLC and serial dilutions of the purified temocillin used to determine MICs.