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Targeting C-reactive protein for the treatment of cardiovascular disease

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Abstract

Complement-mediated inflammation exacerbates the tissue injury of ischaemic necrosis in heart attacks and strokes, the most common causes of death in developed countries. Large infarct size increases immediate morbidity and mortality and, in survivors of the acute event, larger non-functional scars adversely affect long-term prognosis. There is thus an important unmet medical need for new cardioprotective and neuroprotective treatments. We have previously shown that human C-reactive protein (CRP), the classical acute-phase protein that binds to ligands exposed in damaged tissue and then activates complement1, increases myocardial and cerebral infarct size in rats subjected to coronary or cerebral artery ligation, respectively2,3. Rat CRP does not activate rat complement, whereas human CRP activates both rat and human complement4. Administration of human CRP to rats is thus an excellent model for the actions of endogenous human CRP2,3. Here we report the design, synthesis and efficacy of 1,6-bis(phosphocholine)-hexane as a specific small-molecule inhibitor of CRP. Five molecules of this palindromic compound are bound by two pentameric CRP molecules, crosslinking and occluding the ligand-binding B-face of CRP and blocking its functions. Administration of 1,6-bis(phosphocholine)-hexane to rats undergoing acute myocardial infarction abrogated the increase in infarct size and cardiac dysfunction produced by injection of human CRP. Therapeutic inhibition of CRP is thus a promising new approach to cardioprotection in acute myocardial infarction, and may also provide neuroprotection in stroke. Potential wider applications include other inflammatory, infective and tissue-damaging conditions characterized by increased CRP production, in which binding of CRP to exposed ligands in damaged cells may lead to complement-mediated exacerbation of tissue injury.

Keywords

Targeting, reactive, protein, for, treatment, cardiovascular, disease

Disciplines

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Targeting C-reactive protein for the treatment of cardiovascular disease

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Complement-mediated inflammation exacerbates the tissue injury of ischaemic necrosis in heart attacks and strokes, the most common causes of death in developed countries. Large infarct size increases immediate morbidity and mortality and, in survivors of the acute event, larger non-functional scars adversely affect long-term prognosis. There is thus an important unmet medical need for new cardioprotective and neuroprotective treatments. We have previously shown that human C-reactive protein (CRP), the classical acute-phase protein that binds to ligands exposed in damaged tissue and then activates complement[1], increases myocardial and cerebral infarct size in rats subjected to coronary or cerebral artery ligation, respectively[2,3]. Rat CRP does not activate rat complement, whereas human CRP activates both rat and human complement[4]. Administration of human CRP to rats is thus an excellent model for the actions of endogenous human CRP[2,3]. Here we report the design, synthesis and efficacy of 1,6-bis(phosphocholine)-hexane as a specific small-molecule inhibitor of CRP. Five molecules of this palindromic compound are bound by two pentameric CRP molecules, crosslinking and occluding the ligand-binding B-face of CRP and blocking its functions. Administration of 1,6-bis(phosphocholine)-hexane to rats undergoing acute myocardial infarction abrogated the increase in infarct size and cardiac dysfunction produced by injection of human CRP. Therapeutic inhibition of CRP is thus a promising new approach to cardioprotection in acute myocardial infarction, and may also provide neuroprotection in stroke. Potential wider applications include other inflammatory, infective and tissue-damaging conditions characterized by increased CRP production, in which binding of CRP to exposed ligands in damaged cells may lead to complement-mediated exacerbation of tissue injury.

No inhibitors of calcium-dependent binding of human CRP to phosphocholine, the natural ligand for which it has highest affinity, were detected in a screen of the comprehensive 500,000 small-molecule library of a pharmaceutical company. We therefore rationally designed novel ligands for CRP based on the crystal structure of the CRP–phosphocholine complex[5] and the structure of a potent drug we have developed that targets the homologous protein, serum amyloid P component (SAP)[6]. The SAP inhibitor consists of two D-proline residues linked by a hexanoyl chain, and the SAP–drug complex contains two SAP molecules crosslinked by five drug molecules, with each D-proline located in the calcium-dependent ligand-binding pocket of an SAP protomer[6].

In the CRP–phosphocholine complex, one phosphocholine molecule is positioned parallel to the pentamer surface on each of the five CRP subunits, and is oriented with the choline moieties, pinned between Glu 81

and Phe 66, towards the fivefold axis[5]. Two oxygen atoms of the phosphocholine phosphate group directly coordinate the two bound calcium ions of CRP, but the third is oriented away from the protein surface[5], providing a suitable exit point to direct a crosslinking chain towards a putative twofold-axis-related subunit of an adjacent CRP pentamer. We selected a six-carbon linker to connect the phosphate groups, expecting a greater separation between binding sites of a putative CRP decamer caused in part by rotation of ~20^{o} of each CRP subunit towards the fivefold axis of the pentamer compared to SAP. This new chemical entity, composed of two phosphocholines with a hexane linker, 1,6-bis[{[(trimethylammonium) ethoxy]phosphinyl}-oxy]hexane, colloquially 1,6-bis(phosphocholine)-hexane (bis(PC)-H, Fig. 1), was synthesized at high yield (see Supplementary Information).

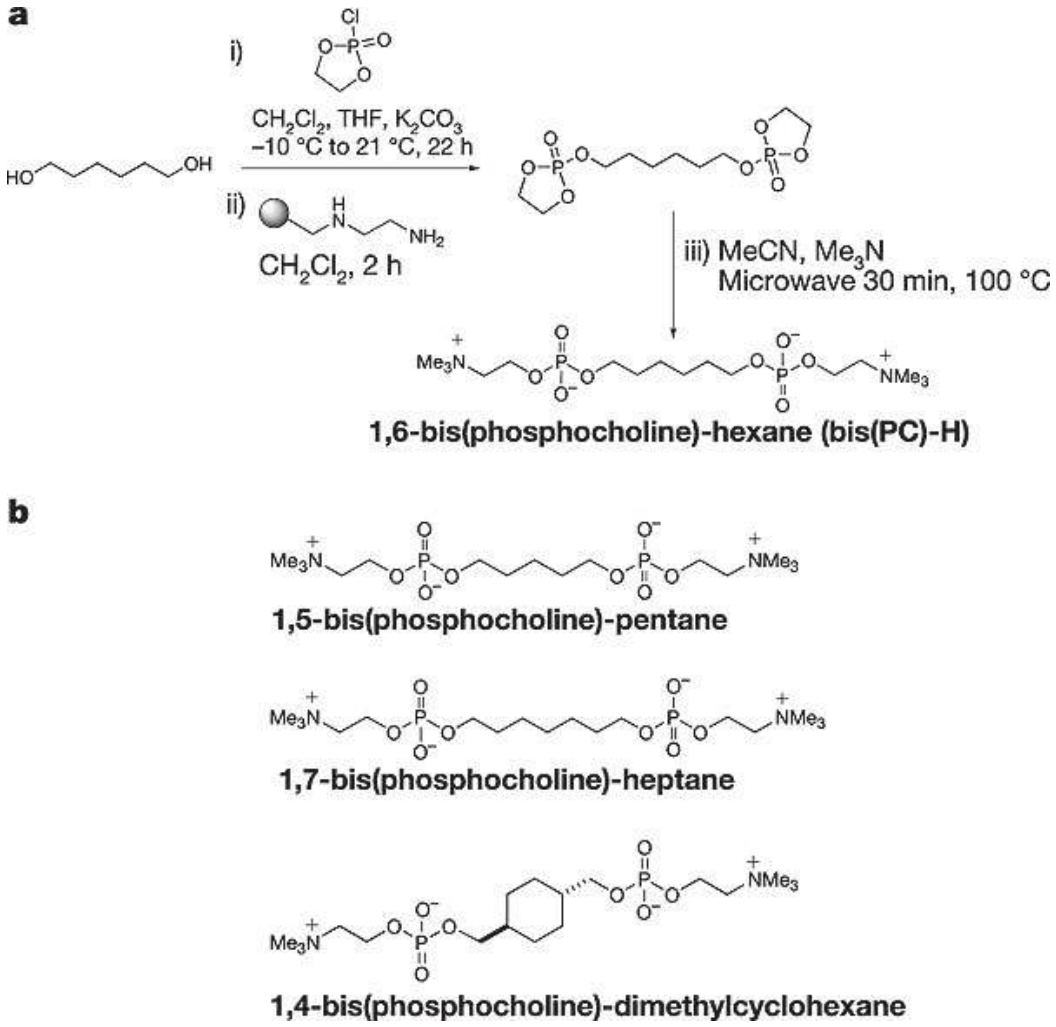

Figure 1. Synthesis and structure of bis-phosphocholine compounds. a. Synthesis and structure of bis(phosphocholine)-hexane. b. Structures of bis-phosphocholine compounds with different linkers. See Supplementary Information.

Bis(PC)-H was bound by human CRP in a calcium-dependent manner in physiological saline solution, with a dissociation constant (K_d) of ~300nM by isothermal titration calorimetry. Bis(PC)-H inhibited calcium-dependent binding of CRP to all of its other known ligands, including phosphoethanolamine, modified lowdensity lipoprotein, and late apoptotic or necrotic cells. The IC_{50} for inhibition by bis(PC)-H of CRP binding to immobilized pneumococcal C-polysaccharide was ~2 μM, compared to ~20 μM for free phosphocholine. Bis(PC)-H also blocked complement activation by human CRP and C-polysaccharide in

human serum. In mixtures of drug and CRP at molar ratios between 1:1 and 1:1,000 with respect to CRP protomers, all CRP molecules became associated in pairs, as shown by size-exclusion chromatography, electron microscopy and mass spectrometry (Fig. 2). Addition of bis(PC)-H to isolated CRP, or to CRP in whole serum, resulted in disappearance of CRP immunoreactivity using a monoclonal antibody assay (Roche) specific for a calcium-dependent epitope on the binding (B) face of the CRP molecule[7], suggesting that this epitope was occluded in the CRP–drug complex. The IC_{50} was at the ratio of three bis(PC)-H per two pentameric CRP molecules. Covalent crosslinking of CRP molecules in the CRP–drug complex, followed by fragmentation and mass spectrometric analysis (data not shown), confirmed the B face–B face association.

The X-ray crystal structure of the CRP–bis(PC)-Hcomplex revealed two pentameric CRP molecules lying face-to-face with a common fivefold symmetry axis, crosslinked via their phosphocholine-binding sites by five drug molecules (Fig. 3; see Supplementary Information). The CRP pentamers are displaced by a relative rotation of 20^{o} about this axis, separating the binding sites by 15Å and inclining the axis of the bound bis(PC)-H from that of the protein complex. The structures of the protein subunits and the bound phosphocholine component of bis(PC)-Hare very similar to those observed in the CRP complex with free phosphocholine[5] (r.m.s. best fit over all Cα = 0.4Å). However, a considerably worse fit (r.m.s. all Cα = 0.8Å) was observed upon overlaying complete pentamers. This was due to a systematic movement of ß-strands by ~1Å towards the fivefold axis, resulting in a contraction of ~2Å in the pore diameter at the centre of the pentamer in the CRP-bis(PC)-H complex. There are ~25% more inter-atomic contacts of less than 3.5Å per CRP subunit than in the complex with phosphocholine. Identical cryopreservation methods were used during data collection, indicating that this effect is a specific consequence of bis(PC)-H binding by CRP. There are also two additional calcium ions bound per subunit: one by the side chain of Asp 60 and the carbonyl of Asn 59, close to the ligand-binding double calcium site, and the other by the main-chain carbonyl of Glu 70 and the side-chain carboxylate of the same residue from the twofold-symmetry-related subunit of the adjacent pentamer, providing decamer stabilization. The ligand fields of these calcium ions are limited, and they are likely to be occupied only by virtue of the high calcium ion concentration (50mM) in the crystallization cocktail. Further decamer stability is provided by ten inter-pentamer ion pairs between Lys 69 Nε and Glu 85'Oδ. All of these interactions between pentamers are orthogonal to the observed direction of contraction, and are unlikely to be its cause.

The electron density for the phosphocholine component of bis(PC)-H and the first carbon of the crosslinker is very good but, not unexpectedly for such a flexible linker, the density for the central four carbon atoms is weaker (Fig. 3c). In order to fit these atoms within the space available and to achieve the required approach path to the phosphate groups, there is an unfavourable eclipsed rotamer about the C3–C4 bond of the linker. However, 1,5-bis(phosphocholine)-pentane, with a shorter linker, was bound with substantially lower affinity (K_d ~3.7 μM), and there was no binding at all to the more rigid bis(phosphocholine)-dimethylcyclohexane (Fig. 1 and Supplementary Information). In contrast 1,7-bis(phosphocholine)-heptane (Fig. 1; Kd ~300 nM) evidently had sufficient linker length and flexibility to permit this mechanism of drug–protein interaction.

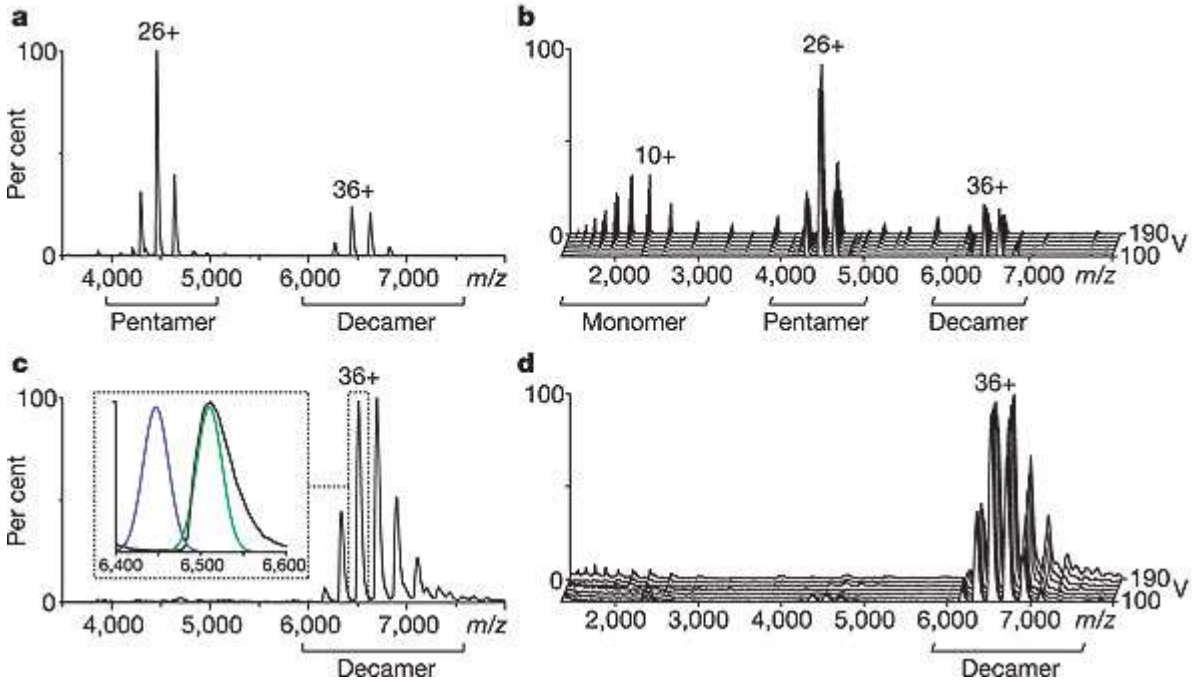

Figure 2. Nano electrospray mass spectra of CRP.
a, **b**, CRP alone yielded peaks corresponding to the native pentamer and traces of decamer, reflecting the known tendency of native CRP to aggregate. Accelerating the ions (with increasing cone voltage in the atmospheric pressure region of the mass spectrometer) increases their internal energy and causes the decamers and pentamers to dissociate, yielding predominantly free protomers at the highest cone voltages. **c**, **d**, Addition of bis(PC)-H yields exclusively decameric CRP, which is resistant to gas phase collision-induced dissociation. The peaks in the mass spectrum are shifted towards higher *m/z* values, indicating drug binding. The inset in **c** compares the 36 + charge state in the mass spectrum of the decamer in the absence of bis(PC)-H (blue) and the peak anticipated for the addition of five bis(PC)-H molecules (green), demonstrating the stoichiometry of binding (five bis(PC)-H molecules and two pentameric CRP molecules).

Bolus intravenous or intraperitoneal injections in mice and rats of up to 1 mmol kg^{-1} bis(PC)-H in physiological saline solution, or continuous infusion at 1mmol kg^{-1} per day for seven days via subcutaneous osmotic pump, were tolerated without noticeable adverse effects, and inhibited binding of injected human CRP to other ligands and its reactivity using the Roche assay[7]. The plasma half-life of bis(PC)-H in mice was ~90 min. Continuous infusion of 1 mmol bis(PC)-H per kg per day in rats completely blocked the effects of daily subcutaneous injections of 40 mg kg^{-1} human CRP (~1.74 µmol CRP protomer), despite the presence of rat CRP, which binds the drug with K_d ~150 nM, circulates at 300–500 mg l^{-1}, and is produced at the rate of ~10 µmol protomer per kg per day.

Clinical treatment with a CRP inhibitor could be started immediately upon admission to hospital following acute myocardial infarction— this would precede the acute phase CRP response, which starts about 6 h after onset of pain and peaks at about 50 h (refs 8, 9). We therefore initiated infusion of bis(PC)-H before coronary artery ligation in rats, and gave the first of five daily subcutaneous injections of human CRP immediately after recovery from surgery, closely replicating the initial dynamics of the endogenous human CRP response. Administration of human CRP was associated with increased mortality compared to vehicle-only controls, as we have previously reported[2]. In contrast, there were no deaths among the rats receiving bis(PC)-H in addition to CRP (Table 1) (Fisher's exact test for comparison of mortality in all groups, P = 0.08). Infarct size on day 5 was substantially larger in the rats treated with CRP (unpaired t-test, P = 0.0001

compared to vehicle-treated rats), but in rats receiving bis(PC)-H as well as CRP, infarct size was the same as in vehicle-only controls (Table 1). Electro- and echocardiographic indices of cardiac function on day 5 were consistent with the larger infarcts in CRP-treated rats and with a protective effect of bis(PC)-H (Table 1).

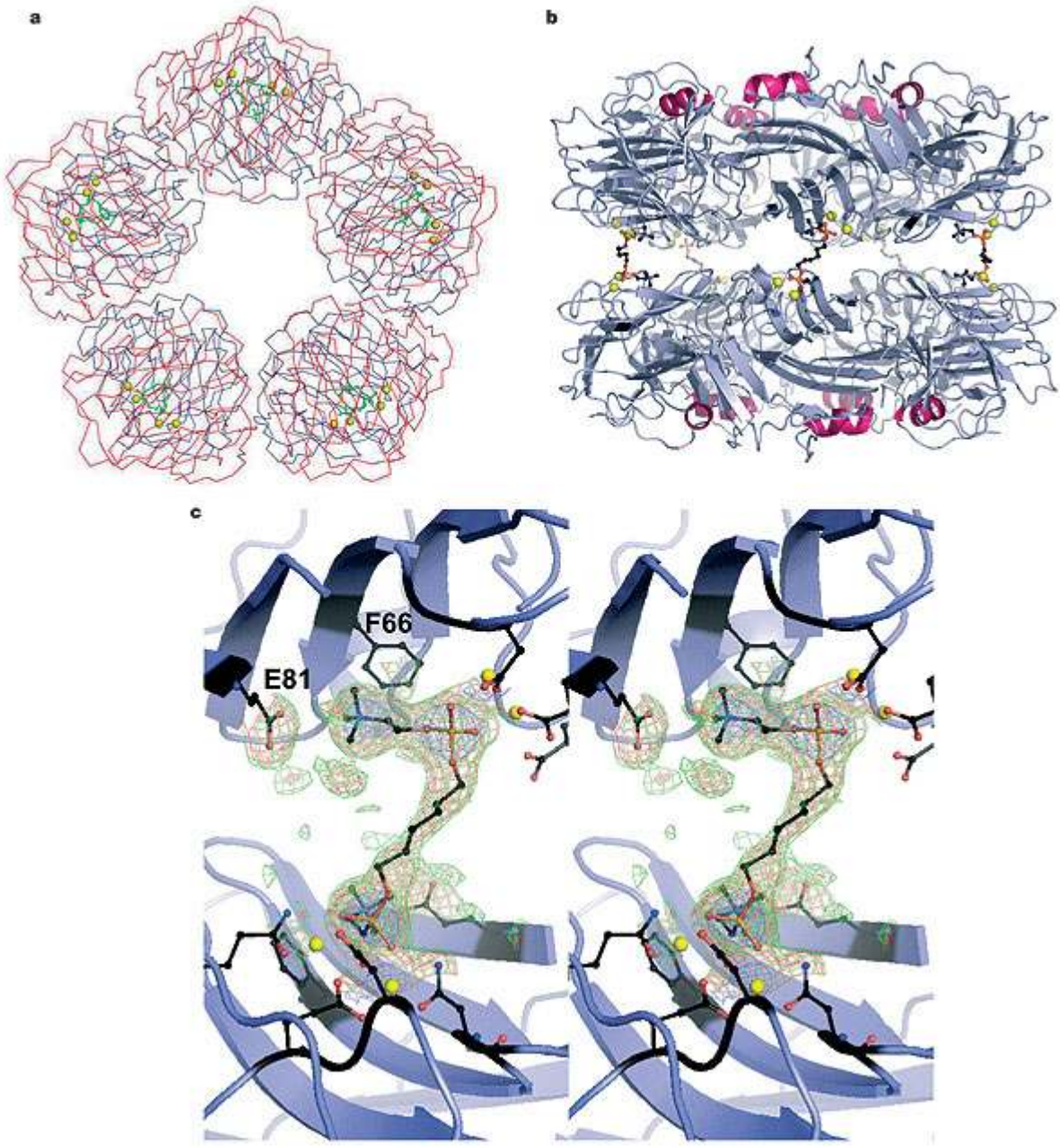

Figure 3. Structure of the complex of CRP with 1,6-bis(phosphocholine)-hexane.
a, Two CRP pentamers viewed down the fivefold axis, one in red and one in blue, crosslinked via their B-faces by five molecules of bis(PC)-H (green). **b**, View of the complex perpendicular to the fivefold axis and along the local twofold axis, relating pairs of subunits. A-face helices are in pink. Both views show the rotation of pentamers relative to each other, with corresponding displacement of calcium ions (yellow) and inclination of the drug molecules. **c**, Stereo view of electron density ($2|F_o| - |F_c|$) contoured at 3σ (blue), 1σ (red) and 0.75σ (green), and the fitted bis(PC)-H molecule showing how the buckled linker chain directs the phosphocholine head group into the double calcium site, with the choline moiety sandwiched between Phe 66 and Glu 81, and two phosphate oxygens coordinating the calcium ions. Images were prepared using PyMol.[23]

Table 1. Effect of human CRP and bis(PC)-H on of myocardial infarct size and cardiac dysfunction.

Group (*n*)	Sham-operated (5)	Coronary artery ligation			*P* values		
Treatment	None	A (12) Vehicle	B (15) CRP	C (11) CRP+Bis(PC)-H	A versus B	A versus C	B versus C
Infarct size (p) (%)	0	17.0 ± 3.8	24.8 ± 3.3	18.6 ± 3.8	0.0001	0.32	0.0002
Infarct size (w) (%)	0	17.7 ± 4.7	29.5 ± 5.7	18.4 ± 3.7	0.0001	0.68	0.0001
ST voltage (μV)	−44.8 ± 24.1	14.9 ± 82.3	95.0 ± 61.8*	16.2 ± 74.3	0.006	0.97	0.008
Elevated ST, *n* (%)	0	7 (58.3)	14 (100)*	7 (63.6)	0.007	1.00	0.06
Ejection fraction (%)	72.67 ± 3.91	37.41 ± 15.95	28.75 ± 14.82	38.40 ± 11.47	0.16	0.87	0.08
LVEDP (mm Hg)	4.4 ± 1.8	8.8 ± 4.1	12.9 ± 3.8	4.2 ± 6.3	0.01	0.05	0.0002
LVEDD (mm)	6.54 ± 0.24	7.43 ± 0.87	8.06 ± 0.74	7.19 ± 1.08	0.05	0.56	0.02
LVESD (mm)	3.1 ± 0.51	5.94 ± 1.39	6.70 ± 1.21	5.60 ± 1.16	0.14	0.53	0.03

Values shown are mean ± s.d except for ST elevation, where the number of individuals with ST elevation and their proportion as a percentage of each group are shown. *P* values are from unpaired t-tests. Infarct size on day 5 is shown as a percentage of the left ventricle, measured by planimetry (p) and by weight (w). VEDD, left ventricular end diastolic diameter; LVEDP, left ventricular end diastolic pressure; LVESD, left ventricular end systolic diameter; ST, ST segment of the electrocardiogram.
*n =14 for these measurements.

At bleed-out on day 5, 24 h after the last dose of human CRP, the mean (±.d.) concentration of human CRP in the serum of CRP-treated rats was 16.7 ^ 10.6 mg l21, but human CRP was detectable by standard immunoassay in only 4 of the 11 CRP-treated rats receiving bis(PC)-H (mean 3.3 ^ 1.5mg 2l), and was not detected at all by the Roche assay, demonstrating complex formation between bis(PC)-H and CRP. Continuous infusion of bis(PC)-H thus resulted in accelerated clearance of human CRP and blocked its function.

In a separate experiment, administration of the same dose of bis(PC)-H to rats (n = 11) that underwent coronary artery ligation but did not receive human CRP had no effect on infarct size at day 5, compared to coronary artery ligation controls receiving vehicle alone (n = 6). The mean infarct size (±s.d.) as a percentage of the left ventricle was 19.0 ± 1.9% for the bis(PC)-H group and 20.0 ± 2.0% for the vehicle group. There was also no difference in haemodynamic measures, indicating that bis(PC)-H had no significant cardiovascular effect in the absence of human CRP.

The physiological role of human CRP is unknown because no deficiency or structural polymorphism in human CRP, or experimental CRP knockout, has yet been reported. Experimental animal studies suggest that CRP may contribute to innate immunity, can be anti-inflammatory, and may protect against autoimmunity, and we have shown that the administration of human CRP exacerbates pre-existing tissue damage in a complement-dependent fashion[2,3]. Previously reported pro-inflammatory effects of human CRP preparations on cells in vitro were due to bacterial endotoxin and other contaminants rather than CRP itself[10–12], and pure human CRP is not pro-inflammatory when injected into healthy animals[12,13]. Furthermore, transgenic human CRP is not pro-atherogenic or pro-thrombotic in apolipoprotein-E-knockout mice[14]. However, the evolutionary conservation of CRP does not exclude potentially harmful effects—although CRP may have evolved to promote beneficial functions, it might also enhance lesion severity, especially in post-reproductive-age diseases such as atherothrombosis, autoimmune and other chronic inflammatory conditions, which are not subject to evolutionary pressure. Our rationally designed CRP-targeting drug demonstrates that CRP inhibition is a valid therapeutic strategy that is unlikely to have adverse effects, and that may prove informative about the physiological and pathological roles of human CRP.

METHODS

Reagents and assays. Human CRP was isolated from malignant ascites fluid as reported[3,13]. Human CRP was assayed by the Roche[7] and Dade-Behring methods[15] and by electroimmunoassay[16]. Rat CRP and complement C3 were measured by electroimmunoassay[4]. Calcium-dependent binding of ^{125}I-labelled CRP to pneumococcal C-polysaccharide (Statens Serum Institut) and modified human low-density lipoprotein[17], which were covalently immobilized on Corning Costar N-hydroxysuccinimide microtitre plates, was compared in the presence and absence of inhibitor compounds. Binding of CRP to phosphoethanolamine-Sepharose was determined as reported previously for SAP[18]. Activation of complement in whole human serum by CRP and C-polysaccharide in the presence and absence of bis(PC)-H was monitored by two-dimensional immunolectrophoresis[4] with monospecific antiserum against human C3.

Binding affinity of CRP for ligands in solution in 0.01M Tris, 0.14M NaCl, 0.002M $CaCl_2$, 0.1% NaN_3 pH 8.0 (TC buffer plus azide) was measured at 37°C by isothermal titration calorimetry[6]. The effects of bis(PC)-H on CRP molecules were monitored by chromatography on a Superdex 200 HR10/30 column in the *Å*KTA Explorer 100 HPLC system (Amersham Biosciences) eluted with TC buffer, by uranyl acetate negative-staining electron microscopy on carbon grids, and by electrospray mass spectrometry (Supplementary Information).

Myocardial infarction. ALZET osmotic mini-pumps, delivering 10 µl h^{-1} for seven days, were implanted subcutaneously in male Wistar rats (200–220 g) two days before coronary artery ligation. Groups A and B received TC buffer; and group C received 1.0Mbis(PC)-H in TC buffer, providing 1 mmol kg^{-1} per day. Coronary artery ligation or sham operations were performed under intraperitoneal anaesthesia with 75 mg kg^{-1} ketamine, 0.6 mg kg^{-1} xylazine and 0.2mg kg^{-1} atropine[19,20], and post-operative atipamezole 0.5 mg kg^{-1} two days after pump implantation. Five daily subcutaneous injections of either TC buffer alone (group A) or human CRP at 40mg kg^{-1} per day in TC buffer (groups B and C) were given, starting immediately after recovery from coronary surgery. Echocardiography (10–22MHz probe, Dynamic Imaging)[21,22], right carotid artery cannulation (using a pressure-transducer tipped catheter 1.4 F, Millar Instrument Inc.) and cardiac catheterization were performed on day 5, with the rats under isofluorane anaesthesia. The rats were then bled, and their hearts excised, cleaned, weighed and frozen. Frozen hearts were then cut transversely into 2.5-mm slices and stained with 1% (w/v) 2,3,5-triphenyl tetrazolium chloride in phosphate buffer. Infarct size was measured by planimetry (using an MCID image analysis system, Imaging Research Inc.) on formalin-fixed, glass-mounted sections, and confirmed by dissection and weighing. All treatments and measurements were performed by an experimenter blind to the treatment group.

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Supplementary Information

METHODS

Synthesis.

1,6-bis(phosphocholine)-hexane (bis(PC)-H)

Systematic name: 1,6-bis[{[(trimethylammonium)ethoxy]phosphinyl}-oxy]hexane. A solution of ethylene chlorophosphate (15.1 g, 106.4 mmol) in dichloromethane (5 ml) was added to a stirred suspension of potassium carbonate (14 g, 107.6 mmol) in dichloromethane (15 ml) at –10°C. A solution of 1,6-hexanediol (2.5 g, 21.1 mmol) in tetrahydrofuran (10 ml) was added dropwise over 10 min, and the reaction was allowed to warm to room temperature over 4 h and stirred for 18 h. *N*-(2-Aminoethyl)aminomethyl polystyrene resin (22 g, loading *ca* 3 mmol/g) and dichloromethane (10 ml) were added and the mixture was agitated for 15 min before being filtered through Celite®, eluting with dichloromethane (250 ml). The filtrate was concentrated *in vacuo* to afford the intermediate bis-phosphate as a pale yellow oil (9.5 g). The bis-phosphate was dissolved in anhydrous acetonitrile (10 ml/g) and divided amongst Smith Process Vials™ (2 ml/vial). Trimethylamine (2 ml/vial) was added and the vials were sealed before being heated to 100°C for 30 min under microwave irradiation (300W). The supernatant liquid was decanted and the residues were dissolved in methanol (5 ml/vial) and combined. The mixture was concentrated under vacuum to afford 1,6-bis(phosphocholine)-hexane (10 g) as a thick yellow oil.

(*Intermediate bisphosphate*): ^{1}H-NMR (400 MHz, CD_3CN): δ 4.4 (8H, m, OCH_2CH_2O); 4.1 (4H, m, CH_2OP); 1.7 (4H, m, CH_2CH_2OP); 1.4 (4H, m, $CH_2CH_2CH_2OP$); ^{13}C-NMR (100 MHz, CD_3CN): δ 68.4, 66.4, 29.8, 24.5.

(*1,6-bis(phosphocholine)-hexane*): ^{1}H-NMR (400 MHz, CD_3CN): δ 4.3 (4H, m, CH_2N); 4.1 (4H, m, CH_2CH_2N); 3.8 (4H, m, CH_2OP); 3.3 (18H, s, $N(CH_3)_3$); 1.6 (4H, m, CH_2CH_2OP); 1.3 (4H, m, $CH_2CH_2CH_2OP$); ^{13}C-NMR (100 MHz, D_2O): δ 65.9, 65.8, 54.4, 45.1, 29.6, 25.0; $[M]^+$ calculated for $C_{16}H_{39}N_2O_8P_2$, 449.2182; found 449.2196.

1,7-bis(phosphocholine)-heptane

Systematic name: 1,7-bis[{[(trimethylammonium)ethoxy]phosphinyl}-oxy]heptane. A solution of ethylene chlorophosphate (5.11 g, 35.9 mmol) in dichloromethane (5 ml) was added to a stirred suspension of potassium carbonate (4.76 g, 36.6 mmol) in dichloromethane (15 ml) at –10°C. A solution of 1,7-heptanediol (0.95 g, 7.98 mmol) in tetrahydrofuran (10 ml) was added dropwise over 10 min, and the reaction mixture was allowed to warm to room temperature over 4 h and then stirred for further 18 h. *N*-(2-Aminoethyl)aminomethyl polystyrene resin (7.18 g, loading *ca* 3 mmol/g) and dichloromethane (10 ml) were added and the mixture was agitated for 2 h before being filtered through Celite®, eluting with diethyl ether (100 ml). The filtrate was concentrated *in vacuo* to afford intermediate bis-phosphate as an orange oil. The bis-phosphate was dissolved in anhydrous acetonitrile (10 ml/g) and divided amongst Smith Process Vials™ (2 ml/vial). Trimethylamine (2 ml/vial) was then added and the vials sealed before being heated to 100°C for 30 min under microwave irradiation (300W). The supernatant liquid was decanted and the dark

yellow residues were dissolved in methanol (5 ml/vial) and combined. The mixture was concentrated under vacuum to afford 1,7-bis(phosphocholine)-heptane (4.4 g) as a thick orange oil.

(*Intermediate bisphosphate*): ^{1}H-NMR (400 MHz, CD_3CN): δ 4.5 (8H, m, OCH_2CH_2O); 4.1 (4H, m, CH_2OP); 1.7 (4H, m, CH_2CH_2OP); 1.4 (6H, m, CH_2CH_2CH_2CH_2OP); ^{13}C-NMR (100 MHz, D_2O): δ 68.5, 67.3, 66.4, 66.3, 29.8, 28.1, 24.8; $[M]^+$ calculated for $C_{11}H_{22}O_8P_2Na$, 367.0688; found 367.0681.

(*1,7-bis(phosphocholine)-heptane*): ^{1}H-NMR (400 MHz, MeOD): δ 4.2 (4H, m, CH_2N); 3.9 (4H, m, CH_2CH_2N); 3.7 (4H, m, CH_2OP); 3.2 (18H, s, N(CH_3)$_3$); 1.7 (4H, m, CH_2CH_2OP); 1.4 (6H, m, CH_2CH_2CH_2CH_2OP); ^{13}C NMR (100 MHz, DMSO): 66.5, 60.7, 55.1, 45.7, 32.1, 30.3, 27.2; $[M]^+$ calculated for $C_{17}H_{41}N_2O_8P_2$, 463.2260; found 463.2242.

1,5-bis(phosphocholine)-pentane

Systematic name: 1,5-bis[{[(trimethylammonium)ethoxy]phosphinyl}-oxy]pentane). A solution of ethylene chlorophosphate (6.84 g, 48 mmol) in dichloromethane (5 ml) was added to a stirred suspension of potassium carbonate (6.4 g, 49 mmol) in dichloromethane (15 ml) at –10°C. A solution of 1,5-pentanediol (1 g, 9.6 mmol) in tetrahydrofuran (10 ml) was added dropwise over 10 min and the reaction was allowed to warm to room temperature over 4 h and then stirred for further 18 h. *N*-(2-Aminoethyl)aminomethyl polystyrene resin (9.6 g, loading *ca* 3 mmol/g) and dichloromethane (15 ml) were added and the mixture was agitated for 2 h before being filtered through Celite®, eluting with diethyl ether (100 ml). The filtrate was concentrated *in vacuo* to afford intermediate bis-phosphate as a colourless oil. The bis-phosphate was dissolved in anhydrous acetonitrile (10 ml/g) and divided amongst Smith Process Vials™ (2 ml/vial). Trimethylamine (2 ml/vial) was then added and the vials were sealed before being heated to 100°C for 30 min under microwave irradiation (300W). The supernatant liquid was decanted and the dark yellow residues were dissolved in methanol (5 ml/vial) and combined. The mixture was concentrated under vacuum to afford 1,5-bis(phosphocholine)-pentane (1.3 g) as a thick dark yellow oil.

(*Intermediate bisphosphate*): ^{1}H-NMR (400 MHz, CD_3CN): δ 4.4 (8H, m, OCH_2CH_2O); 4.1 (4H, m, CH_2OP); 1.7 (4H, m, CH_2CH_2OP); 1.5 (2H, m, CH_2CH_2CH_2OP); ^{13}C-NMR (100 MHz, CD_3CN): δ 67.0, 66.2, 29.0, 20.7; $[M]^+$ calculated for $C_9H_{18}O_8P_2Na$, 339.0375; found 339.2989.

(*1,5-bis(phosphocholine)-pentane*): ^{1}H-NMR (400 MHz, CD_3CN): δ 4.3 (4H, m, CH_2N); 3.9 (4H, m, CH_2CH_2N); 3.5 (4H, m, CH_2OP); 2.9 (18H, s, N(CH_3)$_3$); 1.8 (4H, m, CH_2CH_2OP); 1.5 (2H, m, CH_2CH_2CH_2OP); ^{13}C-NMR (100 MHz, DMSO): δ 66.3, 63.2, 50.0, 45.7, 20.0; $[M]^+$ calculated for $C_{15}H_{37}N_2O_8P_2$, 435.2025; found 435.2043.

1,4-bis(phosphocholine)-dimethylcyclohexane

Systematic name: *trans*-1,4-bis[{[(trimethylammonium)ethoxy]phosphinyl}-oxymethyl]-cyclohexane. A solution of *trans*-1,4-cyclohexanedimethanol (1 g, 6.9 mmol) in tetrahydrofuran (10 ml) was added dropwise to a stirring solution of ethylene chlorophosphate (1.9 ml, 20.8 mmol) in a mixture of dichloromethane (40 ml) and pyridine (1.68 ml, 20.8 mmol) at –30°C. The reaction mixture was allowed to warm to room temperature over 4 h and then allowed to stir for 18 h at room temperature. *N*-(2-Aminoethyl)aminomethyl polystyrene resin (4.6 g, loading *ca* 3 mmol/g) and dichloromethane (10 ml) were added and the reaction was then shaken for 2 h before filtration through Celite®, eluting with diethyl ether (50 ml). The filtrate was

concentrated *in vacuo* to afford intermediate bis-phosphate as an orange oil. The bis-phosphate was dissolved in anhydrous acetonitrile (10 ml/g) and divided amongst Smith Process Vials™ (2 ml/vial). Trimethylamine (2 ml/vial) was added and the vials were sealed before being heated to 100°C for 30 min under microwave irradiation (300W). The supernatant liquid was decanted and the dark yellow residues were dissolved in methanol and combined. The mixture was concentrated under vacuum to afford 1,4-bis-(phosphocholine)-dimethylcyclohexane (0.9 g).

(*Intermediate bisphosphate*): ^{1}H-NMR (400 MHz, MeOD): δ 4.5 (8H, m, OC*H*$_2$C*H*$_2$O); 4.0 (4H, m, C*H*$_2$OP); 1.9 (4H, m, C*H*$_2$CHCH$_2$OP); 1.8 (2H, m, C*H*); 1.1 (2H, m, C*H*$_2$CHCH$_2$OP); ^{13}C-NMR (100 MHz, DMSO): δ 68.5, 62.4, 29.7, 29.6; $[M]^+$ calculated for $C_{12}H_{22}O_8P_2Na$, 379.0688; found 379.0688.

(*1,4-bis-(phosphocholine)-dimethylcyclohexane*): ^{1}H-NMR (400 MHz, MeOD): δ 4.2 (2H, m, C*H*$_2$N); 4.9 (2H, m, C*H*$_2$N); 3.7 (8H, m, C*H*$_2$CH$_2$N and C*H*$_2$OP); 2.9 (18H, s, N(C*H*$_3$)$_3$); 1.8 (4H, m, C*H*$_2$CHCH$_2$OP); 1.7 (2H, m, C*H*); 1.1 (4H, m, C*H*$_2$CHCH$_2$OP); ^{13}C-NMR (100 MHz, DMSO): δ 72.6, 68.9, 63.1, 56.3, 40.5, 30.2; $[M]^+$ calculated for $C_{18}H_{40}N_2O_8P_2Na$, 497.2158; found 497.2136.

Electrospray mass spectrometry. CRP at 10 □M in 200 mM NH_4Ac, 1 mM $CaCl_2$, pH 7.0 was analysed in the presence and absence of 1 mM 1,6-bis(phosphocholine)-hexane on an LCT mass spectrometer (Waters, UK) modified to allow higher pressures in the ion-transfer stage of the instrument, and from conditions which allow the preservation of non-covalent interactions in the gas-phase: capillary 1550 V, sample cone 120 V, extractor cone 8 V, ion transfer stage pressure 900 Pa. Results were processed (MassLynx) and spectra shown are without background subtraction and with minimal smoothing. Peaks were simulated in SigmaPlot and Gaussian curves constructed using the measured mass of CRP with and without the addition of five 1,6-bis(phosphocholine)-hexane molecules, and a peak width at half height identical to that observed for CRP alone.

X-ray analysis. Crystals of the CRP-drug complex were grown by hanging drop vapour diffusion from 11 mg/ml CRP with a ten-fold molar excess of 1,6-bis(phosphocholine)-hexane in 150 mM NaAc pH 4.6, 50 mM $CaCl_2$, and 52% v/v 2-methyl-2,4-pentanediol.. The crystals were orthorhombic, space group $P2_12_12_1$ with unit cell dimensions a=96.2, b=158.9, c=165.1 Å. X-ray diffraction data from a single crystal at 100 K to a resolution of 2.3 Å on beam line ID14.1 (ESRF, Grenoble) were processed with MOSFLM[1,2]. Molecular replacement with MOLREP[3] used a previously derived CRP pentamer as the search model. There were two CRP pentamers in the asymmetric unit with a solvent content of 50%. The structure was refined with CNS[4] and REFMAC[5] and progress monitored with WHATIF and PROCHECK[6,7]. The final model showed no residues within the disallowed region of the Ramachandran plot.

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Table 1 Data collection and refinement statistics for the complex of CRP with 1,6-bis(phosphocholine)-hexane (bis(PC)-H)

Parameter	Value
Space Group	$P2_12_12_1$
Unit Cell (Å)	a=96.17 b=158.94 c=165.12
Resolution range (Å)	60.0-2.3
Measured reflections	842,376
Unique reflections	112,504
Multiplicity	7.5 (7.6)
Completeness (%)	99.7 (99.6)
Rmerge (%)	11.7 (73.8)
Mean (I)/sd(I)	17.0 (2.5)
Solvent content (%)	50.17
Model Rfactor (%)	18.9
Model Rfree (%)	24.3
r.m.s. bond lengths (Å)	0.02
r.m.s. bond angles (degree)	2.4