Targeted pharmacological depletion of serum amyloid P component for treatment of human amyloidosis

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The normal plasma protein serum amyloid P component (SAP) binds to fibrils in all types of amyloid deposits, and contributes to the pathogenesis of amyloidosis. In order to intervene in this process we have developed a drug, *R*-1-[6-[*R*-2-carboxy-pyrrolidin-1-yl]-6-oxo-hexanoyl]pyrrolidine-2-carboxylic acid, that is a competitive inhibitor of SAP binding to amyloid fibrils. This palindromic compound also crosslinks and dimerizes SAP molecules, leading to their very rapid clearance by the liver, and thus produces a marked depletion of circulating human SAP. This mechanism of drug action potently removes SAP from human amyloid deposits in the tissues and may provide a new therapeutic approach to both systemic amyloidosis and diseases associated with local amyloid, including Alzheimer's disease and type 2 diabetes.

Amyloidosis is a disorder of protein folding in which normally soluble globular proteins are deposited extracellularly in the tissues as abnormal insoluble cross-β-sheet fibrils, leading to tissue damage and disease[1,2]. Treatments that reduce the supply of fibril precursor proteins can lead to regression of amyloid deposits[3–6], but such an approach is not possible in most forms of either acquired or hereditary amyloidosis, and these disorders are usually fatal. There is no treatment that specifically promotes regression of amyloid deposits, and systemic amyloidosis—in which the deposits are unequivocally the direct cause of disease—is responsible for about one per thousand of all deaths in developed countries. Amyloidosis is also a common and serious complication of long-term haemodialysis for end-stage renal failure. Furthermore, amyloid is always associated with the neurodegeneration of Alzheimer's disease and with the islet failure of type 2 diabetes. Although it is not known whether amyloid deposition itself causes the tissue dysfunction in these latter diseases, new treatments to promote removal of the damaging deposits are urgently required.

The normal non-fibrillar plasma glycoprotein serum amyloid P component, (SAP), a member of the pentraxin family, is universally present in amyloid deposits[7]. This reflects its specific calcium-dependent binding to motifs displayed by all types of amyloid fibrils. SAP itself is highly resistant to proteolysis, and binding of SAP to amyloid fibrils *in vitro* protects them from degradation by phagocytic cells and proteolytic enzymes[8]. Furthermore, SAP persists within human amyloid deposits for prolonged periods and is completely unmodified with respect to circulating SAP[9]. In mice and hamsters, plasma concentrations of SAP are closely related to amyloidogenesis[7]. We have therefore suggested that SAP may contribute to the failure to clear amyloid deposits *in vivo*, leading to tissue damage and disease. When we first identified a chemically defined ligand for SAP of low molecular mass—the cyclic pyruvate acetal of galactose, methyl 4,6-*O*-(1-carboxyethylidene)-β-D-galactopyranoside (MOβDG)—and showed that it could completely dissociate SAP from amyloid deposits *in vitro*, we proposed that such molecular dissection of the deposits might be an approach to therapy of amyloidosis[10]. Our recent finding that mice with targeted deletion of the SAP gene show retarded and reduced induction of experimental reactive systemic (AA type) amyloidosis confirmed

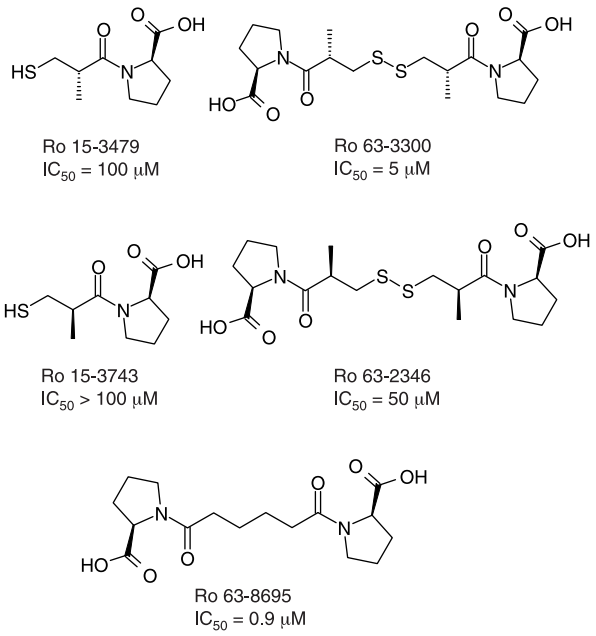

Figure 1 Inhibitors of SAP binding. The original screening hits were Ro 15-3479 and Ro 63-2346. Ro 63-3300 was synthesized and was more potent. The optimal product of the subsequent chemistry programme was Ro 63-8695 (*R*-1-[6-[*R*-2-carboxy-pyrrolidin-1-yl]-6-oxo-hexanoyl]pyrrolidine-2-carboxylic acid), which we abbreviate here as CPHPC. The IC_{50} values shown are as measured in the primary screening assay.

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that SAP does indeed contribute to pathogenesis of amyloidosis[11] and is a valid therapeutic target[12].

Identification and development of inhibitors of SAP binding

A high throughput assay for inhibitors of SAP binding to Alzheimer's disease amyloid-β (Aβ) amyloid fibrils[12] immobilized in microtitre plates was used to screen the Roche compound library. Two hits were identified: Ro 15-3479, 1-[*S*-3-mercapto-2-methylpropionyl]-D-proline, an epimer of captopril, and Ro 63-2346, *R*-1-[*R*-3-[*R*-3-[*R*-2-carboxy-pyrrolidin-1-yl]-2-methyl-3-oxo-propyldisulphanyl]-2-methyl-propionyl]-pyrrolidine-2-carboxylic acid, the oxidative disulphide dimer of the diastereomer 1-[*R*-3-mercapto-2-methylpropionyl]-D-proline (Ro 15-3743; Fig. 1). Captopril, 1-[*S*-3-mercapto-2-methylpropionyl]-L-proline, was inactive, demonstrating the key importance of the D-proline configuration. The dimer of the first hit was prepared—*R*-1-[*S*-3-[*S*-3-[*R*-2-carboxy-pyrrolidin-1-yl]-2-methyl-3-oxo-propyldisulphanyl]-2-methyl-propionyl]-pyrrolidine-2-carboxylic acid, Ro 63-3300 (Fig. 1)—and was a more potent inhibitor of SAP binding than the monomeric thiol. The molecular mechanism responsible for this greater potency was suggested by our recent observation that the complex between SAP and dAMP (2′-deoxyadenosine 5′-phosphate) contained pairs of pentameric SAP molecules, interacting face to face as a result of base-stacking interactions between the bound dAMP molecules[13]. The pairing of SAP molecules bridged by the palindromic compound Ro 63,3300 would both enhance the avidity of the protein–ligand interaction and occlude the binding (B) face[14] of the SAP molecule. Gel filtration experiments confirmed that pairs of SAP molecules were indeed formed in mixtures with Ro 63-3300 at appropriate molar ratios. Ro 63-3300 was selected as the lead compound for synthetic work to optimize the half-maximal inhibitory concentration (IC_{50}) for inhibition of SAP binding to amyloid fibrils *in vitro*, enhance the capacity to inhibit/dissociate SAP binding to amyloid deposits *in vivo*, and to minimize toxicity *in vivo*. *In vivo* evaluation of the best candidate compounds led to selection of *R*-1-[6-[*R*-2-carboxy-pyrrolidin-1-yl]-6-oxohexanoyl]pyrrolidine-2-carboxylic acid[15] (Ro 63-8695), abbreviated to CPHPC, as the optimal compound for clinical proof of concept testing.

Molecular interaction between SAP and CPHPC

SAP is a pentamer with five identical non-covalently associated protomers each bearing a single calcium-dependent ligand-binding site on one planar face, the binding (B) face, of the molecule[16]. The palindromic structure of CPHPC, with two D-proline residues joined by an aliphatic linker chain, enables it, like Ro 63-3300, not only to block the ligand-binding sites on individual SAP protomers, but also to crosslink pairs of pentameric SAP molecules to form B-face to B-face decamers. Gel filtration analysis confirmed that in mixtures of isolated SAP with CPHPC, at ratios between equimolar and 100-fold molar excess of CPHPC (relative molecular mass (M_r) of 340) over SAP protomers (M_r 25,462), all the SAP was decameric. However at 128-fold or greater molar excess of CPHPC, all the SAP was in its single pentameric form, presumably because each binding site was then occupied by a different individual CPHPC molecule, preventing crosslinking of pentamers. Addition of CPHPC to whole serum, at a concentration equimolar with SAP or greater, also produced stable decameric SAP. The multi-point binding of CPHPC between pairs of pentameric SAP molecules greatly increases the avidity of the interaction, and this was reflected in an apparent affinity constant of 10 nM, measured by isothermal calorimetry. This contrasted with affinity constants of just 50 μM for MOβDG and 15 μM for *N*-acetyl D-proline. Interestingly, the dissociation constant for binding of human SAP to isolated amyloid fibrils is also about 10 nM[17].

Table 1 Data collection and refinement statistics for the SAP–CPHCP complex

Parameter	Value
Space group	$P4_32_12$
Unit cell (Å)	$a = b = 230.9$, $c = 281.4$
Resolution range (Å)	25–3.3
Measured reflections	423,005
Unique reflections	110,179
Multiplicity	3.8 (3.2)
Completeness (%)	96.7 (90.9)
R_{merge} (%)	8.2 (46.0)
$I/\sigma I$ (% >2.0)	74.0 (16.2)
Solvent content (%)	83.3
Model R_{factor} (%)	22.3
Model R_{free} (%)	22.9
r.m.s. bond lengths (Å)	0.010
r.m.s. bond angles (degree)	1.60

Values in parentheses denote the highest resolution shell statistics between 3.42–3.3 Å. r.m.s., root mean square. $I/\sigma I$, intensity/standard deviation of intensity.

The X-ray crystal structure of the SAP–CPHPC complex determined at 3.3 Å resolution (Table 1 and Fig. 2) confirmed that it was a decamer composed of two pentameric SAP molecules reversibly crosslinked by five CPHPC molecules. The CPHPC terminal carboxylates are bound in the calcium-dependent ligand-binding pockets of the SAP subunits and bridge across between the adjacent B faces of the two SAP molecules (Fig. 2). There are no close contacts between the two pentameric SAP molecules and they are separated by a clear solvent region. Further stabilization of the complex derives from the snug binding of the pyrrolidine ring of the drug into a hydrophobic pocket formed by Tyr 74, Leu 62 and Tyr 64 adjacent to the bound calcium ions. The 2.4 Å resolution structure of SAP co-crystallized with *N*-acetyl-D-proline shows a very close superposition with the drug head group in the CPHPC complex, with the proline side-chain pucker enabling packing against the Tyr 74 ring. The alkyl chain linking the two proline head groups of the drug is too long to fit the electron density in an extended conformation and adopts a kinked rotamer with eclipsed substituents about the C2–C3 bond. This displaces the two half-drug components (the halves of the palindromic CPHPC molecule) from a common long axis and facilitates approach of the head groups to the binding sites of two-fold axis related SAP protomers. Furthermore, the best fit to the electron density is achieved when both of the peptide bonds preceding the proline residues adopt the *cis*-configuration. On energetic grounds this *cis*-peptide bond probably occurs in about 25% of the drug head groups in solution, but the species carrying two *cis* proline head groups is probably much less abundant. The impact of this observation on drug potency is the subject of further studies.

Experimental studies of CPHPC *in vivo*

CPHPC was not metabolized in mice and was very rapidly excreted, predominantly in the urine, with a small amount in the bile. However, intraperitoneal or subcutaneous injection of CPHPC inhibited uptake of radiolabelled human SAP tracer into experimentally induced mouse AA amyloid deposits. Continuous infusion of CPHPC for 5 days accelerated whole-body clearance of radiolabelled human SAP tracer, with which the amyloid deposits had previously been loaded, and removed all the endogenous mouse SAP from the deposits (Fig. 3). Even 50 μg per kg per day of CPHPC significantly dissociated human SAP from the deposits (not shown), but 1.5 mg per kg per day was required to dissociate any endogenous mouse SAP significantly, and there was a clear dose response effect up to 15 mg per kg per day, which removed all of the mouse SAP (Fig. 3). Injection of just 100 μg CPHPC into mice transgenic for human SAP reduced circulating human SAP values by greater than 95% within 3 h, whereas the same dose of the monomeric thiol Ro 15-3479 (Fig. 1) had no effect at all on the plasma concentration of human SAP despite being able to inhibit uptake of radiolabelled human SAP into murine AA amyloid deposits *in vivo* (not shown). The capacity of the palindromic CPHPC molecule to crosslink pairs of SAP molecules is evidently critical for depletion of circulating SAP. Despite very limited oral bioavailability, administration of

CPHPC in the drinking water also rapidly induced sustained depletion of circulating human SAP in the transgenic mice (Fig. 3). However, none of these CPHPC treatments reduced the plasma concentration of mouse SAP in wild-type animals. The human SAP–CPHPC complex is evidently recognized as abnormal *in vivo* and is swiftly removed from the circulation, but mouse SAP–CPHPC complexes are apparently formed and/or cleared less efficiently *in vivo*.

Murine AA amyloidosis, induced experimentally using chronic inflammation to produce persistent high levels of serum amyloid A protein (SAA), the precursor of AA amyloid fibrils[2], is not suitable for rigorous studies of amyloid regression because, once initiated, amyloid deposition proceeds unpredictably throughout life. Nevertheless we investigated the effect of CPHPC on this model, but, to specifically target human SAP, we used SAP knockout mice transgenic for human SAP that express human SAP instead of mouse SAP. Amyloid was induced with a single intravenous injection of amyloid-enhancing factor followed by four subcutaneous injections of casein over the next 7 days[11]. Seven days later treatment was started in 21 mice with 0.25–1.0 mg ml^{-1} CPHPC in the drinking water, sufficient to reduce and maintain circulating human SAP values at 5 mg l^{-1} or less, while a control group ($n = 11$) received no treatment. After 40 days treatment, amyloid deposition was quantified by three independent observers (who were blind to the treatment regime) using Congo red histochemical staining[11]. There was significantly less amyloid in the treated group ($P < 0.0001$, Wilcoxon Mann–Whitney test), and 5 of the 11 untreated controls had a substantial amyloid load compared with only 2 of the 21 CPHPC-treated mice ($P = 0.032$, Fisher's exact test), suggesting that treatment with CPHPC had reduced the amyloid burden.

Formal toxicological assessment of CPHPC in rats and dogs revealed no adverse effects during 28 days intravenous adminis-

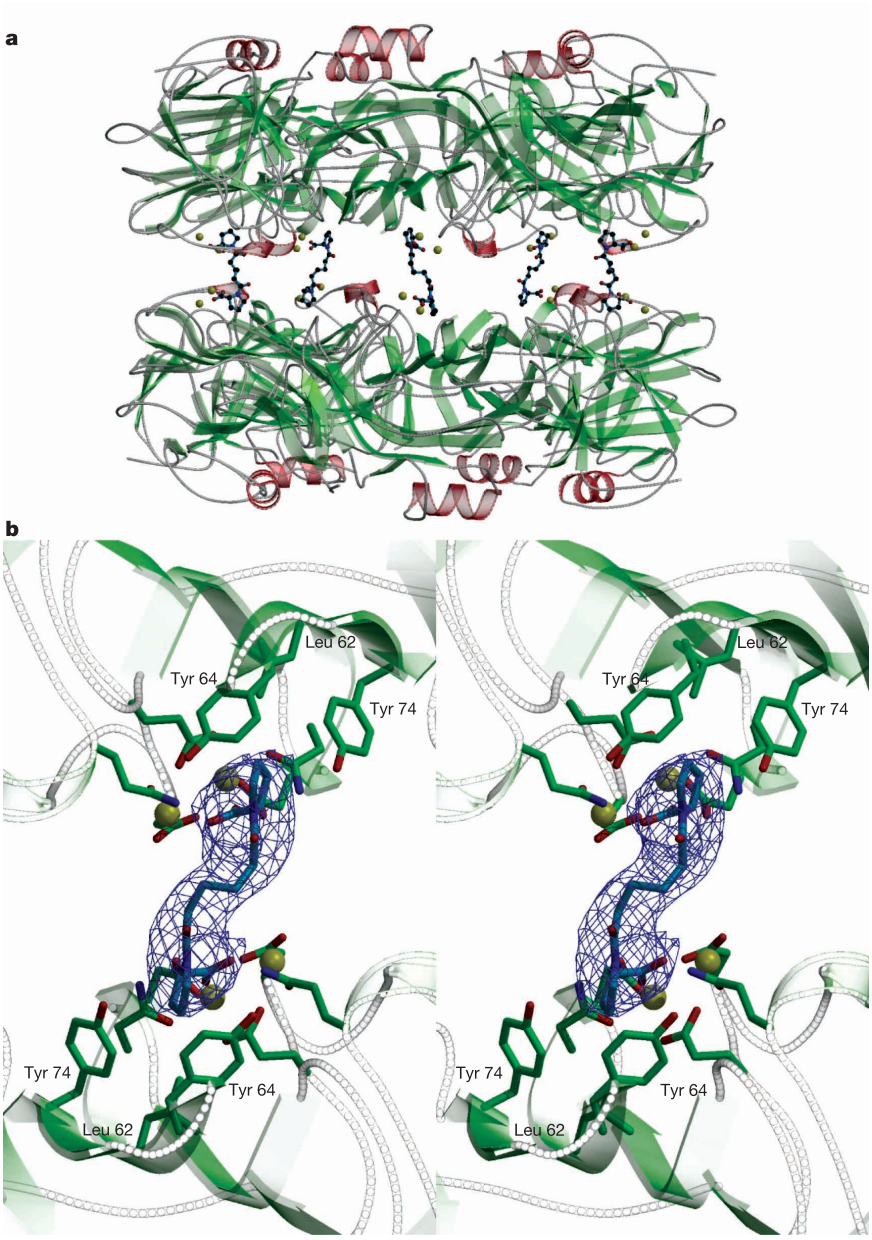

Figure 2 The structure of the complex of CPHPC with SAP. **a**, Two SAP pentamers crosslinked by means of their B faces by five molecules of CPHPC (blue), viewed perpendicular to the fivefold axis. A-face helices are shown in red. The two calcium ions bound to each SAP subunit are yellow. **b**, Stereo view of annealed omit map electron density (($F_o - F_c$) contoured at 3σ) and the fitted CPHPC molecule showing the kinked alkyl linker and binding of the drug head groups into the double calcium site of two-fold axis related SAP subunits. Images were prepared with Bobscript[39] and Raster3d[40].

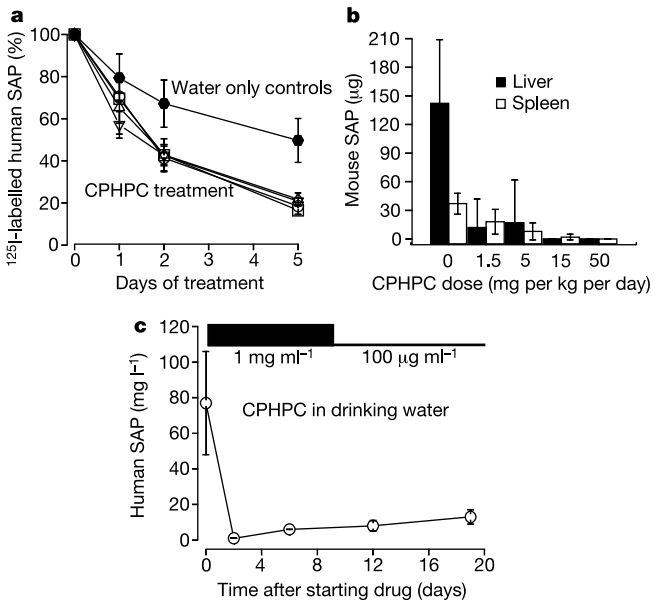

Figure 3 Effects of CPHPC on human and mouse SAP in mice *in vivo*. **a**, **b**, Groups of ten age-, sex- and weight-matched mice with experimentally induced reactive systemic AA amyloidosis were given a single loading dose of ^{125}I-labelled human SAP on day −1, and received implanted osmotic mini-pumps delivering the doses shown of CPHPC. Clearance from the amyloid deposits, catabolism and excretion of the human SAP tracer were monitored globally by whole-body counting of the mice (**a**). Each point represents the mean (s.d.) of all animals in each group; there was no significant difference between the groups receiving different doses of CPHPC: 1.5 mg per kg per day (squares); 5.0 mg per kg per day (circles); 15 mg per kg per day (triangles); 50 mg per kg per day (inverted triangles). The total amount of mouse SAP in the amyloidotic organs was determined after killing the animals on day 5 (**b**). **c**, Effect of administration of CPHPC in the drinking water (at the concentrations shown) on circulating human SAP values in human SAP transgenic mice. Mice of approximately 20 g body weight consume about 3 ml of water per day.

tration of up to 400 mg per kg per day. The drug was not metabolized and 95% of a single dose was excreted within 24 h. During prolonged administration, a mean of 99% (s.d. 1%) of the drug was recovered, 90% (s.d. 3%; range 85–93%) from renal excretion and 8% (s.d. 3%; range 6–13%) in the faeces. CPHPC did not inhibit the five main human cytochrome P450 isoenzymes and was, therefore, unlikely to affect the metabolism of other drugs. We thus proceeded to study directly the action of CPHPC in patients with amyloidosis.

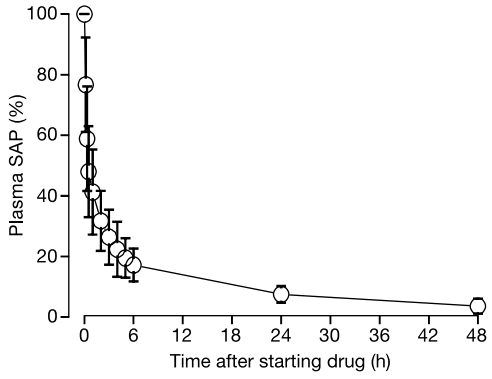

Figure 4 Effect of intravenous infusion of CPHPC on plasma SAP values in patients with systemic amyloidosis. Six patients with AL amyloidosis and one with AA type received doses of CPHPC of between 0.25–6.0 mg per kg per day for 48 h. The SAP concentration was measured immediately before and at the intervals shown during the infusion. Each point represents the mean (s.d.) of all patients.

Clinical studies of CPHPC in human amyloidosis

CPHPC was administered for 48 h by intravenous infusion to seven patients with systemic amyloidosis (6 monoclonal immunoglobulin light chain (AL) type and one AA type), and one patient with minor localized AL amyloidosis. There was rapid and consistent depletion of circulating SAP in all subjects—the SAP concentration started to fall when about 2 mg CPHPC had been given—and at all doses between 0.25–6 mg per kg per day, SAP was almost completely cleared from the plasma by the end of the infusion (Fig. 4). Circulating SAP values were already falling sharply when the ratio of plasma drug concentration to that of SAP pentamer approached equimolar levels. Gel filtration analysis confirmed that this SAP:CPHPC ratio is sufficient to generate some decameric SAP, but presumably with pairs of pentameric SAP molecules crosslinked by just two CPHPC molecules rather than by five as in the crystal structure (Fig. 2). These pairs of SAP molecules were evidently recognized as abnormal and were promptly cleared. In one further subject—a carrier of the amyloidogenic Ala 60 transthyretin variant but with no clinical amyloidosis—a bolus of 2 mg CPHPC followed by infusion at just 0.1 mg per kg per day produced slower and less complete SAP depletion.

The plasma SAP concentration returned to normal by 48 h after stopping the drug in the individuals with little or no amyloid, but in subjects with significant amyloidosis the SAP value remained low for prolonged periods of time. In the patient in the group with the heaviest whole-body amyloid load, the plasma SAP concentration was still below 25% of its initial value 20 days after the infusion. This indicates that most of the 50–100 mg of SAP newly produced per day[18] was distributing into the amyloid deposits before becoming available to replete the plasma pool, and it is very strong indirect evidence that even the brief 48-h infusion of CPHPC had substantially depleted the amyloid-associated SAP pool.

Direct evidence for depletion of SAP from amyloid in the organs and for the mechanism of action of CPHPC was obtained by quantitative whole-body scintigraphy using ^{123}I-labelled SAP as a tracer. Each patient received a standard dose of ^{123}I-labelled SAP 24 h before the CPHPC infusion started, and was scanned immediately before treatment to provide a baseline image and values for localization of tracer to the amyloid deposits. Patients were then scanned at intervals thereafter, up to the end of the 48-h infusion. By 6 h after starting treatment the blood pool signal had virtually disappeared (Fig. 5), and there was marked accumulation of tracer in the liver, identifying this organ as the site to which CPHPC caused clearance of the circulating SAP. At the same time there was a

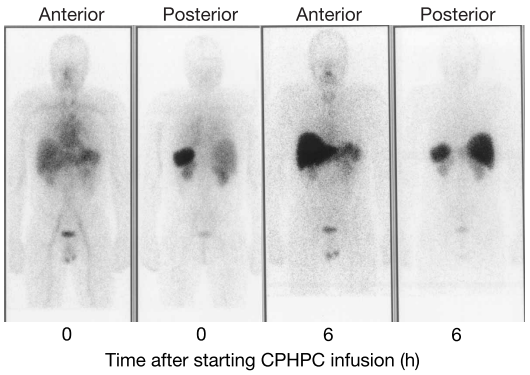

Figure 5 Whole-body ^{123}I-labelled SAP scintigraphy before and 6-h after starting infusion of CPHPC. The figure shows a patient with a modest load of AL amyloid in the spleen and kidneys, with notable blood pool background of tracer in the heart and circulation before administration of the drug (left panel). At 6 h the blood pool background is completely absent and the liver, which is the only site of catabolism of SAP *in vivo*, has taken up the tracer while the intensity of SAP signal from the amyloidotic spleen and kidneys is significantly reduced.

marked decrease in the retention of tracer in amyloid deposits elsewhere, exemplified by the spleen, in contrast to the usual situation in untreated (control) amyloidosis patients (Table 2).

We have previously demonstrated in mice that the liver, and specifically the hepatocyte, is the only significant site of clearance and catabolism of both mouse and human SAP *in vivo*[19]. Furthermore, asialo human SAP is instantly cleared by the liver in humans by means of the hepatocyte asialoglycoprotein receptor, and we have imaged this process using ^{123}I-labelled asialo SAP[9]. CPHPC apparently triggers similarly rapid hepatic uptake of SAP *in vivo*, leading to virtually total depletion of circulating SAP. This promotes redistribution of SAP from the tissues to the plasma, supplementing the effect of CPHPC as a competitive inhibitor of SAP binding to amyloid fibrils, and enhancing the efficiency of removal of SAP from amyloid deposits. Importantly from a clinical viewpoint, subcutaneous injection of as little as 0.25 mg per kg per day of CPHPC, given in doses every 12 hours, had the same effect on plasma SAP as intravenous infusion of the drug.

In an open label study we have now infused CPHPC intravenously, or injected it subcutaneously, at 30–40 mg per day (mean, 38.7 mg) continuously for periods of 1.2–9.5 months (mean, 2.6 months) in 19 systemic amyloidosis patients aged 43–67 years (mean 56 $\pm$ 7 years). There were 12 patients with AL amyloidosis, two with hereditary transthyretin type, one with AA type, and one each with hereditary apolipoprotein AI, fibrinogen A α-chain, gelsolin and lysozyme amyloidosis[2]. Plasma SAP values were reduced to a mean of 5 $\pm$ 2.5% of pre-treatment levels in all patients throughout treatment. There were no adverse clinical effects, either locally from twice daily subcutaneous injections, or systemically, and no biochemical, haematological, immunological or organ function abnormalities were detected that could be attributed to CPHPC. Although many of these patients had end-stage disease and were offered CPHPC because they had either already failed to respond to all other possible treatments, or no other treatment exists for their type of amyloidosis, most of them remained clinically stable throughout treatment. One individual with rapidly progressive, terminal, AL amyloidosis (affecting mainly the heart, kidneys, spleen and autonomic nervous system) who had not responded to maximal cytotoxic chemotherapy, lived for 6 months after starting treatment with CPHPC. At autopsy, the organs still contained substantial amyloid deposits but their SAP content was markedly reduced, to about 15% of the amount typically associated with comparable amyloid loads. Prolonged treatment with CPHPC is thus likely to be required to completely clear SAP from major amyloid deposits and to thereby maximally enhance amyloid regression.

Discussion

We report here the first example, to our knowledge, of a low molecular mass drug that profoundly depletes a specific plasma protein from the circulation and the tissues. The physiological mechanisms for recognizing extremely subtle, abnormal molecular conformations in plasma proteins are exquisitely sensitive and provide highly effective clearance from the circulation. The present compound, CPHPC, takes advantage of this phenomenon and specifically targets human SAP to produce a 'knockout' for therapeutic purposes[20].

SAP in amyloid deposits derives entirely from circulating SAP, which is produced, and eventually cleared and catabolized, exclusively in the liver[7]. The plasma and amyloid pools of SAP are in free dynamic equilibrium, and our original goal was to produce a low molecular mass compound that would remove SAP from amyloid deposits *in vivo* by inhibiting binding of SAP to amyloid fibrils and dissociating SAP that was already bound. Indeed, CPHPC and the related molecules from which it was developed (Ro 15-3479 and Ro 63-3300, Fig. 1) do precisely that with respect to mouse SAP in murine systemic amyloidosis. However, human SAP binds more avidly than mouse SAP to all their known ligands including amyloid fibrils[21], and binding of the palindromic CPHPC molecule by human SAP has an additional and powerful effect *in vivo*. CPHPC crosslinks pairs of native pentameric human SAP molecules, creating a decameric assembly that is swiftly cleared from the circulation by the liver. The resulting profound depletion of plasma SAP markedly shifts the distribution of SAP between the plasma and amyloid pools, with SAP flowing from the tissues into the circulation where it is immediately targeted by the drug for clearance in the liver. Coupled with direct inhibition of SAP binding by CPHPC, this leads to rapid and extensive removal of SAP from the amyloid deposits.

Binding of SAP stabilizes amyloid fibrils *in vitro* and protects them from proteolytic degradation. We therefore hope that efficient removal of SAP *in vivo* will reduce the stability of amyloid deposits and promote their regression. The absence of SAP may also retard new amyloid deposition, as it does in SAP-deficient mice[11]. We are currently testing this hypothesis by long-term treatment of systemic amyloidosis patients with CPHPC. Elimination of new fibril formation by removal of the supply of fibril precursor proteins is associated with regression of deposits and with clinical benefit in all forms of amyloidosis in which it has been achieved[3–6], indicating that clearance of human amyloid deposits can occur *in vivo*. Clearance of experimental amyloid deposits has been reported in transgenic mice expressing human Aβ, the amyloid fibril protein of Alzheimer's disease, after production or administration of anti-Aβ antibodies[22,23], and inhibition or reversal of amyloid fibrillogenesis are also being investigated. However, CPHPC has potential advantages over other therapeutic approaches and could also be combined with them. It is an easily administered, non-toxic, well tolerated and highly potent low molecular mass compound. Importantly, the drug may not need to gain access to the tissues as it exerts its desired biochemical effect within the circulation. Thus if CPHPC does indeed promote amyloid regression, it should be readily applicable not only in systemic amyloidosis where disease is unequivocally caused by the deposits, but also in both Alzheimer's disease and type 2 diabetes, in which local amyloid deposits are implicated in pathogenesis. □

Table 2 CPHPC rapidly clears circulating SAP to the liver and depletes SAP from visceral amyloid deposits

	^{123}I-labelled SAP retention (%)					
	Liver			Spleen		
Treatment	Time after tracer injection (h)		Number of patients	Time after tracer injection (h)		Number of patients
	24 h	48 h		24 h	48 h	
None	100	78 (8)	12	100	86 (25)	14
CPHPC	100	125 (20)	7	100	54 (18)	7
P-value		$P < 0.0001$			$P = 0.008$	

Values are mean values with s.d. in parentheses.
All patients had systemic AL amyloidosis and received a standard intravenous tracer dose of ^{123}I-labelled SAP at time zero. After whole-body quantitative scintigraphic imaging at 24 h, uptake in liver and spleen were taken as 100% for each individual. Intravenous infusion of CPHPC was then started and scintigraphy with organ counting was repeated at 48 h. The controls received no treatment. Significance of differences between the two groups was evaluated by a *t*-test.

Methods

Protein studies

Human and mouse SAP were isolated, purified and assayed as previously described[17,24–26]. Gel filtration analysis to determine the molecular assembly of SAP–CPHPC complexes was performed as reported elsewhere[27]. Isothermal calorimetry to measure heat of binding between SAP and its ligands, and calculation of affinity constants were performed as previously reported[28] using a VP–ITC system from MicroCal Inc. Forty injections of 5 μl of ligand were titrated at 5.4 min intervals into SAP, in solution in 10 mM Tris-buffered 0.14 M NaCl containing 10 mM EDTA and 200 mM $CaCl_2$, pH 8.0, within a 1.3-ml sample cell. Ligand concentration was varied between 1.6 mM and 800 μM, and protein between 40 μM and 100 μM, so that a heat change of approximately 1 μcal was observed on initial injections.

X-ray crystal structure

Crystals of the SAP–CPHPC complex were grown by hanging-drop vapour diffusion at pH 7.6 and 4 °C, using polyethylene glycol monomethyl ether 550 as the precipitant in the presence of a tenfold molar excess of CPHPC. The crystals were tetragonal, space group $P4_32_12$, with unit cell dimensions $a = b = 230.9$ Å and $c = 281.4$ Å. X-ray diffraction data were collected from a single crystal at 100 K to a resolution of 3.2 Å on beamline ID14-3 at the European Synchrotron Radiation Facility (ESRF), Grenoble, and were processed using DENZO and SCALEPACK[29]. Molecular replacement was performed in MOLREP[30] using a previously derived SAP pentamer[16] as the search model. There were two SAP pentamers in the asymmetric unit, with an unusually high solvent content of 83%. The structure was refined using CNS[31] with tight tenfold NCS restraints between the protomers. During the course of the refinement the stereochemical properties were checked using WHAT IF[32] and PROCHECK[33], with the final model showing no residues within the disallowed regions of the Ramachandran plot. A summary of the data collection and refinement statistics is shown in Table 1.

In vivo mouse studies

Reactive systemic AA amyloidosis was induced in CBA mice by repeated subcutaneous injections of casein, and studies of the localization, retention and turnover of radiolabelled human and mouse SAP, and of amyloid load, were conducted precisely as described previously[11,21]. There was complete concordance between the rank scores, 0–5, assigned by different observers to the amount of amyloid demonstrable by Congo red staining in the spleen and liver. The sum of these scores for each animal, as well as simply the presence or absence of substantial deposits, were analysed by appropriate nonparametric statistical tests. Pure line C57BL/6 mice transgenic for human SAP were bred in London from embryos of the strain previously reported[34].

In vivo human studies

SAP scintigraphy studies, including whole-body imaging, region of interest counting of specific organs, and grading of whole-body amyloid load, were performed as described elsewhere[35–38]. Plasma concentration of CPHPC was determined by Multiple Reaction Monitoring on a QuattRo II mass spectrometer (Micromass) after protein precipitation, Oasis HLB extraction (Waters Ltd), dimethylation with diazomethane and high-performance liquid chromatography (10 cm × 2 cm; RPB) (acetonitrile:water:formic acid, 40:60:0.1 v/v/v, 0.14 ml min^{-1}). Transitions were mass/charge ratio m/z 369.4 to 240.2 (CPHPC) and 377.4 to 248.2 ($^2H_8$-CPHPC).

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Competing interests statement

The authors declare competing financial interests: details accompany the paper on *Nature*'s website (http://www.nature.com).

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