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Chaperone-like activity of the acute-phase component human serum α_1-acid glycoprotein: Inhibition of thermal- and chemical-induced aggregation of various proteins

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Dedicated to the memory of my parents

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ABSTRACT

In vitro chaperone-like activity of the acute-phase component and plasma drug transporter human α_1-acid glycoprotein (AAG) has been shown for the first time. AAG suppressed thermal aggregation of a variety of unrelated enzymatic (e.g., aldolase, catalase, enolase, carbonic anhydrase) and non-enzymatic proteins (β-lactoglobulin, ovotransferrin) and it also prevented dithiothreitol induced aggregation of insulin. The anti-aggregation ability of AAG was abolished/reduced upon drug binding suggesting that protein–protein interactions established between the lipocalin β-barrel fold of AAG and hydrophobic surfaces of the stressed proteins are involved in the chaperone-like activity. The results shed some light on the possible biological function of this enigmatic protein and suggest that besides haptoglobin, clusterin, fibrinogen and α_2-macroglobulin AAG can be considered as a novel member of the extracellular molecular chaperones found in human body fluids.

Human α_1-acid glycoprotein (AAG) is a major acute-phase protein in the plasma (0.4–1.1 mg/ml) the level of which increases under variety of physiological and pathological conditions including stress, infections, inflammatory and neoplastic diseases.[1,2] The definitive biological role of AAG has not yet been elucidated; however, as an immunocalin it is thought to be involved in modulation of immuno- and inflammatory responses.[3,4] AAG exhibits characteristic drug binding ability which renders it to be one of the most important drug transporters in plasma, showing affinity especially for basic and neutral compounds.[1,2] Similarly to other lipocalins, AAG possesses a central hydrophobic cavity, which is the primary binding site for ligand molecules.[5] Due to its high sugar content (*N*-acetylglucosamine, *N*-acetylneuraminic acid) which accounts for about 40% of the total mass (40 kDa) of the protein, AAG is highly water soluble with low isoelectric point (p*I* ≈ 2–3). AAG is synthesized mainly in the liver but extrahepatic synthesis has also been reported in leukocytes,[3,6] endothelial[7] and even in cancer cells.[8]

As noted above, AAG is the integrant member of the extracellular protein pool at considerably high concentration which might increase up to fivefold upon various noxa.[2] Currently four proteins are known to exhibit chaperone function within the plasma proteome: clusterin,[9] haptoglobin,[10,11] α_2-macroglobulin,[12] and fibrinogen.[13] These are all secreted glycoproteins and similarly to AAG the three latter ones are acute-phase reactants too. Clusterin, haptoglobin, and α_2-macroglobulin have been shown to inhibit amyloid fibril formation in vitro.[14,15] Within the lipocalin superfamily, amyloid β-chaperone activity of prostaglandin D synthase has been demonstrated.[16] AAG has been identified as the aggregation inhibitor of the amyloidogenic human α-atrial natriuretic peptide.[17] In addition, AAG has been described to form a function-stabilizing complex with plasminogen activator inhibitor **1**[18] and to participate in collagen self-assembly.[19] Overall, these facts suggest that AAG might exhibit chaperone-like properties. In an attempt to assess this hypothesis, effect of AAG on heat- and chemical-induced aggregation of several unrelated proteins has been investigated in the current study.

According to the widely applied protocol[20] aggregation assays were performed in 40 mM pH 7.5 Hepes-KOH buffer by using various proteins susceptible for thermal or chemical stresses (see experimental information in the Supplementary data). The kinetics of aggregation was monitored at different temperatures as a function of time by detecting light scattering of the samples at 500 nm

Abbreviations: AAG, α_1-acid glycoprotein; ALD, aldolase; ADH, alcohol dehydrogenase; BLG, β-lactoglobulin; CA, carbonic anhydrase; CHL, chlorpromazine; CS, citrate synthase; CTL, catalase; DTT, DL-dithiothreitol; ENL, enolase; GPDH, glyceraldehyde-3-phosphate dehydrogenase; IMT, imatinib; INS, insulin; LDH, L-lactate dehydrogenase; MFP, Mifepristone; OVT, ovotransferrin; STR, staurosporine.

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(INS was detected at 360 nm). Thermolabile proteins frequently used in chaperone assays were selected (Table 1). For BLG, which is a thermostable protein showing aggregation only at 70 °C in a concentrated solution (pH 7.5, $c = 273$ μM),[21] Zn^{2+} ions were used to promote the aggregation at lower temperature and concentration[22] (cf. Table 1). AAG itself showed no aggregation at the applied temperatures (data not shown), which is in agreement with previous circular dichroism and infrared spectroscopic studies.[23] DTT-induced aggregation of INS is based on the reduction of three interchain disulfide bonds of the protein and subsequent dissociation/aggregation of the α and β chains. AAG also contains two disulfide bridges (Cys5-Cys147 and Cys72-Cys165) but DTT did not alter either its secondary or tertiary structure as showed by circular dichroism spectra (see experimental information).

Addition of AAG into the sample solutions markedly inhibited aggregation of the test proteins in a dose dependent manner (Table 1, Fig. 1 and Supplementary data). For most proteins light scattering intensities decreased by more than 50% and in the cases of CA, CTL, ENL, and INS the aggregation was almost completely suppressed (Table 1). The AAG/test protein ratio required for limiting thermal aggregation of OVT was far below the values found in the other assays.

The lipocalin fold of the AAG molecule defines a large hydrophobic cavity where a variety of guest molecules can be bound.[5,24] It can be assumed that this binding pocket may interact with the exposed hydrophobic groups of the test proteins and thus steric hindrance of the cavity would limit the chaperone-like effect of AAG. In order to address this issue, STR, a bulky anticancer agent (Fig. 2) was selected first, which binds to AAG with very high affinity ($K_a = 1.1 \times 10^7\ M^{-1}$).[25] Previous drug competition experiments indicated that the AAG binding site of UCN-01 (7-hydroxystaurosporine), the very close structural analogue of STR (Fig. 2), overlaps with that of other cavity-binder drug molecules.[26] Results of aggregation assays performed in the presence and absence of STR show that the drug completely abolished the anti-aggregation effect of AAG in most cases (Table 2). For OVT, preincubation of AAG with STR reduced the aggregation suppressing ability by about 50%. To obtain more information on the effect of drug binding of AAG on its chaperone-like activity additional drug ligands (Fig. 2) were tested in thermal aggregation assays of ALD and LDH. The amphiphilic drug molecule IMT, a selective tyrosine kinase inhibitor, binds avidly in the central cavity of AAG ($K_a = 1.7 \times 10^6\ M^{-1}$).[27] Similarly, the synthetic anti-progesterone steroid MFP and the phenothiazine drug CHL are high-affinity cavity ligands of the protein ($K_a = 1.7$ and $1.5 \times 10^6\ M^{-1}$, respectively).[28,29] These compounds were preincubated with AAG prior to initiating thermal aggregation by adding ALD or LDH into the sample solution. In both assays MFP totally suppressed the aggregation inhibitory effect of AAG and the same result was obtained in the IMT–LDH experiment (Table 3). IMT was less effective in decreasing anti-aggregation ability of AAG in the ALD assay. CHL did not abolish completely but significantly reduced the protective impact of AAG in both tests. Comparing to drug-free measurements, the light scattering curves of ALD and LDH were not altered by equimolar amounts of the pharmaceutical substances used in the previous experiments (data not shown).

For a better evaluation of the anti-aggregation ability of AAG, the results are compared to analogous data reported for extracellular molecular chaperones identified in the human body (Table 4).[10–13,30,31] Comparison of data in Tables 1 and 4 show that anti-aggregation effect of AAG in DTT-induced aggregation assays is similar to that of clusterin. Though in a lesser extent, AAG inhibited thermal aggregation of OVT at smaller ratios than that was reported for clusterin, haptoglobin and α_2-macroglobulin. With the exception of fibrinogen, higher AAG/test protein ratios were required in CS and ADH assays to achieve significant anti-aggregation effects comparably to that found with other extracellular chaperones.

The results presented here demonstrate that human serum AAG (also known as orosomucoid) is able to inhibit in vitro aggregation of a series of proteins subjected to thermal and chemical stresses (Fig. 1, Table 1). To our knowledge, this is the first study showing the chaperone-like activity of AAG so far. Similarly to the previously identified extracellular chaperone fibrinogen, haptoglobin, and α_2-macroglobulin AAG is also an abundant acute-phase protein of the plasma of which normal level can increase by fivefold during stress response. These results may provide a new avenue for understanding real biological function of AAG. Namely, the

Table 1
Effect of AAG on thermal- and DTT-induced (INS) aggregation of various proteins in 40 mM pH 7.5 Hepes-KOH buffer

	Light scattering decrease (%)	*R*	Test protein cc. (μM)	*t* (°C)
ALD	20	1.0	1.7	54
	40	2.0		
	50	4.0		
	60	6.7		
ADH	50	0.7	2.2	50
	65	1.4		
BLG	20	0.2	2.6	48
	35	0.5		
	60	1.1		
	65	2.2		
CA	65	3.0	2.8	48
	90	4.0		
CS	20	1.0	0.6	43
	25	2.0		
	30	3.7		
	35	7.4		
CTL	15	0.5	0.9	50
	60	1.0		
	90	2.0		
ENL	60	0.5	0.6	50
	80	1.0		
	90	2.0		
GPDH	20	2.0	1.2	45
	25	4.0		
	35	7.4		
INS	50	0.5	34	37
	60	1.0		
	80	2.0		
LDH	20	0.5	0.7	55
	25	1.0		
	40	2.0		
	50	4.0		
	70	8.0		
OVT	40	0.02	1.4	55
	45	0.05		

Decrease of light scattering measured at 500 or 360 nm (INS) is expressed in relation to the light scattering of samples with no added AAG. '*R*' values refer to AAG/test protein molar ratios (molar concentrations of multimeric test proteins were calculated by using MW of their subunits).

Table 2
Effect of AAG on thermal and DTT-induced (INS) aggregation of various proteins in the presence and absence of STR (40 mM pH 7.5 Hepes-KOH buffer)

	Max. scattering decrease (%)		*R*	Test protein cc. (μM)	*t* (°C)
	No STR	With STR			
ALD	30	0	2.5	1.7	54
GPDH	35	0	7.5	1.1	45
INS	50	0	0.9	34	37
LDH	65	0	7.5	0.7	55
OVT	45	20	0.02	1.4	55

STR–AAG equimolar mixtures were preincubated at the temperatures shown. Maximum decrease of light scattering measured at 500 or 360 nm (INS) is expressed in relation to the scattering of samples with no added AAG. '*R*' values refer to AAG/test protein molar ratios.

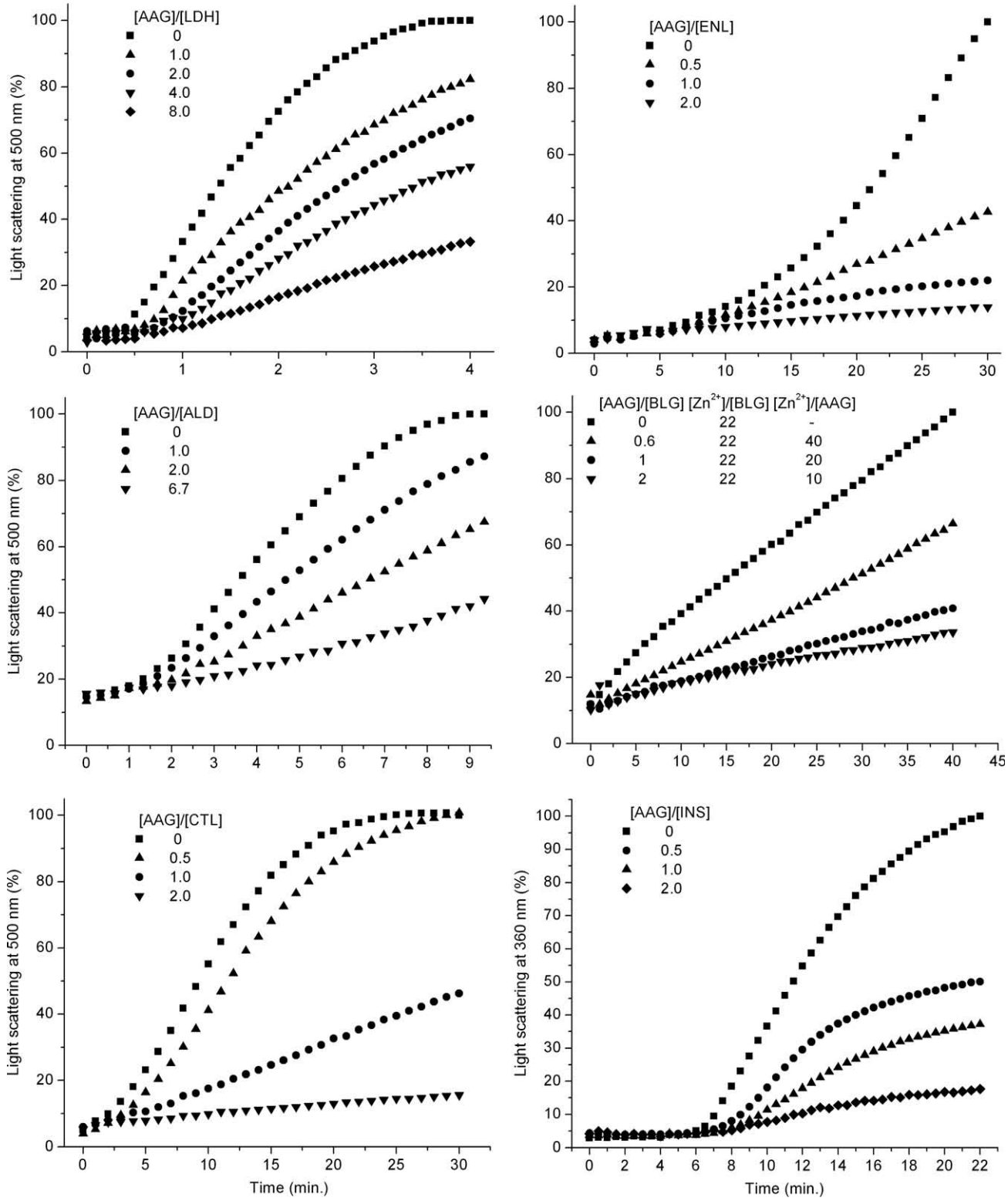

Figure 1. Representative light scattering curves showing inhibition of stress-induced protein precipitation by AAG in 40 mM pH 7.5 Hepes-KOH buffer. Data were measured at 500 or 360 nm (INS) as a function of time. AAG/test protein molar ratios are shown. See Table 1 for further details.

possibility is raised that AAG may exert its anti-inflammatory action by inhibiting inappropriate self-association of extracellular stressed proteins especially under acute phase conditions like infections, burn injuries, and neoplastic diseases.

Aggregation assays performed in the presence of some drug ligands of AAG indicated the impact of drug binding on AAG-stressed protein interactions (Table 2). The opening of the cleft-like central cavity of AAG is about 9–12 Å in diameter[5] and it has been shown that the open entrance of the pocket is able to accommodate bulky compounds such as hemin[32] and gold(I) macrocycles having molecular dimensions of 13 × 14 × 14 Å.[24] Thus, results of the aggregation assays performed in the presence of drugs indicate

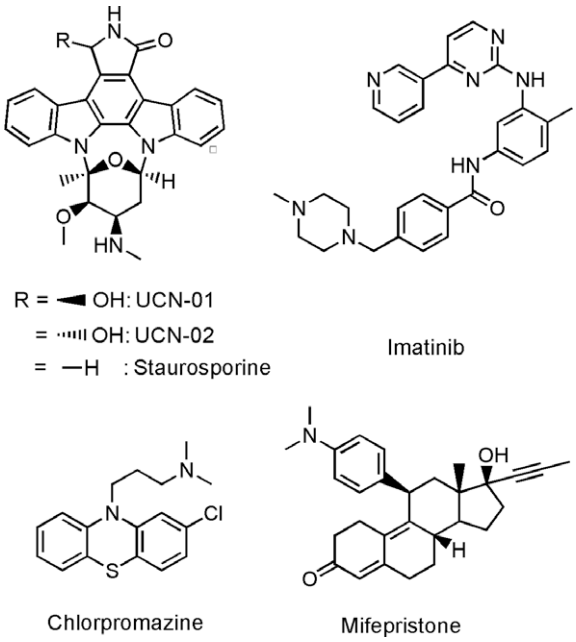

Figure 2. Chemical structures of drug ligands of AAG.

Table 3
Effect of AAG on the thermal aggregation of ALD and LDH in the presence and absence of drug ligands of AAG (40 mM pH 7.5 Hepes-KOH buffer)

	Max. scattering decrease (%)				*R*	Test protein cc. (μM)	*t* (°C)
	No drug	IMT	MFP	CHL			
ALD	40	30	0	20	2.5	1.7	54
LDH	65	0	0	15	7.5	0.7	55

Drug–AAG equimolar mixtures were preincubated at the temperatures shown. '*R*' values refer to AAG/test protein molar ratios.

that non-covalent interactions established between the central pocket and exposed hydrophobic surfaces of stressed proteins might be involved in mediating the chaperone-like activity of AAG. It is to be noted that the hydrophobic probe bisANS preincubated with α_2-macroglobulin completely abolished its ability to inhibit thermal aggregation of CS and creatine phosphokinase.[12] However, an additional mechanism should also be considered regarding the effect of drug binding on the chaperone-like behaviour of AAG. Although CHL is a less bulky compound in relation to the other ligands used in this study, however, it significantly reduced the anti-aggregation ability of AAG (Table 3). Taking into account the size of CHL, steric restriction of AAG–stressed protein interactions is less likely in this case. On the other hand, it is known that AAG binding of CHL enhances structural rigidity of the protein.[33] Since most chaperones rely on conformational flexibility for their activity[34,35] accommodation of CHL inside the β-barrel pocket of AAG may decrease its chaperone effectivity by restricting conformational mobility of AAG. As infrared and circular dichroism studies showed, AAG adopts an intermediate less folded state characterized by a relaxed tertiary structure upon heating to 50–55 °C.[23] Presumably drug binding acts against this process by stabilizing the native fold of AAG. Noteworthy that in the secondary structure of AAG the unordered content is considerably high (≈30%)[36] similarly to several protein chaperones showing disordered fractions from 25% to 55%.[37] Structural disorder has been proposed as an important functional feature of molecular chaperones.[37]

Consideration of the hydrophobic pocket as a potential chaperone binding site raises a novel aspect regarding the promiscuous drug binding feature of AAG. AAG binding of more than 300 pharmaceutical agents has been reported[1] and the β-barrel cavity has been shown to be the primary drug binding site.[5,38] AAG binding affects pharmacokinetic parameters of drugs,[1,39] however, in the light of the above results, the protein bound compounds might also modify the chaperone-like action of AAG by steric restriction of protein–protein interactions and/or by enhancing the conformational rigidity of the protein. Accordingly, high-affinity AAG binding of drug molecules administered in various diseases (e.g., kinase inhibitors,[39] macrolide antibiotics,[40] Vinca alkaloids[39]) may alter chaperone activity of AAG. Analogously, AAG ligands of endogenous origin (e.g., biliverdin,[41] steroids, lysophospholipids[42]) might also affect AAG-misfolded protein interactions. Interestingly, drug binding (paclitaxel) of the extracellular chaperone clusterin has been recently described, however, its effect on the chaperone function has not been investigated.[43]

Beside the prostaglandin D synthase, AAG is the second protein of the lipocalin superfamily which shows chaperone-like properties. Lipocalins generally bind small, hydrophobic ligands yet can also bind to peptides,[44] large soluble macromolecules, and cell-sur-

Table 4
Aggregation suppressing ability of human extracellular chaperones as reported in the literature

	Max. scattering decrease (%)	*R*	Test protein cc. (μM)	*t* (°C)	Buffers	Ref.
CLS-ADH	90	0.3	17	55	10 mM Na_2HPO_4, 150 mM NaCl at pH 7.4	30
CLS-BSA	85	1.5	11	37	50 mM Na-phosphate, 0.1 M NaCl, pH 7.0	31
CLS-CS	80	6.0	0.15	43	Hepes at pH 7.5	13
CLS-CTL	90	0.8	3.3	60	50 mM Na-phosphate pH 7.0	31
CLS-GST	100	0.3	7.7	60		
CLS-LCT	100	0.3	54	37	50 mM Na-phosphate, 0.1 M NaCl, pH 7.0	
CLS-OVT	90	0.1	26	60	50 mM Na-phosphate, 50 mM KCl, 5 mM $MgCl_2$ at pH 7.0	30
FB-CS	70	6.0	0.15	43	Hepes at pH 7.5	13
HP-CTL	50	3.8	2.5	50	Not reported	11
HP-CS	75	1.0	3	43	14 mM Na_2HPO_4, 3.2 mM Tris, 26 mM NaCl, 0.6 mM EDTA, pH 7.4	10
HP-GST	80	1.0	7.8	55	50 mM Na_2HPO_4 at pH 7.4	
HP-OVT	80	0.8	13	60		
α_2M-CS	85	0.8	6	43	50 mM Tris, 2 mM EDTA, pH 8	12
α_2M-CPK	90	0.3	25	43	137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4	
α_2M-GST	80	0.9	8	60		
α_2M-OVT	70	0.6	12.5	60		

Approximate values of reduction in light scattering (%) were estimated from figures in the references. '*R*' values refer to chaperone/test protein molar ratios. BSA and LCT were subjected for DTT-induced aggregation assays. Glutathione S-transferase (GST) was prepared from *Schistosoma japonicum*. Abbreviations used only in Table 4: α_2M, α_2-macroglobulin; BSA, bovine serum albumin; CLS, clusterin; CPK, creatine phosphokinase (rabbit); FB, fibrinogen; HP, haptoglobin; LCT, α-lactalbumin (bovine).

face receptors.[45,46] Therefore, this protein family may be a potential source of additional extra- and/or intracellular molecular chaperones especially in cases when the lipocalin fold defines a wide central calyx (e.g., complement protein C8γ,[44] neutrophil gelatinase associated lipocalin[47]).

In conclusion, it has been demonstrated that human serum AAG possesses a chaperone-like activity suppressing thermal and chemical-induced aggregations of various test proteins. Drug binding of AAG cancels/reduces the chaperone action. AAG is proposed to be a new member of human extracellular molecular chaperones.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.11.132.

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