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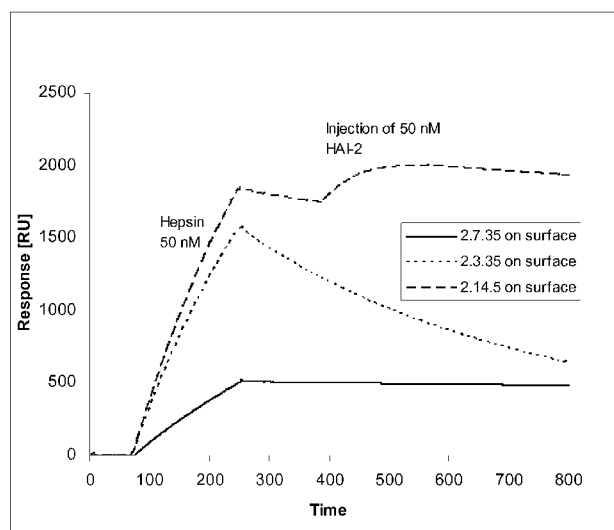
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(54) Title: ANTI-HEPSIN ANTIBODIES AND METHODS OF USE

Fig. 1

(57) Abstract: The present invention relates to antibodies against human hepsin (hepsin antibody), methods for their production, pharmaceutical compositions containing said antibodies, and methods of using the same.



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Anti-Hepsin Antibodies and Methods of Use

The present invention relates to antibodies against human hepsin (hepsin antibody), methods for their production, pharmaceutical compositions containing said antibodies, and methods of using the same.

Background of the Invention

5 Human hepsin (EC 3.4.21.106, hepsin) is a Type II serine transmembrane protease and belongs to trypsin family of serine proteases. Hepsin was identified by homology screening of a human liver cDNA library in 1988 (Leytus, S.P. et al., Biochem. 27 (1988) 1067-1074). It is known that hepsin plays an important role in cell growth and maintenance of cell line morphology (Tsuji, A., et al., J. Biol. hem. 10 266 (1991) 16948-16953, and Torres-Rosado, A., et al., PNAS USA 90 (1993) 7181-7185). Torres-Rosado showed, in addition, that when hepatoma cells were treated with rabbit polyclonal anti-hepsin antibodies, their growth was substantially arrested. Hepsin-specific antisense oligonucleotides showed the same effect. Kazama, Y., et al., in J. Biol. Chem. 270 (1995) 66-72, described that hepsin 15 synthesis may be dramatically upregulated in tissue factor-deficient tumor cells and contribute to coagulation activation of these cells. Li et al showed that Hepsin mediates invasive tumor growth and metastasis which can be inhibited by a pegylated Kunitz domain (KD-1 derived from HAI-1) in vivo (Cancer Res. 2009;69:(21) November 1, 2009).

20 Hepsin is expressed in several normal tissues including liver, kidney, pancreas, prostate, and thyroid, but is overexpressed in several cancers such as ovarian cancer, renal carcinoma, estrogen receptor positive breast cancer, endometrial cancer, and prostate cancer. Overexpresion of Hepsin mRNA is observed in over 90% of prostate cancers, and overexpression of protein has also been demonstrated 25 by immunohistochemical staining showing 30 to 40-fold higher in cancer Vs. benign prostatic hyperplasia or normal prostate tissue. In addition, single nucleotide polymorphisms (SNPs) in the hepsin gene have been shown to be associated with susceptibility to prostate cancer.

30 To date, the physiological role of hepsin remains unclear, however in vitro, hepsin has been shown to cleave factor VII, pro-urokinase plasminogen activator, pro-hepatocyte growth factor, and more recently was shown to cleave laminin-332 (Tripathi, M., et al., JBC 283 (2008) 30576-30584). Activation of factor VII

ultimately leads to thrombin formation, which could promote cell migration and invasion. Similarly, activation of the uPA/uPAR and HGF/C-Met pathways could promote cell growth and invasion/metastasis. Cleavage of laminin-332 has also been demonstrated to promote cell migration/ invasion.

5 WO 01/62271 relates to a method of producing activated T cells directed towards Hepsin. WO 02/064839 refers to a diagnosing method of ovarian cancer by measuring hepsin gene expression. WO 03/016484 refers to a diagnosing method of prostate cancer by measuring hepsin gene expression. WO 2004/033630 refers to a modified Hepsin and an antibody binding against such a modified Hepsin.
10 WO 2004/086035 refers to a human hepsin variant which is associated with cardiovascular disorders, respiratory diseases, gastroenterological disorders, endocrinological disorders, urological disorders, reproduction disorders, metabolic diseases and cancer. Also mentioned are compounds which bind to and/or activate refers to reagents that regulate a human hepsin variant and reagents which bind to
15 said human hepsin gene products for preventing, ameliorating, or correcting dysfunctions or diseases including cardiovascular disorders, endocrinological and hormonal disorders, metabolic disorders (including diabetes), inflammatory disorders, gastrointestinal and liver disorders, cancer, hematological disorders, respiratory disorders, neurological disorders, reproductive disorders, and
20 genitourinary disorders. WO 2005/021582 refers to a diagnosing method of ovarian, prostate or kidney cancer by measuring hepsin mRNA expression. US 7,029,675 refers to a method for treating or preventing infarction of a patient comprising administering a hepsin fragment. WO 2006/014928 refers to a screening method for identification of a substance which is capable of inhibiting
25 hepsin activation of single chain HGF (pro-HGF). Antibodies against hepsin or modified hepsin are mentioned in Torres-Rosado, A., et al., PNAS 90 (1993) 7181-7185; Xuan, J.A., et al., Cancer Res. 66 (2006) 3611-3619; WO 2002/064839; WO 2004/033630, and WO 2007/149932.

Hepatocyte growth factor (HGF) activator is a serine protease responsible for
30 proteolytic activation of HGF in response to tissue injury. There exist two inhibitors of HGF activators (HGF activator inhibitor type 1, HAI-1 and HGF activator inhibitor type 2, HAI-2; UniProt Accession No. O43291; Kawaguchi, T., et al., J. Biol. Chem. 272 (1997) 27558-27564 and Kataoka, H., et al., Biochem. Biophys. Res. Comm. 290 (2002) 1096-1100; Li, W., et al., Cancer Res. 69 (2009)
35 8395-8402; Epub 2009 Oct 20; disclosed that pegylated Kunitz domain inhibitor (a hepsin active site inhibitor derived from hepatocyte growth factor activator

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inhibitor-1) suppresses hepsin-mediated invasive tumor growth and metastasis. Kirchhofer, D., et al., FEBS Lett. 579 (2005) 1945-1950 disclosed that Hepsin activates pro-hepatocyte growth factor and is inhibited by hepatocyte growth factor activator inhibitor-1B (HAI-1B) and HAI-2.

- 5 The object of the invention is to provide antibodies against hepsin which are useful as therapeutic agents for tumor treatment.

Summary of the Invention

10 The invention relates to an antibody binding to human hepsin, characterized in that the heavy chain variable domain comprises a CDR1 region of SEQ ID NO: 33, a CDR2 region of SEQ ID NO:34 and a CDR3 region of SEQ ID NO:35 and in that the light chain variable domain comprises a CDR1 region of SEQ ID NO: 36, a CDR2 region of SEQ ID NO:37 and a CDR3 region of SEQ ID NO:38. (MAB55)

In one embodiment said antibody is characterized in comprising

- 15 the heavy chain variable domain defined by amino acid sequence of SEQ ID NO: 9 and the light chain variable domain defined by amino acid sequence of SEQ ID NO: 10,

20 In one embodiment said antibody is characterized in inhibiting serine protease activity of human hepsin with an IC_{50} value of 20nM or lower and reducing in a concentration of 0.67 μ M cell proliferation of LNCaP cells (ATCC CRL-1740) in the MTT assay for 20% or more in relation to cell proliferation without said antibody.

In one embodiment said antibody is characterized in inhibiting tumor growth in vivo by 80% or more in human xenograft models of LNCaP prostate cancer cells at a dose of 20 mg/kg twice weekly.

- 25 In one embodiment said antibody is characterized in inhibiting tumor growth in vivo by 80% or more in human xenograft models of T47D breast cancer cells at a dose of 20 mg/kg twice weekly.

In one embodiment said antibody is characterized in inhibiting binding of human HGF activator inhibitor type 2 of SEQ ID NO:17 to hepsin.

- 30 In one embodiment the antibody according to the invention binds to human hepsin and is characterized in inhibiting serine protease activity of human hepsin with an

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IC₅₀ value of 20nM or lower and reducing in a concentration of 0.67μM cell proliferation of LNCaP cells (ATCC CRL-1740) in the MTT assay for 20% or more in relation to cell proliferation without said antibody.

5 In one aspect the invention relates to an antibody binding to human hepsin, characterized in reducing in a concentration of 0.67μM cell proliferation of LNCaP cells (ATCC CRL-1740) in the MTT assay for 20% in relation to cell proliferation without said antibody.

10 In one aspect the invention relates to an antibody binding to human hepsin, characterized in inhibiting binding of human HGF activator inhibitor type 2 (HAI-2, SEQ ID NO:17) to hepsin.

In one embodiment the the antibody according to the invention is monoclonal. In one embodiment the the antibody according to the invention is a human or humanized antibody.

15 In one embodiment the the antibody according to the invention is characterized in that the heavy chain variable domain comprises a CDR1 region of SEQ ID NO: 1, a CDR2 region of SEQ ID NO:2 and a CDR3 region of SEQ ID NO:3 and in that the light chain variable domain comprises a CDR1 region of SEQ ID NO: 4, a CDR2 region of SEQ ID NO:5 and a CDR3 region of SEQ ID NO:6 or that the heavy chain variable domain comprises a CDR1 region of SEQ ID NO: 18, a CDR2 region of SEQ ID NO:19 and a CDR3 region of SEQ ID NO:20 and that the light chain variable domain comprises a CDR1 region of SEQ ID NO: 21, a CDR2 region of SEQ ID NO:22 and a CDR3 region of SEQ ID NO:23;

or a humanized variant thereof.

25 In one embodiment the the antibody according to the invention is characterized in that the heavy chain variable domain comprises SEQ ID NO:7 or SEQ ID NO:24 or a humanized variant thereof.

30 In one embodiment the the antibody according to the invention is characterized in that the light chain variable domain comprises SEQ ID NO:8 or SEQ ID NO:25 or a humanized variant thereof.

In one embodiment the the antibody according to the invention is characterized in that the heavy chain variable domain comprises SEQ ID NO:7 or a humanized variant thereof and the light chain variable domain comprises SEQ ID NO:8 or a

humanized variant thereof or that the heavy chain variable domain comprises SEQ ID NO:24 or a humanized variant thereof and the light chain variable domain comprises SEQ ID NO:25 or a humanized variant thereof.

5 Preferably the antibody according to the invention is characterized in being a chimeric or humanized variant of an antibody comprising in the heavy chain variable domain a CDR1 region of SEQ ID NO: 1, a CDR2 region of SEQ ID NO:2 and a CDR3 region of SEQ ID NO:3 and in the light chain variable domain a CDR1 region of SEQ ID NO: 4, a CDR2 region of SEQ ID NO:5 and a CDR3 region of SEQ ID NO:6 or of an antibody comprising in the heavy chain variable domain comprises a CDR1 region of SEQ ID NO: 18, a CDR2 region of SEQ ID NO:19 and a CDR3 region of SEQ ID NO:20 and in the light chain variable domain a CDR1 region of SEQ ID NO: 21, a CDR2 region of SEQ ID NO:22 and a CDR3 region of SEQ ID NO:23.

15 The antibody according to the invention is preferably characterized in that said antibody comprises the heavy chain variable domain defined by amino acid sequence of SEQ ID NO: 7 and the light chain variable domain defined by amino acid sequence of SEQ ID NO: 8. The antibody according to the invention is preferably characterized in that said antibody comprises the heavy chain variable domain defined by amino acid sequence of SEQ ID NO: 9 and the light chain variable domain defined by amino acid sequence of SEQ ID NO: 10. The antibody according to the invention is preferably characterized in that said antibody comprises the heavy chain variable domain defined by amino acid sequence of SEQ ID NO: 11 and the light chain variable domain defined by amino acid sequence of SEQ ID NO: 12. The antibody according to the invention is preferably characterized in that said antibody comprises the heavy chain variable domain defined by amino acid sequence of SEQ ID NO: 24 and the light chain variable domain defined by amino acid sequence of SEQ ID NO: 25.

30 The antibody according to the invention is preferably characterized in binding to human hepsin, in inhibiting binding of human HGF activator inhibitor type 2 (HAI-2, SEQ ID NO:17) to human hepsin, and by the above mentioned amino acid sequence combinations.

35 The invention comprises an antibody binding to the same epitope of hepsin as antibody Mab 2.7.35, comprising as the heavy chain variable domain sequence SEQ ID NO:7 and as the light chain variable domain sequence SEQ ID NO:8. The invention comprises an antibody binding to the same epitope of hepsin as antibody

Mab 2.3.35, comprising as the heavy chain variable domain sequence SEQ ID NO:24 and as the light chain variable domain sequence SEQ ID NO:25.

Preferably the antibody according to the invention is characterized in comprising a constant region selected from the group consisting of SEQ ID NO:26-32.

- 5 The antibody according to the invention binds human hepsin (active two-chain form). The antibody according to the invention neutralizes serine protease activity (enzymatic activity) of hepsin.

Preferably the antibody according to the invention is characterized in binding to the enzymatically active two-chain form of human hepsin and in inhibiting the enzymatic serine protease activity of hepsin with an IC_{50} value of 20 nM or lower, preferably 10nM or lower, preferably between 1 and 10 nM or preferably 2 nM or lower. The antibody according to the invention do not inhibit trypsin or chymotrypsin activity, which means that it inhibits trypsin or chymotrypsin activity with an IC_{50} value of 200 nM or more. The antibody according to the invention shows an in vitro cell potency of 100 μ g/ml or higher (IC_{20} , MW 150.000).

The antibody according to the invention is not cross-reactive with mouse hepsin, which means that the antibody inhibits mouse hepsin enzymatic activity with IC_{50} 200 nM or more. The antibody according to the invention is cross-reactive with Cynomolgus hepsin, which means that the antibody inhibits Cynomolgus hepsin enzymatic activity with IC_{50} of 20 nM or lower, preferably 2 nM or lower.

The antibody according to the invention preferably comprises a Fc part derived from human origin. Preferably the antibody according to the invention is a monoclonal antibody. Preferably the antibody according to the invention is a chimeric, humanized or human antibody. Preferably the antibody according to the invention is characterized in being a humanized antibody derived from hamster. Preferred humanized variants are Mab53 and Mab55, which are derived from of Mab 2.7.35 and comprise the same CDRs as Mab 2.7.35.

The antibody is preferably an immunoconjugate comprising the antibody of the invention and a cytotoxic agent.

- 30 The antibody according to the invention is especially characterized by selectively blocking the serine protease activity of hepsin (hepsin enzymatic activity).

The antibody according to the invention is preferably of human isotype (antibody class) IgG1, IgG2, IgG3, or IgG4, whereby IgG1 is preferred.

A further embodiment of the invention is a nucleic acid encoding an antibody according to the invention.

5 A further embodiment of the invention is a nucleic acid encoding a heavy chain of an antibody binding to hepsin, characterized in comprising a CDR1 region of SEQ ID NO: 1, a CDR2 region of SEQ ID NO:2 and a CDR3 region of SEQ ID NO:3 or a CDR1 region of SEQ ID NO: 18, a CDR2 region of SEQ ID NO:19 and a CDR3 region of SEQ ID NO:20.

10 More preferably the nucleic acid encodes a hepsin antibody heavy chain variable region of SEQ ID NO:7 or 24 or a humanized variant thereof and a heavy chain constant region of human IgG1 type.

A further embodiment of the invention is a nucleic acid encoding a light chain of an antibody binding to hepsin, characterized in comprising a CDR1 region of SEQ ID NO: 4, a CDR2 region of SEQ ID NO:5 and a CDR3 region of SEQ ID NO:6 or
15 comprises a CDR1 region of SEQ ID NO: 21, a CDR2 region of SEQ ID NO:22 and a CDR3 region of SEQ ID NO:23. More preferably the nucleic acid encodes a hepsin antibody light chain variable region of SEQ ID NO:8 or 25 or a humanized variant thereof and a light chain constant region of human IgG1 type.

20 A further embodiment of the invention is a nucleic acid encoding an antibody binding to hepsin being characterized in comprising a heavy chain variable region of SEQ ID NO:7 and light chain variable region of SEQ ID NO:8.

A further embodiment of the invention is a nucleic acid encoding an antibody binding to hepsin being characterized in comprising a heavy chain variable region
25 of SEQ ID NO:9 and light chain variable region of SEQ ID NO:10.

A further embodiment of the invention is a nucleic acid encoding an antibody binding to hepsin being characterized in comprising a heavy chain variable region of SEQ ID NO:11 and light chain variable region of SEQ ID NO:12.

30 A further embodiment of the invention is a nucleic acid encoding an antibody binding to hepsin being characterized in comprising a heavy chain variable region of SEQ ID NO:24 and light chain variable region of SEQ ID NO:25.

A further embodiment of the invention is a nucleic acid encoding an antibody according to the invention, characterized in comprising a constant region selected from the group consisting of SEQ ID NO:26-32.

5 A further embodiment of the invention is a host cell comprising the nucleic acid of an antibody according to the invention.

The invention further provides expression vectors containing nucleic acid according to the invention capable of expressing said nucleic acid in a prokaryotic or eukaryotic host cell, and host cells containing such vectors for the recombinant production of such an antibody.

10 The invention further comprises a prokaryotic or eukaryotic host cell comprising a vector according to the invention.

The invention further comprises a method for the production of a recombinant human or humanized antibody according to the invention, characterized by expressing a nucleic acid according to the invention in a prokaryotic or eukaryotic
15 host cell and recovering said antibody from said cell or the cell culture supernatant. The invention further comprises the antibody obtainable by such a recombinant method.

The invention further comprises a method for the generation of anti-hepsin antibodies according to the invention, characterized by immunizing a host animal
20 like mouse, hamster, rabbit or rat by the use of an enzymatically active hepsin over three months or more, preferably followed by an i.v. boost on day 4 before fusion and isolation of an antibody according to the invention, preferably by use of a hybridoma method.

Antibodies according to the invention show benefits for patients in need of a hepsin
25 targeting therapy. The antibodies according to the invention have new and inventive properties causing a benefit for a patient suffering from cancer, especially from breast or prostate cancer. The invention comprises also a method for the treatment of a patient suffering from such disease.

The invention comprises a method for the treatment of a patient in need of therapy,
30 characterized by administering to the patient an antibody according to the invention.

5 A further embodiment of the invention is a method for the treatment of a patient suffering from cancer, especially from solid tumors such as prostate cancer, breast cancer, ovarian cancer, endometrial cancer, renal cell carcinoma, and hepatocellular carcinoma and liver tumors, characterized by administering to the patient an antibody according to the invention.

The invention comprises the use of an antibody according to the invention for therapy.

10 The invention comprises the use of an antibody according to the invention for the preparation of a medicament for the treatment of cancer, especially breast or prostate cancer.

The invention comprises the use of an antibody according to the invention for the treatment of cancer, especially breast or prostate cancer

15 The invention further provides a method for treating a patient suffering from cancer, especially from breast or prostate cancer, comprising administering to a patient diagnosed as having such a disease (and therefore being in need of an such a therapy) an effective amount of an antibody binding to hepsin according to the invention. The antibody is administered preferably in a pharmaceutical composition.

20 A further embodiment of the invention is a pharmaceutical composition comprising an antibody according to the invention.

The invention further comprises a pharmaceutical composition containing an antibody according to the invention in a pharmaceutically effective amount, optionally together with a buffer and/or an adjuvant useful for the formulation of antibodies for pharmaceutical purposes.

25 The invention further provides pharmaceutical compositions comprising such antibodies in a pharmaceutically acceptable carrier. In one embodiment, the pharmaceutical composition may be included in an article of manufacture or kit.

A further embodiment of the invention is the use of an antibody according to the invention for the manufacture of a pharmaceutical composition.

30 A further embodiment of the invention is a method for the manufacture of a pharmaceutical composition comprising an antibody according to the invention.

The invention further provides a method for the manufacture of a pharmaceutical composition comprising an antibody according to the invention together with a pharmaceutically acceptable carrier and the use of the antibody according to the invention for such a method.

5 The invention also provides the use of an antibody according to the invention for the manufacture of a pharmaceutical agent, preferably together with a pharmaceutically acceptable carrier, for the treatment of a patient suffering from cancer, especially from breast or prostate cancer.

10 The invention further comprises the use of an antibody according to the invention for the diagnosis of cancer susceptibility in vitro, preferably by an immunological assay determining the binding between soluble hepsin of a human plasma sample and the antibody according to the invention.

Description of the Figures

15 **Figure 1** shows a sensogram of an epitope mapping experiment. Here an anti-hepsin antibody was immobilized on a CM5 chip and 50 nM hepsin was added. Then HAI-2 (50nM) was injected.

20 **Figure 2** shows a further sensogram of an epitope mapping experiment. Here anti-hepsin antibody 2.7.35, 2.3.35 and 2.14.5 were immobilized on a CM5 chip and 50 nM hepsin was added. Then antibody 2.7.35 was injected with a concentration of 50 nM.

Figure 3 shows a further sensogram of an epitope mapping experiment. Here anti-hepsin antibody 2.7.35, 2.3.35 and 2.14.5 were immobilized on a CM5 chip and 50 nM hepsin was added. Then antibody 2.3.35 was injected with a concentration of 50 nM.

25 **Figure 4** Comparison of inhibition and affinity of hepsin antibodies. **(A)** Inhibition of hepsin activity by antibody mouse Mab 2.7.35 (= mH35) (squares), chimeric Mab 2.7.35 (= chH35) (circles) and Mab55 (= hH35) (triangles). After preincubation of antibodies with hepsin for 30 minutes, hydrolysis reaction was started with addition of Ac-KQLR-AMC peptide. Fluorescence increase was measured after 40 minutes incubation. The IC₅₀ values were calculated after fitting the % inhibition data to a four-parameter equation. Error bars represent standard deviation from three experiments. **(B)** Adjusted surface plasmon resonance (Biacore) sensogram analyzing binding of hepsin to immobilized antibody

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Mab55 (= hH35). Hepsin was injected at concentrations 0-200 nM. Curve fittings using a 1:1 Langmuir binding model are shown by black lines. **(C)** Comparison of binding and dissociation constants for mouse Mab 2.7.35 (= mH35), chimeric Mab 2.7.35 (= chH35) and Mab5(= hH35) antibodies analyzed by surface-plasmon resonance measurement.

Figure 5

Binding of Mab55 (= hH35) to cell surface hepsin. **(A)** HEK293 cell lines that stably overexpress full length hepsin with a C-terminal GFP fusion tag were analyzed by flow cytometry. By incubation with increasing amounts of Mab55, specific and saturable surface staining could be detected. **(B)** Surface staining is confirmed by confocal laser scanning microscopy analysis. Untransfected cells (HEK293 wt) did not display any detectable binding.

Figure 6

Mab55 (= hH35) is protease and species specific and shows a non-linear inhibition type. **(A)** Activity of hepsin antibodies against other serine proteases. For assessing the inhibitory potential of the antibodies on other serine proteases the same test conditions as for hepsin were used. Hepsin (1 nM), Matrilysin (2 nM), HAT (2 nM), Enteropeptidase (0.7 nM), Trypsin (1.8 nM) were incubated with antibody (500 nM) for 30 minutes and reaction was started with 5 μ M Ac-KQLR-AMC peptide. Data are shown for three replicate experiments. **(B)** Activity of antibody Mab55 analyzed in a FRET activity assay against human hepsin (blue), cynomolgus (brown) and mouse (magenta). Cross reactivity was found against cynomolgus, but not against mouse hepsin. IC₅₀ values are stated accordingly. **(C)** Progress curve of hepsin inhibition by Mab55 (= hH35). Hepsin (1 nM) was added to a mixture of Ac-KQLR-AMC peptide (10 μ M) and 0, 111.1, 333.3 and 1000 nM of Mab55. The increase of fluorescence was monitored at 10-second intervals over 200 seconds. The amidolytic activity of hepsin in absence of antibody shows a straight line ($R^2 = 0.9946$). **(D)** Eadie-Hofstee plot of hepsin inhibition by mouse Mab 2.7.35. Hepsin (1 nM) was preincubated without (filled circles) or with Mab55 (= hH35) (20-0.31 nM in 2-fold dilution steps) for 15 min. After addition of Ac-KQLR-AMC peptide (40, 20, 10, 5, 2.5 μ M) the linear rates

of the increase in fluorescence were measured on a kinetic microplate reader. The plot for the top-ranked mixed tight inhibition model is shown ($V_{\max}=0.81\pm0.01\ \mu\text{M AMC/min}$ and $K_m=13.65\pm0.44\ \mu\text{M}$ for control; $\alpha=1.3\pm0.3$). Data are plotted as mean $\pm$ SD for $n=3$.

Figure 7

Multiple-sequence alignments of Mab55 (= hH35) variable regions and hepsin. **(A)** Alignment of F_v sequences for Mab55 (= hH35) antibody, IMGT germlines and mouse Mab 2.7.35 (= mH35) antibody. Invariant and conserved residues are highlighted in green and yellow, respectively. Residues are numbered according to the Kabat [37] numbering systems and secondary structure elements for Mab55 variable sequences indicated above the sequence (spirals, α - and $3_{10}(\eta)$ -helices; arrows, β -strands). Residues important for humanization and CDR sequences are marked. **(B)** Alignment and structural conservation of hepsin, HGFA and trypsin. Secondary structure elements are indicated for hHepsin in structure PDB 1Z8G and complex hHepsin-Mab55 above the sequences (spirals, α - and $3_{10}(\eta)$ -helices; arrows, β -strands; lines, ordered but without secondary structure; dashed lines, disordered regions). Residues 160-166, 297-306, 343-350 and 377-382 were disordered in the hHepsin-Mab55 crystal structure. Sequence alignments were created with STRAP [63] and MUSCLE [64], figures were prepared with ESPript [65].

Figure 8

Analysis of protein hHepsin-Mab55 (= hH35)-Fab structure. **(A)** Surface-view of hHepsin in complex with Mab55 Fab fragment. The protease domain of hHepsin is coloured in violet, the SRCR domain in light violet. Heavy and light chains of Mab55-Fab fragment are coloured in blue and green, respectively. The surface is semitransparent to show the underlying residues in a ribbon model. **(B)** Residues following hHepsin $\alpha 3$ helix reach into a specific, deep and hydrophobic pocket formed by Mab55 (= hH35). The hydrophobicity-surface of Mab55-Fab is shown semitransparently, with colours ranging from dodger blue for the most hydrophilic to white at 0.0 and to orange red for the most hydrophobic. A zoom into the pocket illustrates involved residues. **(C)** Ribbon model illustration of antibody CDRs

(H1-H3 and L1-L3) coloured in orange. Disordered parts in the crystal structure are indicated by dashed lines.

Figure 9

Comparison of recognition regions with published HGFA-Fab complex structures. **(A)** Ribbon model representation of hHepsin-Mab55 (= hH35). Colouring is as in Figure 5. The epitope region is located about 15 Å away from the hepsin active centre cleft. **(B)** Ribbon model superposition of hHepsin (violet, light violet) with structure PDB 3K2U [34]. hHGFA is coloured in red, antibody Fab40 fragment in yellow. **(C)** Ribbon model superposition of hHepsin (violet, light violet) with structure PDB 2R0K [50]. hHGFA is coloured in red, antibody Fab58 fragment in ochre.

Figure 10

Induced structural movements. **(A)** Liquorice-view superposition of Mab55 (= hH35) V_L with structure PDB 1NL0 [66]. Usually, residues V_H (blue) Gln³⁹ and V_L (green) Gln⁴⁰ (or according) form a double hydrogen bond at approximately 2.9 Å distance as illustrated by structure PDB 1NL0 in grey. Residue V_L Phe⁴⁴ (orange) causes in structure hHepsin-Mab55 (= hH35) a widening of this hydrogen bond distance to approximately 5.2 Å. This leads to structural rearrangements creating a recognition pocket for hHepsin at the top cleft between V_L and V_H chains. **(B)** Ribbon model superposition of hHepsin in complex hHepsin-Mab55 (= hH35) and hHepsin in structure PDB 1Z8G. The catalytic triad Asp²⁵⁷-His²⁰³-Ser³⁵³ and other important residues are represented as sticks. Hydrogen bonds (dashed blue lines) are omitted in the catalytic triad zoom window for clarity. Substrate binding pockets are marked by S1-S4.

Detailed Description of Embodiments of the Invention

In one embodiment the invention relates to an antibody binding to human hepsin designated Mab55, which is characterized in that the heavy chain variable domain comprises a CDR1 region of SEQ ID NO: 33, a CDR2 region of SEQ ID NO:34 and a CDR3 region of SEQ ID NO:35 and in that the light chain variable domain comprises a CDR1 region of SEQ ID NO: 36, a CDR2 region of SEQ ID NO:37 and a CDR3 region of SEQ ID NO:38.

According to the 2008 GLOBOCAN study (www.globocan.iarc.fr), prostate cancer is the second most common cancer in men worldwide. While this number may be partially biased due to increased screening efforts, prostate cancer remains still the second most common cause of cancer death in the developed countries. More extensive diagnosis and treatment has led to an increase in long-time survival rate, but still a huge effort needs to be invested, to significantly improve therapy and to cope with this type of cancer especially in ageing populations.

Allosteric regulation is very widespread in nature, for instance applied for enzymatic activity regulation, receptor signalling or even regulation of gene transcription. Nevertheless, to date, most enzyme inhibitors are small molecules and target the active sites of enzymes. But this is especially unfavourable in the case of proteases. First, active site topologies are often strongly conserved between distinct proteases and thus difficult to address selectively by small molecules. Second, those inhibitors often mimic the transition state of enzyme catalysis with often undesired pharmacokinetic properties. Thus, especially for the protease enzyme class, there is an unmet need for alternative inhibitors such as inhibitory antibodies. Active site inhibition using antibodies with a very long CDR-loop reaching into the active centre cleft may be an alternative as shown for the type II transmembrane serine-protease matriptase (MT-SP1), but may be restricted in use to only one or two CDR-loops. In contrast, allosteric inhibition may use the full variability spectrum of all CDRs.

Here, we present an antibody (named Mab55) directed against human hepsin that achieves full inhibition already at nanomolar concentrations and functionally binds also to native hepsin on the cell surface. Moreover, the humanized antibody Mab55 is highly protease and species specific.

The kinetic studies for binding of Mab55 to the enzyme and initial inhibition of the enzyme activity indicate a slow onset of hepsin inhibition by Mab55 which is typical for slow binding inhibition. This could be simply due to a slow association and slow dissociation of the enzyme-inhibitor (EI) complex as a possible second-order interaction. However applying the conditions for a tight binding inhibition we observed a significant decrease of hydrolysis rates at increasing concentrations of inhibitor measured after formation of EI-complex. The nature of this mixed/noncompetitive inhibition indicates a more step mechanism possibly due to allosteric influences. Under steady-state conditions a pre-equilibrium complex of EI could be initially formed which may undergo interconversion to a tightly bound

slow dissociating EI*-complex ($E+I \rightleftharpoons EI \rightleftharpoons EI^*$). Based on this slow tight binding mechanism the apparent K_i^* is influenced by the stabilization of the EI* complex through the binding of the inhibitory antibody.

5 A hyperbolic dependence of observed rate constants on inhibitor concentration was recently described for selective active-site antibodies with different inhibition mechanisms. They were either acting by steric hindrance of substrate access (e.g. anti-MT-SP1/FabE2) or exhibiting an “allosteric switch” mechanism e.g. anti-HGFA antibody Fab40. The enzyme kinetics with the KQLR peptide substrate used in this study indicate that Mab55 does not inhibit competitively by steric
10 hindrance but in a noncompetitive way by underlying possible conformational changes in the active site. Therefore we tried to elucidate the allosteric nature of this inhibition by studying the complex by X-ray crystallography.

And indeed, the crystal structure of the hHepsin- Mab55 complex revealed binding about 15 Å away from the active centre, thus strongly arguing for allosteric
15 inhibition. We further detected significant structural changes both at the active centre and the binding pockets when comparing our hepsin structure to previously published crystal structure conformations. More detailed analysis revealed, that binding of the Mab55 antibody induces allosteric changes through a cascade of conformational changes. This is initiated in human hepsin by the turn of the
20 $F^{327}Y^{328}$ -motif containing loop following the $\alpha 3$ -helix towards the antibody cleft.

In mixed type inhibition, the apparent V_{max} is changed and the inhibitor prevents catalysis regardless of whether the substrate is present or not at the enzyme. The observed mixed mode inhibition in our kinetic experiments is likewise confirmed in the crystal structure since the recognized key residues of human hepsin by
25 antibody Mab55 are located far from the active centre. At least for peptide substrates it is easily conceivable, that our antibody is able to bind no matter whether a substrate is bound or not.

The completeness of inhibition likely results from the observed distortion of the active centre and disorder of binding pockets, which may also explain why we
30 could not absolutely distinguish between mixed-mode and noncompetitive tight binding inhibition. A more detailed investigation would require more elaborate studies such as stopped-flow analysis. It is however well possible, that for the macromolecular natural substrates, additional steric hindrance by the bound antibody can occur.

To explain the observed slow tight binding characteristic from the crystal structure is more complex, and especially for the slowness we can currently only speculate – for instance this may result from slow allosteric change or more step kinetic. But for the tightness of binding, our crystal structure gives a clearer explanation:

5 During antibody humanization, the majority of the CDRs amino acids were kept as from the murine candidate ensuring high affinity. The variable framework was adopted by forward- and back-mutations and those mutations did not interfere with recognition as revealed by our crystal structure. In fact, we observed even improved antibody expression in the presence of V_L Phe⁴⁴. Further analysis showed

10 that this is a key residue in inducing the unique binding pocket in Mab55 antibody and thus led to improved affinity as revealed by the crystal structure as well. Due to its large and hydrophobic character, a widening of the distance between residues V_H Gln³⁹ and V_L Gln³⁸ is caused, that usually form a double hydrogen bond. This leverage movement then propagates to the top cleft between V_H and V_L antibody

15 chains, where a deep and narrow binding pocket is formed that is fundamental for the observed tight binding. Using our crystal structure as a reference, good three dimensional Fab homology models may be derived that could enable to apply this effect also for the targeted design of other antibodies.

The affinity and specificity increase for human hepsin upon humanization is best

20 explained, by the specific allosteric movement of the hepsin F³²⁷Y³²⁸-loop. In rodents, this change is not possible due to different conformations and possibly also flexibility of the loop, especially due to hepsin residues 324-325, which are serine-proline. Apparently, it was unexpected to us to observe this effect. Generally, an affinity increase due to conformational changes of the antigen has been observed

25 before, for instance for a antibody-rheumatoid factor complex Mab55.

A series of well recognized studies of protease inhibition by antibodies was conducted for HGFA, of which pro-HGF is a substrate alike for hepsin, too. The crystal structure of the HGFA-Fab40 complex revealed similarly to our study also an allosteric mode of inhibition. However, structural alignment clarified, that the

30 recognized regions are very distinct. Noticeably, this applies also for the actual allosteric mechanism leading to protease inhibition. In case of Ab40 HGFA antibody, the 99-loop is sandwiched between the substrate and the antibody-binding side, serving as a mobile conduit between these sites. Antibody H3 residue Trp⁹⁶ is inserted into a large hydrophobic pocket of HGFA and locks the 99-loop in

35 a non-competent conformation that is characterized by a partial collapse of the S2 pocket and loss of stabilizing P4-S4 interactions. The conformation of the catalytic

5 triad is not significantly changed in comparison with other known structures of HGFA. For hepsin antibody Mab55, we found an opposite situation, where hydrophobic antigen residues insert into a hydrophobic pocket on the antibody surface. The induced structural rearrangements result in small changes in S2 and S4 pockets, but the catalytic triad adopts a distorted conformation and pockets S1 and S3 are highly disordered.

10 In order to evaluate, whether the observed antigen loop flexibility results as a crystal packing artefact or represents the actual in-solution state, we ended up with three arguments supporting the latter interpretation. First, our crystal contact analysis did not locate residues involved in crystal contact closely to the observed and putative residues, that are changed in conformation and involved in the enzymes' activity. Second, our biochemical assays (Figures 4 and 7) agree very well with the observed mode of action judging from the crystal structure. Third, we analyzed the published literature and found, that not only antigen-induced
15 conformational change of the antibody, but also antibody-induced conformational change of the antigen upon binding is a well known phenomenon.

However, strong disorder of the antigen induced by the antibody never occurred to us before. Therefore, we looked also for other examples in the literature, but could not find a similar study. Thus, this study has to be considered as unique in that
20 aspect. More common are examples in which the antibody orders antigen conformations, especially those that are naturally disordered. The adoption of such a conformation results from a shift of native state probability through ligand binding. In this respect, the observed hepsin structure represents a native state that became energetically most favourable.

25 In conclusion, we have created a unique antibody, that exhibits desirable properties such as affinity, protease specificity and a well defined mode of action. Thus this novel antibody has a valuable potential to be exploited in future therapeutic use such as prostate cancer treatment.

I. Definitions

30 An "acceptor human framework" for the purposes herein is a framework comprising the amino acid sequence of a light chain variable domain (VL) framework or a heavy chain variable domain (VH) framework derived from a human immunoglobulin framework or a human consensus framework, as defined below. An acceptor human framework "derived from" a human immunoglobulin

framework or a human consensus framework may comprise the same amino acid sequence thereof, or it may contain amino acid sequence changes. In some embodiments, the number of amino acid changes are 10 or less, 9 or less, 8 or less, 7 or less, 6 or less, 5 or less, 4 or less, 3 or less, or 2 or less. In some embodiments, the VL acceptor human framework is identical in sequence to the VL human immunoglobulin framework sequence or human consensus framework sequence.

“Affinity” refers to the strength of the sum total of noncovalent interactions between a single binding site of a molecule (e.g., an antibody) and its binding partner (e.g., an antigen). Unless indicated otherwise, as used herein, “binding affinity” refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair (e.g., antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (K_d).

An “affinity matured” antibody refers to an antibody with one or more alterations in one or more CDRs, compared to a parent antibody which does not possess such alterations, such alterations resulting in an improvement in the affinity of the antibody for antigen.

The terms “anti-Hepsin antibody” and “an antibody that binds to Hepsin” herein refer to an antibody that is capable of binding Hepsin with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting Hepsin.

The term “antibody” herein is used in the broadest sense and encompasses various antibody structures, including but not limited to monoclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), affinity matured antibodies, and antibody fragments, as long as the characteristic properties according to the invention are retained. The antibody according to the invention is preferably a humanized antibody, chimeric antibody, or further genetically engineered antibody. Preferably the antibody is a “homogeneous antibody” and/or a “naked antibody”.

The term “antigen-binding portion of an antibody” when used herein refer to the amino acid residues of an antibody which are responsible for antigen-binding. The antigen-binding portion of an antibody comprises amino acid residues from the “complementarity determining regions” or “CDRs”. “Framework” or “FR” regions are those variable domain regions other than the hypervariable region residues as herein defined. Therefore, the light and heavy chain variable domains of an antibody comprise from N- to C-terminus the domains FR1, CDR1, FR2, CDR2,

FR3, CDR3, and FR4. Especially, CDR3 of the heavy chain is the region which contributes most to antigen binding and defines the antibody's properties. CDR and FR regions are determined according to the standard definition of Kabat, et al., Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991) and/or those residues from a "hypervariable loop".

The term "amino acid" as used within this application denotes the group of naturally occurring carboxy α -amino acids comprising alanine (three letter code: ala, one letter code: A), arginine (arg, R), asparagine (asn, N), aspartic acid (asp, D), cysteine (cys, C), glutamine (gln, Q), glutamic acid (glu, E), glycine (gly, G), histidine (his, H), isoleucine (ile, I), leucine (leu, L), lysine (lys, K), methionine (met, M), phenylalanine (phe, F), proline (pro, P), serine (ser, S), threonine (thr, T), tryptophan (trp, W), tyrosine (tyr, Y), and valine (val, V).

An "antibody fragment" refers to a molecule other than an intact antibody that comprises a portion of an intact antibody that binds the antigen to which the intact antibody binds. Examples of antibody fragments include but are not limited to Fv, Fab, Fab', Fab'-SH, F(ab')₂; diabodies; linear antibodies; single-chain antibody molecules (e.g. scFv); and multispecific antibodies formed from antibody fragments. ScFv antibodies are, e.g., described in Huston, J.S., Methods in Enzymol. 203 (1991) 46-88). In addition, antibody fragments comprise single chain polypeptides having the characteristics of a V_H domain, namely being able to assemble together with a V_L domain, or of a V_L domain binding to hepsin, namely being able to assemble together with a V_H domain to a functional antigen binding site and thereby providing an antibody with the properties of specifically binding to human hepsin and inhibiting serine protease activity .

The term "binding to hepsin" as used herein means binding of the antibody to human hepsin in a cellular binding assay measured by FACS. Binding is found if the antibody causes an S/N (signal/noise) ratio of 400 or more at an antibody concentration of 1 μ g/ml (MW 150.000). The antibody according to the invention binds therefore specifically to human hepsin. Preferably the antibody according to the invention is characterized in binding to human and cynomolgus hepsin with a binding affinity of at least 10^{-8} M^{-1} , preferably 10^{-8} M^{-1} to 10^{-12} M^{-1} .

As used herein, the expressions "cell", "cell line", and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and

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cultures derived there from without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Variant progeny that have the same function or biological activity as screened for in the originally transformed cell are included.

5 The term "chimeric" antibody refers to an antibody in which a portion of the heavy and/or light chain is derived from a particular source or species, while the remainder of the heavy and/or light chain is derived from a different source or species.

10 The "class" of an antibody refers to the type of constant domain or constant region possessed by its heavy chain. There are five major classes of antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α , δ , ϵ , γ , and μ , respectively.

15 The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents a cellular function and/or causes cell death or destruction. Cytotoxic agents include, but are not limited to, radioactive isotopes (e.g., At211, I131, I125, Y90, Re186, Re188, Sm153, Bi212, P32, Pb212 and radioactive isotopes of Lu);
20 chemotherapeutic agents or drugs (e.g., methotrexate, adriamycin, vinca alkaloids (vincristine, vinblastine, etoposide), doxorubicin, melphalan, mitomycin C, chlorambucil, daunorubicin or other intercalating agents); growth inhibitory agents; enzymes and fragments thereof such as nucleolytic enzymes; antibiotics; toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof; and the various
25 antitumor or anticancer agents disclosed below.

30 The term "diagnosis of cancer susceptibility" as used herein refers to, preferably by an immunological assay determining in vitro the binding between soluble hepsin of a human plasma sample (Tsimanis, A., Immunology Letters 96 (2005) 55-61) and the antibody according to the invention. Expression of hepsin has a correlation with disease progression, and can be used to identify low or high risk individuals for cancer susceptibility. For diagnostic purposes, the antibodies or antigen binding fragments can be labeled or unlabeled. Typically, diagnostic assays entail detecting the formation of a complex resulting from the binding of an antibody or antibody fragment to hepsin.

“Effector functions” refer to those biological activities attributable to the Fc region of an antibody, which vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity (CDC); Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor); and B cell activation.

An "effective amount" of an agent, e.g., a pharmaceutical formulation, refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result.

The term “epitope” denotes a protein determinant capable of specifically binding to an antibody. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually epitopes have specific three dimensional structural characteristics, as well as specific charge characteristics. Conformational and nonconformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents. Preferably an antibody according to the invention binds specifically to native but not to denatured hepsin.

The term “Fc region” or “constant region” herein is used to define a C-terminal region of an immunoglobulin heavy chain. The term includes native sequence Fc regions and variant Fc regions. In one embodiment, a human IgG heavy chain Fc region extends from Cys226, or from Pro230, to the carboxyl-terminus of the heavy chain. However, the C-terminal lysine (Lys447) of the Fc region may or may not be present. Unless otherwise specified herein, numbering of amino acid residues in the Fc region or constant region is according to the EU numbering system, also called the EU index, as described in Kabat, et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991). The “Fc part” of an antibody is not involved directly in binding of an antibody to an antigen, but exhibit various effector functions. A “Fc part of an antibody” is a term well known to the skilled artisan and defined on the basis of papain cleavage of antibodies. Depending on the amino acid sequence of the constant region of their heavy chains, antibodies or immunoglobulins are divided in the antibody classes.

The antibody according to the invention is preferably characterized in that the constant regions are of human origin. Such constant regions are well known in the state of the art and e.g. described by Kabat (see e.g. Johnson, G., and Wu, T.T.,

Nucleic Acids Res. 28 (2000) 214-218). For example a useful human heavy chain constant region comprises an amino acid of SEQ ID NO: 13, 14 or 15. For example an useful human light chain constant region comprises an amino acid sequence of a kappa-light chain constant region of SEQ ID NO: 16.

5 "Framework" or "FR" refers to variable domain residues other than CDR residues. The FR of a variable domain generally consists of four FR domains: FR1, FR2, FR3, and FR4. Accordingly, the CDR and FR sequences generally appear in the following sequence in VH (or VL): FR1-CDR1(L1)-FR2-CDR2(L2)-FR3-CDR3(L3)-FR4.

10 The terms "full length antibody" and "intact antibody," are used herein interchangeably to refer to an antibody having a structure substantially similar to a native antibody structure or having heavy chains that contain an Fc region as defined herein.

15 The term "HAI-2" herein means human Kunitz-type protease inhibitor 2, also named as hepatocyte growth factor activator inhibitor type 2 (UniProt Accession No. O43291). For the purpose of inhibition and competition experiments described herein the extracellular domain of HAI-2 (amino acids 28-197, SEQ ID NO:17) was used. Inhibiting binding of human HAI- 2 means therefore inhibiting binding of extracellular domain of human HAI- 2.

20 The terms "host cell," "host cell line," and "host cell culture" are used interchangeably and refer to cells into which exogenous nucleic acid has been introduced, including the progeny of such cells. Host cells include "transformants" and "transformed cells," which include the primary transformed cell and progeny derived therefrom without regard to the number of passages. Progeny may not be
25 completely identical in nucleic acid content to a parent cell, but may contain mutations. Mutant progeny that have the same function or biological activity as screened or selected for in the originally transformed cell are included herein.

A "human consensus framework" is a framework which represents the most commonly occurring amino acid residues in a selection of human immunoglobulin
30 VL or VH framework sequences. Generally, the selection of human immunoglobulin VL or VH sequences is from a subgroup of variable domain sequences. Generally, the subgroup of sequences is a subgroup as in Kabat, et al., Sequences of Proteins of Immunological Interest, Fifth Edition, NIH Publication 91-3242, Bethesda MD (1991), vols. 1-3. In one embodiment, for the VL, the

subgroup is subgroup kappa I as in Kabat et al., supra. In one embodiment, for the V_H, the subgroup is subgroup III as in Kabat et al., supra.

A “humanized” antibody refers to a chimeric antibody comprising amino acid residues from non-human CDRs and amino acid residues from human FRs. A humanized antibody, e.g., a non-human antibody, refers to an antibody that has undergone humanization. Therefore the term “humanized antibody” or “humanized variant” refers to antibodies in which the framework and/or “complementarity determining regions” (CDR) have been modified to comprise the CDR of an immunoglobulin of different species as compared to that of the parent immunoglobulin. In a preferred embodiment, a mouse CDR is grafted into the framework region of a human antibody to prepare the “humanized antibody” or “humanized variant”. In certain embodiments, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDRs correspond to those of a non-human antibody, and all or substantially all of the FRs correspond to those of a human antibody. A humanized antibody optionally may comprise at least a portion of an antibody constant region derived from a human antibody. In one embodiment, a non-human (e.g. mouse) CDR is grafted into the framework region of a human antibody to prepare the “humanized antibody”. See, e.g., Riechmann, L., et al., Nature 332 (1988) 323-327; and Neuberger, M.S., et al., Nature 314 (1985) 268-270. In one embodiment a “humanized variant of an antibody according to the invention” (which is of non-human origin (e.g. mouse)) refers to an antibody, which is based on the non-human antibody sequences in which the V_H and V_L are humanized by standard techniques (including CDR grafting) and optionally subsequent mutagenesis of certain amino acids in the framework region and the CDR. In one embodiment one to five amino acids (e.g. up to three) in the framework region and/or one to three amino acids (e.g. up to two) in the CDRs can be modified by further mutations. For example the mutagenesis can be based upon molecular modeling as described by Riechmann, L., et al., Nature 332 (1988) 323-327 and Queen, C., et al., Proc. Natl. Acad. Sci. USA 86 (1989) 10029-10033, or others. The suited positions for such mutations can be identified e.g. by sequence or homology analysis, by choosing the human framework (fixed frameworks approach; homology matching or best-fit), by using consensus sequences, by selecting FRs from several different germ lines, or by replacing non-human residues on the three dimensional surface with the most common residues found in human antibodies or based on sterical optimized interactions. In one embodiment such humanized variant is chimerized with a human constant region.

An “immunoconjugate” is an antibody conjugated to one or more heterologous molecule(s), including but not limited to a cytotoxic agent.

5 An “individual” or “subject” is a mammal. Mammals include, but are not limited to, domesticated animals (e.g., cows, sheep, cats, dogs, and horses), primates (e.g., humans and non-human primates such as monkeys), rabbits, and rodents (e.g., mice and rats). In certain embodiments, the individual or subject is a human.

10 The term “inhibiting binding of HAI-2 to hepsin” herein is used to define inhibition of binding of HAI-2 to hepsin by an antibody according to the invention. Preferably an antibody according to the invention, characterized in inhibiting binding of HAI-2 to hepsin, binds to the same epitope of hepsin as antibody MAB 2.7.35 or MAB 2.3.35 does or is inhibited in binding to hepsin due to steric hindrance of binding by one or both of these reference antibodies. Inhibition of binding between HAI-2 and hepsin by an antibody to be investigated can be detected by SPR (BIAcore) assay using immobilized antibody to be investigated at an immobilization level of 18000 RU and hepsin and HAI-2 at a concentration of 15 50 nM. First hepsin is injected for 180 sec onto the antibody coated surface to preform a hepsin-antibody surface. For analysis, HAI-2 is injected at a concentration of 50 nM onto this hepsin-antibody surface. Upon injection, any signal reduction or no change in signal indicates that the antibody to be investigated inhibits binding of HAI-2 to hepsin. In contrast, an increase of at least 20 5 % in signal derived by HAI-2 injection, shows that the antibody to be investigated does not inhibit binding of HAI-2 to hepsin.

25 An “isolated” antibody is one which has been separated from a component of its natural environment. In some embodiments, an antibody is purified to greater than 95% or 99% purity as determined by, for example, electrophoretic (e.g., SDS-PAGE, isoelectric focusing (IEF), capillary electrophoresis) or chromatographic (e.g., ion exchange or reverse phase HPLC). For review of methods for assessment of antibody purity, see, e.g., Flatman, S., et al., J. Chromatogr. B 848 (2007) 79-87.

30 An “isolated” nucleic acid refers to a nucleic acid molecule that has been separated from a component of its natural environment. An isolated nucleic acid includes a nucleic acid molecule contained in cells that ordinarily contain the nucleic acid molecule, but the nucleic acid molecule is present extrachromosomally or at a chromosomal location that is different from its natural chromosomal location.

The terms “nucleic acid” or “nucleic acid molecule”, as used herein, are intended to include DNA molecules and RNA molecules. A nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA. “Isolated nucleic acid encoding an anti-Hepsin antibody” refers to one or more
5 nucleic acid molecules encoding antibody heavy and light chains (or fragments thereof), including such nucleic acid molecule(s) in a single vector or separate vectors, and such nucleic acid molecule(s) present at one or more locations in a host cell.

The term "monoclonal antibody" as used herein refers to an antibody obtained from
10 a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical and/or bind the same epitope, except for possible variant antibodies, e.g., containing naturally occurring mutations or arising during production of a monoclonal antibody preparation, such variants generally being present in minor amounts. In contrast to polyclonal
15 antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. Thus, the modifier “monoclonal” indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be
20 construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including but not limited to the hybridoma method, recombinant DNA methods, phage-display methods, and methods utilizing transgenic animals containing all or part of the human
25 immunoglobulin loci, such methods and other exemplary methods for making monoclonal antibodies being described herein.

A “naked antibody” refers to an antibody that is not conjugated to a heterologous moiety (e.g., a cytotoxic moiety) or radiolabel. The naked antibody may be present in a pharmaceutical formulation.

"Native antibodies" refer to naturally occurring immunoglobulin molecules with
30 varying structures. For example, native IgG antibodies are heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light chains and two identical heavy chains that are disulfide-bonded. From N- to C-terminus, each heavy chain has a variable region (VH), also called a variable heavy domain or a
35 heavy chain variable domain, followed by three constant domains (CH1, CH2, and

CH3). Similarly, from N- to C-terminus, each light chain has a variable region (VL), also called a variable light domain or a light chain variable domain, followed by a constant light (CL) domain. The light chain of an antibody may be assigned to one of two types, called kappa (κ) and lambda (λ), based on the amino acid sequence of its constant domain.

A nucleic acid is “operably linked” when it is placed into a functional relationship with another nucleic acid. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, “operably linked” means that the DNA sequences being linked are colinear, and, in the case of a secretory leader, contiguous and in reading frame. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

The term “package insert” is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, combination therapy, contraindications and/or warnings concerning the use of such therapeutic products.

The “parent” antibody herein is one, which is encoded by an amino acid sequence used for the preparation of the variant. Preferably, the parent antibody has a human framework region and, if present, has a human antibody constant region or human antibody constant domains. For example, the parent antibody may be a humanized or a human antibody.

“Percent (%) amino acid sequence identity” with respect to a reference polypeptide sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign

(DNASTAR) software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc., and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available from Genentech, Inc., South San Francisco, California, or may be compiled from the source code. The ALIGN-2 program should be compiled for use on a UNIX operating system, including digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as

100 times the fraction X/Y

where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. Unless specifically stated otherwise, all % amino acid sequence identity values used herein are obtained as described in the immediately preceding paragraph using the ALIGN-2 computer program.

The term "pharmaceutical formulation" refers to a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered.

A "pharmaceutically acceptable carrier" refers to an ingredient in a pharmaceutical formulation, other than an active ingredient, which is nontoxic to a subject., A

pharmaceutically acceptable carrier includes, but is not limited to, a buffer, excipient, stabilizer, or preservative.

As used herein, "treatment" (and grammatical variations thereof such as "treat" or "treating") refers to clinical intervention in an attempt to alter the natural course of the individual being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of treatment include, but are not limited to, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. In some embodiments, antibodies of the invention are used to delay development of a disease or to slow the progression of a disease.

The term "variable region" or "variable domain" refers to the domain of an antibody heavy or light chain that is involved in binding the antibody to antigen. The variable domains of the heavy chain and light chain (VH and VL, respectively) of a native antibody generally have similar structures, with each domain comprising four conserved framework regions (FRs) and three hypervariable regions (CDRs) (see, e.g., Kindt, T.J., et al., Kuby Immunology, 6th ed., W.H. Freeman and Co., New York (2007), p. 91. The framework regions adopt a β -sheet conformation and the CDRs may form loops connecting the β -sheet structure. The CDRs in each chain are held in their three-dimensional structure by the framework regions and form together with the CDRs from the other chain the antigen binding site. The antibody's heavy chain CDR3 region plays a particularly important role in the binding specificity/affinity of the antibodies according to the invention and therefore provides a further object of the invention. A single VH or VL domain may be sufficient to confer antigen-binding specificity. Furthermore, antibodies that bind a particular antigen may be isolated using a VH or VL domain from an antibody that binds the antigen to screen a library of complementary VL or VH domains, respectively (see, e.g., Portolano, S., et al., J. Immunol. 150 (1993) 880-887; Clarkson, et al., Nature 352 (1991) 624-628).

Exemplary CDRs (CDR-L1, CDR-L2, CDR-L3, CDR-H1, CDR-H2, and CDR-H3) occur at amino acid residues 24-34 of L1, 50-56 of L2, 89-97 of L3, 31-35B of H1, 50-65 of H2, and 95-102 of H3 (Kabat, et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991)). With the exception of CDR1 in VH, CDRs

generally comprise the amino acid residues that form the hypervariable loops. CDRs also comprise "specificity determining residues," or "SDRs," which are residues that contact antigen. SDRs are contained within regions of the CDRs called abbreviated-CDRs, or a-CDRs. Exemplary a-CDRs (a-CDR-L1, a-CDR-L2, 5 a-CDR-L3, a-CDR-H1, a-CDR-H2, and a-CDR-H3) occur at amino acid residues 31-34 of L1, 50-55 of L2, 89-96 of L3, 31-35B of H1, 50-58 of H2, and 95-102 of H3 (see Almagro, J.C. and Fransson, J., *Front. Biosci.* 13 (2008) 1619-1633). Unless otherwise indicated, CDR residues and other residues in the variable domain (e.g., FR residues) are numbered herein according to Kabat et al., *supra*. 10 The term "comprises a heavy chain CDR3 region of SEQ ID NO:1" denotes that the antibody comprises as sequence of its heavy chain CDR3 region the amino acid sequence of SEQ ID NO:1. The same denotes for the other five CDR regions of the antibody.

The term "vector," as used herein, refers to a nucleic acid molecule capable of 15 propagating another nucleic acid to which it is linked. The term includes the vector as a self-replicating nucleic acid structure as well as the vector incorporated into the genome of a host cell into which it has been introduced. Certain vectors are capable of directing the expression of nucleic acids to which they are operatively linked. Such vectors are referred to herein as "expression vectors."

20 The antibodies according to the invention include, in addition, such antibodies having "conservative sequence modifications" (variant antibodies), nucleotide and amino acid sequence modifications which do not affect or alter the above-mentioned characteristics of the antibody according to the invention. Modifications can be introduced by standard techniques known in the art, such as site-directed 25 mutagenesis and PCR-mediated mutagenesis. Conservative amino acid substitutions include ones in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g. lysine, arginine, histidine), acidic side 30 chains (e.g. aspartic acid, glutamic acid), uncharged polar side chains (e.g. glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), nonpolar side chains (e.g. alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (e.g. threonine, valine, isoleucine) and aromatic side chains (e.g. tyrosine, phenylalanine, tryptophan, histidine). Thus, a 35 predicted nonessential amino acid residue in a human anti- hepsin antibody can be preferably replaced with another amino acid residue from the same side chain

family. A “variant” anti-hepsin antibody, refers therefore herein to a molecule which differs in amino acid sequence from a “parent” anti-hepsin antibody amino acid sequence by up to ten, preferably from about two to about five, additions, deletions and/or substitutions in one or more variable region of the parent antibody.

5 Amino acid substitutions can be performed by mutagenesis based upon molecular modeling as described by Riechmann, L., et al., Nature 332 (1988) 323-327 and Queen, C., et al., Proc. Natl. Acad. Sci. USA 86 (1989) 10029-10033.

II. COMPOSITIONS AND METHODS

A. Exemplary Anti-Hepsin Antibodies

10 In any of the above embodiments, an anti-hepsin antibody is humanized. In one embodiment, an anti-Hepsin antibody comprises CDRs as in any of the above embodiments, and further comprises an acceptor human framework, e.g. a human immunoglobulin framework or a human consensus framework.

15 In a further aspect of the invention, an anti-Hepsin antibody according to any of the above embodiments is a monoclonal antibody, including a chimeric, humanized or human antibody. In one embodiment, an anti-Hepsin antibody is an antibody fragment, e.g., a Fv, Fab, Fab', scFv, diabody, or F(ab')₂ fragment. In another embodiment, the antibody is a full length antibody, e.g., an intact IgG1 antibody or other antibody class or isotype as defined herein.

20 In a further aspect, an anti-Hepsin antibody according to any of the above embodiments may incorporate any of the features, singly or in combination, as described in Sections 1-4 below:

1. Antibody Affinity and Inhibition Activity

25 An antibody according to the invention is characterized by Biacore analysis (K_d, preferably between 1 and 10 nM), inhibition of enzymatic activity of Hepsin protein (human; preferably an IC₅₀ of 10 -20 nM and murine; preferably an IC₅₀ of 200 nM or more) and inhibition of enzymatic activity of related serine proteases trypsin and chymotrypsin (preferably an IC₂₀ of 200 nM or more). The antibody therefore shows specific and selective inhibition of Hepsin enzymatic activity.

2. Antibody Fragments

In certain embodiments, an antibody provided herein is an antibody fragment. Antibody fragments include, but are not limited to, Fab, Fab', Fab'-SH, F(ab')₂, Fv, and scFv fragments, and other fragments described below. For a review of certain antibody fragments, see Hudson, P.J., et al., Nat. Med. 9 (2003) 129-134. For a review of scFv fragments, see, e.g., Pluckthuen, In: The Pharmacology of Monoclonal Antibodies, Vol. 113, Rosenberg and Moore (eds.), Springer-Verlag, New York (1994), pp. 269-315; see also WO 93/16185; and U.S. Patent Nos. 5,571,894 and 5,587,458. For discussion of Fab and F(ab')₂ fragments comprising salvage receptor binding epitope residues and having increased in vivo half-life, see U.S. Patent No. 5,869,046.

Diabodies are antibody fragments with two antigen-binding sites that may be bivalent or bispecific (see, for example, EP 0 404 097; WO 1993/01161; Hudson, et al., Nat. Med. 9 (2003) 129-134; and Holliger, P., et al., Proc. Natl. Acad. Sci. USA 90 (1993) 6444-6448). Triabodies and tetrabodies are also described in Hudson et al., Nat. Med. 9 (2003) 129-134.

Single-domain antibodies are antibody fragments comprising all or a portion of the heavy chain variable domain or all or a portion of the light chain variable domain of an antibody. In certain embodiments, a single-domain antibody is a human single-domain antibody (Domantis, Inc., Waltham, MA; see, e.g., U.S. Patent No. 6,248,516 B1).

Antibody fragments can be made by various techniques, including but not limited to proteolytic digestion of an intact antibody as well as production by recombinant host cells (e.g. E. coli or phage), as described herein.

3. Chimeric and Humanized Antibodies

In certain embodiments, an antibody provided herein is a chimeric antibody. Certain chimeric antibodies are described, e.g., in U.S. Patent No. 4,816,567; and Morrison, S.L., et al., Proc. Natl. Acad. Sci. USA 81 (1984) 6851-6855. In one example, a chimeric antibody comprises a non-human variable region (e.g., a variable region derived from a mouse, rat, hamster, rabbit, or non-human primate, such as a monkey) and a human constant region. In a further example, a chimeric antibody is a "class switched" antibody in which the class or subclass has been

changed from that of the parent antibody. Chimeric antibodies include antigen-binding fragments thereof.

5 In certain embodiments, a chimeric antibody is a humanized antibody. Typically, a non-human antibody is humanized to reduce immunogenicity to humans, while retaining the specificity and affinity of the parental non-human antibody. Generally, a humanized antibody comprises one or more variable domains in which CDRs, (or portions thereof) are derived from a non-human antibody, and FRs (or portions thereof) are derived from human antibody sequences. A humanized antibody optionally will also comprise at least a portion of a human constant
10 region. In some embodiments, some FR residues in a humanized antibody are substituted with corresponding residues from a non-human antibody (e.g., the antibody from which the CDR residues are derived), e.g., to restore or improve antibody specificity or affinity.

Humanized antibodies and methods of making them are reviewed, e.g., in
15 Almagro, J.C. and Fransson, J., *Front. Biosci.* 13 (2008) 1619-1633, and are further described, e.g., in Riechmann, et al., *Nature* 332 (1988) 323-329; Queen, et al., *Proc. Natl. Acad. Sci. USA* 86 (1989) 10029-10033; US Patent Nos. 5,821,337, 7,527,791, 6,982,321, and 7,087,409; Kashmiri, S.V., et al., *Methods* 36 (2005) 25-34 (describing SDR (a-CDR) grafting); Padlan, E.A., *Mol. Immunol.* 28 (1991)
20 489-498 (describing "resurfacing"); Dall'Acqua, W.F., et al., *Methods* 36 (2005) 43-60 (describing "FR shuffling"); and Osbourn, J., et al., *Methods* 36 (2005) 61-68 and Klimka, A., et al., *Br. J. Cancer* 83 (2000) 252-260 (describing the "guided selection" approach to FR shuffling).

Human framework regions that may be used for humanization include but are not
25 limited to: framework regions selected using the "best-fit" method (see, e.g., Sims, M.J., et al., *J. Immunol.* 151 (1993) 2296-2308); framework regions derived from the consensus sequence of human antibodies of a particular subgroup of light or heavy chain variable regions (see, e.g., Carter, et al., *Proc. Natl. Acad. Sci. USA*, 89 (1992) 4285; and Presta, L.G., et al., *J. Immunol.* 151 (1993) 2623-2632);
30 human mature (somatically mutated) framework regions or human germline framework regions (see, e.g., Almagro, J.C. and Fransson, J., *Front. Biosci.* 13 (2008) 1619-1633); and framework regions derived from screening FR libraries (see, e.g., Baca, M., et al., *J. Biol. Chem.* 272 (1997) 10678-10684, and Rosok, M.J., et al., *J. Biol. Chem.* 271 (1996) 22611-22618).

4. Multispecific Antibodies

In certain embodiments, an antibody provided herein is a multispecific antibody, e.g. a bispecific antibody. Multispecific antibodies are monoclonal antibodies that have binding specificities for at least two different sites. In certain embodiments, one of the binding specificities is for Hepsin and the other is for any other antigen. In certain embodiments, bispecific antibodies may bind to two different epitopes of Hepsin. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express Hepsin. Bispecific antibodies can be prepared as full length antibodies or antibody fragments.

Techniques for making multispecific antibodies include, but are not limited to, recombinant co-expression of two immunoglobulin heavy chain-light chain pairs having different specificities (see Milstein, C. and Cuello, A.C., Nature 305 (1983) 537-540, WO 93/08829, and Traunecker, A., et al., EMBO J. 10 (1991) 3655-3659), and “knob-in-hole” engineering (see, e.g., U.S. Patent No. 5,731,168). Multi-specific antibodies may also be made by engineering electrostatic steering effects for making antibody Fc-heterodimeric molecules (WO 2009/089004 A1); cross-linking two or more antibodies or fragments (see, e.g., US Patent No. 4,676,980, and Brennan, M., et al., Science 229 (1985) 81-83); using leucine zippers to produce bi-specific antibodies (see, e.g., Kostelny, S.A., et al., J. Immunol. 148 (1992) 1547-1553); using “diabody” technology for making bispecific antibody fragments (see, e.g., Holliger, P., et al., Proc. Natl. Acad. Sci. USA 90 (1993) 6444-6448); and using single-chain Fv (sFv) dimers (see, e.g. Gruber, M., et al., J. Immunol. 152 (1994) 5368-5374); and preparing trispecific antibodies as described, e.g., in Tutt, A., et al., J. Immunol. 147 (1991) 60-69).

Engineered antibodies with three or more functional antigen binding sites, including “Octopus antibodies,” are also included herein (see, e.g. US 2006/0025576 A1).

The antibody or fragment herein also includes a “Dual Acting FAb” or “DAF” comprising an antigen binding site that binds to Hepsin as well as another, different antigen (see, US 2008/0069820, for example).

a) Glycosylation variants

In certain embodiments, an antibody provided herein is altered to increase or decrease the extent to which the antibody is glycosylated. Addition or deletion of

glycosylation sites to an antibody may be conveniently accomplished by altering the amino acid sequence such that one or more glycosylation sites is created or removed.

Where the antibody comprises an Fc region, the carbohydrate attached thereto may be altered. Native antibodies produced by mammalian cells typically comprise a branched, biantennary oligosaccharide that is generally attached by an N-linkage to Asn297 of the CH2 domain of the Fc region (see, e.g., Wright, A., et al., TIBTECH 15 (1997) 26-32). The oligosaccharide may include various carbohydrates, e.g., mannose, N-acetyl glucosamine (GlcNAc), galactose, and sialic acid, as well as a fucose attached to a GlcNAc in the “stem” of the biantennary oligosaccharide structure. In some embodiments, modifications of the oligosaccharide in an antibody of the invention may be made in order to create antibody variants with certain improved properties.

In one embodiment, antibody variants are provided having a carbohydrate structure that lacks fucose attached (directly or indirectly) to an Fc region. For example, the amount of fucose in such antibody may be from 1% to 80%, from 1% to 65%, from 5% to 65% or from 20% to 40%. The amount of fucose is determined by calculating the average amount of fucose within the sugar chain at Asn297, relative to the sum of all glycostructures attached to Asn 297 (e. g. complex, hybrid and high mannose structures) as measured by MALDI-TOF mass spectrometry, as described in WO 2008/077546, for example. Asn297 refers to the asparagine residue located at about position 297 in the Fc region (Eu numbering of Fc region residues); however, Asn297 may also be located about ± 3 amino acids upstream or downstream of position 297, i.e., between positions 294 and 300, due to minor sequence variations in antibodies. Such fucosylation variants may have improved ADCC function. See, e.g., US Patent Publication Nos. US 2003/0157108 (Presta, L.); US 2004/0093621 (Kyowa Hakko Kogyo Co., Ltd). Examples of publications related to “defucosylated” or “fucose-deficient” antibody variants include: US 2003/0157108; WO 2000/61739; WO 2001/29246; US 2003/0115614; US 2002/0164328; US 2004/0093621; US 2004/0132140; US 2004/0110704; US 2004/0110282; US 2004/0109865; WO 2003/085119; WO 2003/084570; WO 2005/035586; WO 2005/035778; WO 2005/053742; WO 2002/031140; Okazaki, A., et al., J. Mol. Biol. 336 (2004) 1239-1249; Yamane-Ohnuki, N., et al., Biotech. Bioeng. 87 (2004) 614-622. Examples of cell lines capable of producing defucosylated antibodies include Lec13 CHO cells deficient in protein fucosylation (Ripka, J., et al., Arch. Biochem. Biophys. 249 (1986) 533-545; US Pat Appl No

US 2003/0157108 A1, Presta, L.G; and WO 2004/056312 A1, Adams, et al., especially at Example 11), and knockout cell lines, such as alpha-1,6-fucosyltransferase gene, FUT8, knockout CHO cells (see, e.g., Yamane-Ohnuki, N., et al., Biotech. Bioeng. 87 (2004) 614-622; Kanda, Y., et al., Biotechnol. Bioeng. 94 (2006) 680-688; and WO 2003/085107).

Antibodies variants are further provided with bisected oligosaccharides, e.g., in which a biantennary oligosaccharide attached to the Fc region of the antibody is bisected by GlcNAc. Such antibody variants may have reduced fucosylation and/or improved ADCC function. Examples of such antibody variants are described, e.g., in WO 2003/011878 (Jean-Mairet et al.); US Patent No. 6,602,684 (Umana et al.); and US 2005/0123546 (Umana et al.). Antibody variants with at least one galactose residue in the oligosaccharide attached to the Fc region are also provided. Such antibody variants may have improved CDC function. Such antibody variants are described, e.g., in WO 1997/30087 (Patel et al.); WO 1998/58964 (Raju, S.); and WO 1999/22764 (Raju, S.).

b) Fc region variants

In certain embodiments, one or more amino acid modifications may be introduced into the Fc region of an antibody provided herein, thereby generating an Fc region variant. The Fc region variant may comprise a human Fc region sequence (e.g., a human IgG1, IgG2, IgG3 or IgG4 Fc region) comprising an amino acid modification (e.g. a substitution) at one or more amino acid positions.

In certain embodiments, the invention contemplates an antibody variant that possesses some but not all effector functions, which make it a desirable candidate for applications in which the half life of the antibody in vivo is important yet certain effector functions (such as complement and ADCC) are unnecessary or deleterious. In vitro and/or in vivo cytotoxicity assays can be conducted to confirm the reduction/depletion of CDC and/or ADCC activities. For example, Fc receptor (FcR) binding assays can be conducted to ensure that the antibody lacks FcγR binding (hence likely lacking ADCC activity), but retains FcRn binding ability. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcγR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch, J.V. and Kinet, J.P., Annu. Rev. Immunol. 9 (1991) 457-492. Non-limiting examples of in vitro assays to assess ADCC activity of a molecule of interest is described in U.S. Patent No. 5,500,362 (see, e.g. Hellstrom, I., et al., Proc. Natl. Acad. Sci. USA 83 (1986)

7059-7063) and Hellstrom, I., et al., Proc. Natl. Acad. Sci. USA 82 (1985) 1499-1502; US 5,821,337 (see Bruggemann, M., et al., J. Exp. Med. 166 (1987) 1351-1361). Alternatively, non-radioactive assays methods may be employed (see, for example, ACTITM non-radioactive cytotoxicity assay for flow cytometry (CellTechnology, Inc. Mountain View, CA; and CytoTox 96® non-radioactive cytotoxicity assay (Promega, Madison, WI). Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g., in a animal model such as that disclosed in Clynes, R., et al., Proc. Natl. Acad. Sci. USA 95 (1998) 652-656. C1q binding assays may also be carried out to confirm that the antibody is unable to bind C1q and hence lacks CDC activity (see, e.g., C1q and C3c binding ELISA in WO 2006/029879 and WO 2005/100402). To assess complement activation, a CDC assay may be performed (see, for example, Gazzano-Santoro, H., et al., J. Immunol. Methods 202 (1997) 163-171; Cragg, M.S. et al., Blood 101 (2003) 1045-1052; and Cragg, M.S. and Glennie, M.J., Blood 103 (2004) 2738-2743). FcRn binding and in vivo clearance/half life determinations can also be performed using methods known in the art (see, e.g., Petkova, S.B., et al., Intl. Immunol. 18 (2006) 1759-1769).

Antibodies with reduced effector function include those with substitution of one or more of Fc region residues 238, 265, 269, 270, 297, 327 and 329 (U.S. Patent No. 6,737,056). Such Fc mutants include Fc mutants with substitutions at two or more of amino acid positions 265, 269, 270, 297 and 327, including the so-called "DANA" Fc mutant with substitution of residues 265 and 297 to alanine (US Patent No. 7,332,581).

Certain antibody variants with improved or diminished binding to FcRs are described (see, e.g., U.S. Patent No. 6,737,056; WO 2004/056312, and Shields, et al., J. Biol. Chem. 9 (2001) 6591-6604).

In certain embodiments, an antibody variant comprises an Fc region with one or more amino acid substitutions which improve ADCC, e.g., substitutions at positions 298, 333, and/or 334 of the Fc region (EU numbering of residues).

In some embodiments, alterations are made in the Fc region that result in altered (i.e., either improved or diminished) C1q binding and/or Complement Dependent Cytotoxicity (CDC), e.g., as described in US Patent No. 6,194,551, WO 99/51642, and Idusogie, E.E., et al., J. Immunol. 164 (2000) 4178-4184.

Antibodies with increased half lives and improved binding to the neonatal Fc receptor (FcRn), which is responsible for the transfer of maternal IgGs to the fetus (Guyer, R.L., et al., J. Immunol. 117 (1976) 587-593 and Kim, J.K., et al., European J. Immunol. 24 (1994) 2429-2434), are described in US 2005/0014934 A1 (Hinton, et al.). Those antibodies comprise an Fc region with one or more substitutions therein which improve binding of the Fc region to FcRn. Such Fc variants include those with substitutions at one or more of Fc region residues: 238, 256, 265, 272, 286, 303, 305, 307, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424 or 434, e.g., substitution of Fc region residue 434 (US Patent No. 7,371,826).

See also Duncan, A.R. and Winter, G., Nature 332 (1988) 738-740; U.S. Patent No. 5,648,260; U.S. Patent No. 5,624,821; and WO 94/29351 concerning other examples of Fc region variants.

c) Cysteine engineered antibody variants

In certain embodiments, it may be desirable to create cysteine engineered antibodies, e.g., "thioMAbs," in which one or more residues of an antibody are substituted with cysteine residues. In particular embodiments, the substituted residues occur at accessible sites of the antibody. By substituting those residues with cysteine, reactive thiol groups are thereby positioned at accessible sites of the antibody and may be used to conjugate the antibody to other moieties, such as drug moieties or linker-drug moieties, to create an immunoconjugate, as described further herein. In certain embodiments, any one or more of the following residues may be substituted with cysteine: V205 (Kabat numbering) of the light chain; A118 (EU numbering) of the heavy chain; and S400 (EU numbering) of the heavy chain Fc region. Cysteine engineered antibodies may be generated as described, e.g., in U.S. Patent No. 7,521,541.

d) Antibody Derivatives

In certain embodiments, an antibody provided herein may be further modified to contain additional nonproteinaceous moieties that are known in the art and readily available. The moieties suitable for derivatization of the antibody include but are not limited to water soluble polymers. Non-limiting examples of water soluble polymers include, but are not limited to, polyethylene glycol (PEG), copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane,

ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propylene glycol homopolymers, polypropylene oxide/ethylene oxide copolymers, polyoxyethylated polyols (e.g., glycerol), polyvinyl alcohol, and mixtures thereof. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water. The polymer may be of any molecular weight, and may be branched or unbranched. The number of polymers attached to the antibody may vary, and if more than one polymer are attached, they can be the same or different molecules. In general, the number and/or type of polymers used for derivatization can be determined based on considerations including, but not limited to, the particular properties or functions of the antibody to be improved, whether the antibody derivative will be used in a therapy under defined conditions, etc.

In another embodiment, conjugates of an antibody and nonproteinaceous moiety that may be selectively heated by exposure to radiation are provided. In one embodiment, the nonproteinaceous moiety is a carbon nanotube (Kam, N.W., et al., Proc. Natl. Acad. Sci. USA 102 (2005) 11600-11605). The radiation may be of any wavelength, and includes, but is not limited to, wavelengths that do not harm ordinary cells, but which heat the nonproteinaceous moiety to a temperature at which cells proximal to the antibody-nonproteinaceous moiety are killed.

B. Recombinant Methods and Compositions

Antibodies may be produced using recombinant methods and compositions, e.g., as described in U.S. Patent No. 4,816,567. Recombinant production of antibodies is well-known in the state of the art and is also described, for example, in the review articles of Makrides, S.C., Protein Expr. Purif. 17 (1999) 183-202; Geisse, S., et al., Protein Expr. Purif. 8 (1996) 271-282; Kaufman, R.J., Mol. Biotechnol. 16 (2000) 151-160; Werner, R.G., Drug Res. 48 (1998) 870-880. In one embodiment, isolated nucleic acid encoding an anti-Hepsin antibody described herein is provided. Such nucleic acid may encode an amino acid sequence comprising the VL and/or an amino acid sequence comprising the VH of the antibody (e.g., the light and/or heavy chains of the antibody). In a further embodiment, one or more vectors (e.g., expression vectors) comprising such nucleic acid are provided. In a further embodiment, a host cell comprising such nucleic acid is provided. In one such embodiment, a host cell comprises (e.g., has been transformed with): (1) a vector comprising a nucleic acid that encodes an amino acid sequence comprising the VL

of the antibody and an amino acid sequence comprising the VH of the antibody, or
(2) a first vector comprising a nucleic acid that encodes an amino acid sequence
comprising the VL of the antibody and a second vector comprising a nucleic acid
that encodes an amino acid sequence comprising the VH of the antibody. In one
5 embodiment, the host cell is eukaryotic, e.g. a Chinese Hamster Ovary (CHO) cell
or lymphoid cell (e.g., Y0, NS0, Sp20 cell). In one embodiment, a method of
making an anti-Hepsin antibody is provided, wherein the method comprises
culturing a host cell comprising a nucleic acid encoding the antibody, as provided
above, under conditions suitable for expression of the antibody, and optionally
10 recovering the antibody from the host cell (or host cell culture medium).

For recombinant production of an anti-Hepsin antibody, nucleic acid encoding an
antibody, e.g., as described above, is isolated and inserted into one or more vectors
for further cloning and/or expression in a host cell. Such nucleic acid may be
readily isolated and sequenced using conventional procedures (e.g., by using
15 oligonucleotide probes that are capable of binding specifically to genes encoding
the heavy and light chains of the antibody). The heavy and light chain variable
domains according to the invention are combined with sequences of promoter,
translation initiation, constant region, 3' untranslated region, polyadenylation, and
transcription termination to form expression vector constructs. The heavy and light
20 chain expression constructs can be combined into a single vector, co-transfected,
serially transfected, or separately transfected into host cells which are then fused to
form a single host cell expressing both chains.

Nucleic acid molecules encoding amino acid sequence variants of anti- hepsin
antibody are prepared by a variety of methods known in the art. These methods
25 include, but are not limited to, isolation from a natural source (in the case of
naturally occurring amino acid sequence variants) or preparation by
oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and
cassette mutagenesis of an earlier prepared variant or a non-variant version of
humanized anti- hepsin antibody.

Suitable host cells for cloning or expression of antibody-encoding vectors include
prokaryotic or eukaryotic cells described herein. Expression is performed in
appropriate prokaryotic or eukaryotic host cells, such as CHO cells, NS0 cells,
SP2/0 cells, HEK293 cells, COS cells, yeast, or E. coli cells, and the antibody is
recovered from the cells (from the supernatant or after cells lysis). Antibodies may
35 be produced in bacteria when glycosylation and Fc effector function are not

needed. For expression of antibody fragments and polypeptides in bacteria, see, e.g., U.S. Patent Nos. 5,648,237, 5,789,199, and 5,840,523 (see also Charlton, Methods in Molecular Biology, Vol. 248 (B.K.C. Lo, ed., Humana Press, Totowa, NJ (2003), pp. 245-254, describing expression of antibody fragments in *E. coli*).

5 After expression, the antibody may be isolated from the bacterial cell paste in a soluble fraction and can be further purified.

The antibodies may be present in whole cells, in a cell lysate, or in a partially purified, or substantially pure form. Purification is performed in order to eliminate other cellular components or other contaminants, e.g. other cellular nucleic acids or proteins, by standard techniques, including column chromatography and other well known in the art. See Ausubel, F., et al., (ed.) Current Protocols in Molecular Biology, Greene Publishing and Wiley Interscience, New York (1987).

Expression in NS0 cells is described by, e.g., Barnes, L.M., et al., Cytotechnology 32 (2000) 109-123; Barnes, L.M., et al., Biotech. Bioeng. 73 (2001) 261-270. Transient expression is described by, e.g., Durocher, Y., et al., Nucl. Acids. Res. 30 (2002) E9. Cloning of variable domains is described by Orlandi, R., et al., Proc. Natl. Acad. Sci. USA 86 (1989) 3833-3837; Carter, P., et al., Proc. Natl. Acad. Sci. USA 89 (1992) 4285-4289; Norderhaug, L., et al., J. Immunol. Methods 204 (1997) 77-87. A preferred transient expression system (HEK 293) is described by Schlaeger, E.-J. and Christensen, K., in Cytotechnology 30 (1999) 71-83, and by Schlaeger, E.-J., in J. Immunol. Methods 194 (1996) 191-199.

Monoclonal antibodies are suitably separated from the culture medium by conventional immunoglobulin purification procedures such as, for example, affinity chromatography. DNA and RNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures. The hybridoma cells can serve as a source of such DNA and RNA. Once isolated, the DNA may be inserted into expression vectors, which are then transfected into host cells, such as HEK 293 cells, CHO cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of recombinant monoclonal antibodies in the host cells.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for antibody-encoding vectors, including fungi and yeast strains whose glycosylation pathways have been "humanized," resulting in the production of an antibody with a partially or fully human

glycosylation pattern. See Gerngross, T.U., Nat. Biotech. 22 (2004) 1409-1414, and Li, H., et al., Nat. Biotech. 24 (2006) 210-215.

Suitable host cells for the expression of glycosylated antibody are also derived from multicellular organisms (invertebrates and vertebrates). Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains have been identified which may be used in conjunction with insect cells, particularly for transfection of *Spodoptera frugiperda* cells.

Plant cell cultures can also be utilized as hosts. See, e.g., US Patent Nos. 5,959,177, 6,040,498, 6,420,548, 7,125,978, and 6,417,429 (describing PLANTIBODIESTM technology for producing antibodies in transgenic plants).

Vertebrate cells may also be used as hosts. For example, mammalian cell lines that are adapted to grow in suspension may be useful. Other examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7); human embryonic kidney line (293 or 293 cells as described, e.g., in Graham, F.L., et al., J. Gen Virol. 36 (1977) 59-74); baby hamster kidney cells (BHK); mouse sertoli cells (TM4 cells as described, e.g., in Mather, J.P., Biol. Reprod. 23 (1980) 243-252); monkey kidney cells (CV1); African green monkey kidney cells (VERO-76); human cervical carcinoma cells (HELA); canine kidney cells (MDCK); buffalo rat liver cells (BRL 3A); human lung cells (W138); human liver cells (Hep G2); mouse mammary tumor (MMT 060562); TRI cells, as described, e.g., in Mather, J.P., et al., Annals N.Y. Acad. Sci. 383 (1982) 44-68; MRC 5 cells; and FS4 cells. Other useful mammalian host cell lines include Chinese hamster ovary (CHO) cells, including DHFR- CHO cells (Urlaub, G., et al., Proc. Natl. Acad. Sci. USA 77 (1980) 4216-4220); and myeloma cell lines such as Y0, NS0 and Sp2/0. For a review of certain mammalian host cell lines suitable for antibody production, see, e.g., Yazaki, P.J., et al., Methods in Molecular Biology 248 (2004) 255-268.

C. Assays

Anti-Hepsin antibodies provided herein may be identified, screened for, or characterized for their physical/chemical properties and/or biological activities by various assays known in the art.

1. Binding assays and other assays

In one aspect, an antibody of the invention is tested for its antigen binding activity, e.g., by known methods such as ELISA, Western blot, etc.

5 In another aspect, competition assays may be used to identify an antibody that competes with Mab 2.7.35, Mab53 and/or Mab55 for binding to Hepsin. In certain embodiments, such a competing antibody binds to the same epitope (e.g., a linear or a conformational epitope) that is bound by Mab 2.7.35, Mab53 and/or Mab55. Detailed exemplary methods for mapping an epitope to which an antibody binds are provided in Morris, G.E. (ed.), Epitope Mapping Protocols, In: Methods in
10 Molecular Biology, Vol. 66, Humana Press, Totowa, NJ (1996).

In an exemplary competition assay, immobilized Hepsin is incubated in a solution comprising a first labeled antibody that binds to Hepsin (e.g., Mab 2.7.35, Mab53 and/or Mab55) and a second unlabeled antibody that is being tested for its ability to
15 compete with the first antibody for binding to Hepsin. The second antibody may be present in a hybridoma supernatant. As a control, immobilized Hepsin is incubated in a solution comprising the first labeled antibody but not the second unlabeled antibody. After incubation under conditions permissive for binding of the first antibody to Hepsin, excess unbound antibody is removed, and the amount of label
20 associated with immobilized Hepsin is measured. If the amount of label associated with immobilized Hepsin is substantially reduced in the test sample relative to the control sample, then that indicates that the second antibody is competing with the first antibody for binding to Hepsin. See Harlow and Lane (1988) Antibodies: A Laboratory Manual ch.14 (Cold Spring Harbor Laboratory, Cold Spring Harbor,
25 NY).

2. Activity assays

In one aspect, assays are provided for identifying anti-Hepsin antibodies thereof having biological activity. Biological activity may include, e.g., neutralizing the serine protease activity of Hepsin. Antibodies having such biological activity in
30 vivo and/or in vitro are also provided.

In certain embodiments, an antibody of the invention is tested for such biological activity.

D. Immunoconjugates

The invention also provides immunoconjugates comprising an anti-Hepsin antibody herein conjugated to one or more cytotoxic agents, such as chemotherapeutic agents or drugs, growth inhibitory agents, toxins (e.g., protein
5 toxins, enzymatically active toxins of bacterial, fungal, plant, or animal origin, or fragments thereof), or radioactive isotopes.

In one embodiment, an immunoconjugate is an antibody-drug conjugate (ADC) in which an antibody is conjugated to one or more drugs, including but not limited to a maytansinoid (see U.S. Patent Nos. 5,208,020, 5,416,064 and European Patent
10 EP 0 425 235 B1); an auristatin such as monomethylauristatin drug moieties DE and DF (MMAE and MMAF) (see U.S. Patent Nos. 5,635,483 and 5,780,588, and 7,498,298); a dolastatin; a calicheamicin or derivative thereof (see U.S. Patent Nos. 5,712,374, 5,714,586, 5,739,116, 5,767,285, 5,770,701, 5,770,710, 5,773,001, and 5,877,296; Hinman, L.M., et al., Cancer Res. 53 (1993) 3336-3342; and Lode,
15 H.N., et al., Cancer Res. 58 (1998) 2925-2928); an anthracycline such as daunomycin or doxorubicin (see Kratz, F., et al., Current Med. Chem. 13 (2006) 477-523; Jeffrey, S.C., et al., Bioorganic & Med. Chem. Letters 16 (2006) 358-362; Torgov, M.Y., et al., Bioconj. Chem. 16 (2005) 717-721; Nagy, A., et al., Proc. Natl. Acad. Sci. USA 97 (2000) 829-834; Dubowchik, G.M., et al., Bioorg. &
20 Med. Chem. Letters 12 (2002) 1529-1532; King, H.D., et al., J. Med. Chem. 45 (2002) 4336-4343; and U.S. Patent No. 6,630,579); methotrexate; vindesine; a taxane such as docetaxel, paclitaxel, larotaxel, tesetaxel, and ortataxel; a trichothecene; and CC1065.

In another embodiment, an immunoconjugate comprises an antibody as described
25 herein conjugated to an enzymatically active toxin or fragment thereof, including but not limited to diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolacca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia
30 inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothecenes.

In another embodiment, an immunoconjugate comprises an antibody as described herein conjugated to a radioactive atom to form a radioconjugate. A variety of radioactive isotopes are available for the production of radioconjugates. Examples
35 include At211, I131, I125, Y90, Re186, Re188, Sm153, Bi212, P32, Pb212 and

radioactive isotopes of Lu. When the radioconjugate is used for detection, it may comprise a radioactive atom for scintigraphic studies, for example tc99m or I123, or a spin label for nuclear magnetic resonance (NMR) imaging (also known as magnetic resonance imaging, mri), such as iodine-123 again, iodine-131, indium-111, fluorine-19, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron.

Conjugates of an antibody and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta, E.S., et al., Science 238 (1987) 1098-1104. Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO 94/11026. The linker may be a "cleavable linker" facilitating release of a cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, photolabile linker, dimethyl linker or disulfide-containing linker (Chari, R.V., et al., Cancer Res. 52 (1992) 127-131; U.S. Patent No. 5,208,020) may be used.

The immunoconjugates or ADCs herein expressly contemplate, but are not limited to such conjugates prepared with cross-linker reagents including, but not limited to, BMPS, EMCS, GMBS, HBVS, LC-SMCC, MBS, MPBH, SBAP, SIA, SIAB, SMCC, SMPB, SMPH, sulfo-EMCS, sulfo-GMBS, sulfo-KMUS, sulfo-MBS, sulfo-SIAB, sulfo-SMCC, and sulfo-SMPB, and SVSB (succinimidyl-(4-vinylsulfone)benzoate) which are commercially available (e.g., from Pierce Biotechnology, Inc., Rockford, IL., U.S.A).

E. Methods and Compositions for Diagnostics and Detection

In certain embodiments, any of the anti-Hepsin antibodies provided herein is useful for detecting the presence of Hepsin in a biological sample. The term "detecting" as

used herein encompasses quantitative or qualitative detection. In certain embodiments, a biological sample comprises a cell or tissue, such as a B cell.

5 In one embodiment, an anti-Hepsin antibody for use in a method of diagnosis or detection is provided. In a further aspect, a method of detecting the presence of Hepsin in a biological sample is provided. In certain embodiments, the method comprises contacting the biological sample with an anti-Hepsin antibody as described herein under conditions permissive for binding of the anti-Hepsin antibody to Hepsin, and detecting whether a complex is formed between the anti-Hepsin antibody and Hepsin. Such method may be an in vitro or in vivo method. In 10 one embodiment, an anti-Hepsin antibody is used to select subjects eligible for therapy with an anti-Hepsin antibody, e.g. where Hepsin is a biomarker for selection of patients.

Exemplary disorders that may be diagnosed using an antibody of the invention include cancer, especially breast or prostate cancer.

15 In certain embodiments, labeled anti-Hepsin antibodies are provided. Labels include, but are not limited to, labels or moieties that are detected directly (such as fluorescent, chromophoric, electron-dense, chemiluminescent, and radioactive labels), as well as moieties, such as enzymes or ligands, that are detected indirectly, e.g., through an enzymatic reaction or molecular interaction. Exemplary labels 20 include, but are not limited to, the radioisotopes ^{32}P , ^{14}C , ^{125}I , ^3H , and ^{131}I , fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luciferases, e.g., firefly luciferase and bacterial luciferase (U.S. Patent No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP), alkaline phosphatase, β -galactosidase, glucoamylase, lysozyme, saccharide oxidases, e.g., glucose oxidase, 25 galactose oxidase, and glucose-6-phosphate dehydrogenase, heterocyclic oxidases such as uricase and xanthine oxidase, coupled with an enzyme that employs hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, or microperoxidase, biotin/avidin, spin labels, bacteriophage labels, stable free 30 radicals, and the like.

F. Pharmaceutical Formulations

Pharmaceutical formulations of an anti-Hepsin antibody as described herein are prepared by mixing such antibody having the desired degree of purity with one or more optional pharmaceutically acceptable carriers (Remington's Pharmaceutical

Sciences 16th edition, Osol, A., ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Pharmaceutically acceptable carriers are generally nontoxic to recipients at the dosages and concentrations employed, and include, but are not limited to: buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride; benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as polyethylene glycol (PEG). Exemplary pharmaceutically acceptable carriers herein further include interstitial drug dispersion agents such as soluble neutral-active hyaluronidase glycoproteins (sHASEGP), for example, human soluble PH-20 hyaluronidase glycoproteins, such as rHuPH20 (HYLENEX®, Baxter International, Inc.). Certain exemplary sHASEGPs and methods of use, including rHuPH20, are described in US Patent Publication Nos. 2005/0260186 and 2006/0104968. In one aspect, a sHASEGP is combined with one or more additional glycosaminoglycanases such as chondroitinases.

Exemplary lyophilized antibody formulations are described in US Patent No. 6,267,958. Aqueous antibody formulations include those described in US Patent No. 6,171,586 and WO 2006/044908, the latter formulations including a histidine-acetate buffer.

The formulations to be used for in vivo administration are generally sterile. Sterility may be readily accomplished, e.g., by filtration through sterile filtration membranes.

G. Therapeutic Methods and Compositions

Any of the anti-Hepsin antibodies provided herein may be used in therapeutic methods.

In one aspect, an anti-Hepsin antibody for use as a medicament is provided. In further aspects, an anti-Hepsin antibody for use in treating cancer, especially breast or prostate cancer is provided. In certain embodiments, an anti-Hepsin antibody for use in a method of treatment is provided. In certain embodiments, the invention provides an anti-Hepsin antibody for use in a method of treating an individual having cancer, especially breast or prostate cancer comprising administering to the individual an effective amount of the anti-Hepsin antibody. In one such embodiment, the method further comprises administering to the individual an effective amount of at least one additional therapeutic agent, e.g., as described below. In further embodiments, the invention provides an anti-Hepsin antibody for use cancer therapy. An "individual" according to any of the above embodiments is preferably a human.

In a further aspect, the invention provides for the use of an anti-Hepsin antibody in the manufacture or preparation of a medicament. In one embodiment, the medicament is for treatment of cancer, especially breast or prostate cancer. In a further embodiment, the medicament is for use in a method of treating cancer, especially breast or prostate cancer comprising administering to an individual having cancer, especially breast or prostate cancer an effective amount of the medicament. In one such embodiment, the method further comprises administering to the individual an effective amount of at least one additional therapeutic agent, e.g., as described below.

In a further aspect, the invention provides a method for treating cancer, especially breast or prostate cancer. In one embodiment, the method comprises administering to an individual having such cancer, especially breast or prostate cancer an effective amount of an anti-Hepsin antibody. In one such embodiment, the method further comprises administering to the individual an effective amount of at least one additional therapeutic agent, as described below. An "individual" according to any of the above embodiments may be a human.

In a further aspect, the invention provides pharmaceutical formulations comprising any of the anti-Hepsin antibodies provided herein, e.g., for use in any of the above therapeutic methods. In one embodiment, a pharmaceutical formulation comprises any of the anti-Hepsin antibodies provided herein and a pharmaceutically acceptable carrier. In another embodiment, a pharmaceutical formulation comprises any of the anti-Hepsin antibodies provided herein and at least one additional therapeutic agent, e.g., as described below.

Antibodies of the invention can be used either alone or in combination with other agents in a therapy. For instance, an antibody of the invention may be co-administered with at least one additional therapeutic agent.

5 Such combination therapies noted above encompass combined administration (where two or more therapeutic agents are included in the same or separate formulations), and separate administration, in which case, administration of the antibody of the invention can occur prior to, simultaneously, and/or following, administration of the additional therapeutic agent and/or adjuvant.

10 An antibody of the invention (and any additional therapeutic agent) can be administered by any suitable means, including parenteral, intrapulmonary, and intranasal, and, if desired for local treatment, intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. Dosing can be by any suitable route, e.g. by injections, such as intravenous or subcutaneous injections, depending
15 in part on whether the administration is brief or chronic. Various dosing schedules including but not limited to single or multiple administrations over various time-points, bolus administration, and pulse infusion are contemplated herein.

20 Antibodies of the invention would be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The antibody need not be, but is optionally formulated with one or more agents currently used to
25 prevent or treat the disorder in question. The effective amount of such other agents depends on the amount of antibody present in the formulation, the type of disorder or treatment, and other factors discussed above. These are generally used in the same dosages and with administration routes as described herein, or about from 1 to 99% of the dosages described herein, or in any dosage and by any route that is
30 empirically/clinically determined to be appropriate.

For the prevention or treatment of cancer, especially breast or prostate cancer, the appropriate dosage of an antibody of the invention (when used alone or in combination with one or more other additional therapeutic agents) will depend on the type of disease to be treated, the type of antibody, the severity and course of the
35 disease, whether the antibody is administered for preventive or therapeutic

purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments. Depending on the type and severity of the disease, about 1 µg/kg to 15 mg/kg (MW 150.000; e.g. 0.1mg/kg-10mg/kg) of antibody can be an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. One typical daily dosage might range from about 1 µg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment would generally be sustained until a desired suppression of disease symptoms occurs. One exemplary dosage of the antibody would be in the range from about 0.05 mg/kg to about 10 mg/kg. Thus, one or more doses of about 0.5 mg/kg, 2.0 mg/kg, 4.0 mg/kg or 10 mg/kg (or any combination thereof) may be administered to the patient. Such doses may be administered intermittently, e.g. every week or every three weeks (e.g. such that the patient receives from about two to about twenty, or e.g. about six doses of the antibody). An initial higher loading dose, followed by one or more lower doses may be administered. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

It is understood that any of the above formulations or therapeutic methods may be carried out using an immunoconjugate of the invention in place of or in addition to an anti-Hepsin antibody.

H. Articles of Manufacture

In another aspect of the invention, an article of manufacture containing materials useful for the treatment, prevention and/or diagnosis of the disorders described above is provided. The article of manufacture comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, IV solution bags, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is by itself or combined with another composition effective for treating, preventing and/or diagnosing the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an antibody of the invention. The label or package insert indicates that the composition is used for treating the condition of choice.

Moreover, the article of manufacture may comprise (a) a first container with a composition contained therein, wherein the composition comprises an antibody of the invention; and (b) a second container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent. The article of manufacture in this embodiment of the invention may further comprise a package insert indicating that the compositions can be used to treat a particular condition. Alternatively, or additionally, the article of manufacture may further comprise a second (or third) container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

It is understood that any of the above articles of manufacture may include an immunoconjugate of the invention in place of or in addition to an anti-Hepsin antibody.

The following examples, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

20 **Description of the Sequences**

SEQ ID NO: 1	heavy chain CDR1, Mab 2.7.35
SEQ ID NO: 2	heavy chain CDR2, Mab 2.7.35
SEQ ID NO: 3	heavy chain CDR3, Mab 2.7.35
SEQ ID NO: 4	light chain CDR1, Mab 2.7.35
25 SEQ ID NO: 5	light chain CDR2, Mab 2.7.35
SEQ ID NO: 6	light chain CDR3, Mab 2.7.35
SEQ ID NO: 7	heavy chain variable domain, Mab 2.7.35
SEQ ID NO: 8	light chain variable domain, Mab 2.7.35
SEQ ID NO: 9	heavy chain variable domain, Mab53
30 SEQ ID NO: 10	light chain variable domain, Mab53
SEQ ID NO: 11	heavy chain variable domain, Mab55
SEQ ID NO: 12	light chain variable domain, Mab55
SEQ ID NO: 13	γ 1 heavy chain constant region
SEQ ID NO: 14	γ 4 heavy chain constant region

	SEQ ID NO: 15	γ 4 heavy chain constant region (SPLE mutant)
	SEQ ID NO: 16	λ light chain constant region
	SEQ ID NO: 17	HAI-2 extracellular domain
	SEQ ID NO: 18	heavy chain CDR1, Mab 2.3.35
5	SEQ ID NO: 19	heavy chain CDR2, Mab 2.3.35
	SEQ ID NO: 20	heavy chain CDR3, Mab 2.3.35
	SEQ ID NO: 21	light chain CDR1, Mab 2.3.35
	SEQ ID NO: 22	light chain CDR2, Mab 2.3.35
	SEQ ID NO: 23	light chain CDR3, Mab 2.3.35
10	SEQ ID NO: 24	heavy chain variable domain, Mab 2.3.35
	SEQ ID NO: 25	light chain variable domain, Mab 2.3.35
	SEQ ID NO: 26	human kappa light chain
	SEQ ID NO: 27	human lambda light chain
	SEQ ID NO: 28	human IgG1 (Caucasian Allotype)
15	SEQ ID NO: 29	human IgG1 (Afroamerican Allotype)
	SEQ ID NO: 30	human IgG1 LALA-Mutant (Caucasian Allotype)
	SEQ ID NO: 31	human IgG4
	SEQ ID NO: 32	human IgG4 SPLE-Mutant
	SEQ ID NO: 33	heavy chain CDR1, Mab55
20	SEQ ID NO: 34	heavy chain CDR2, Mab55
	SEQ ID NO: 35	heavy chain CDR3, Mab55
	SEQ ID NO: 36	light chain CDR1, Mab55
	SEQ ID NO: 37	light chain CDR2, Mab55
	SEQ ID NO: 38	light chain CDR3, Mab55
25		

Example 1a**Generation of monoclonal antibodies neutralizing the serine protease activity of Hepsin**

The Hepsin protein immunogen was expressed as an N-terminal EE-tagged (Glu-Phe-Met-Pro-Met-Glu; EFMPME) fusion protein in SF9 insect cells that becomes autocatalytically activated when concentrating the protein.

a) Production of EE-tagged human Hepsin

Hepsin is tagged with Glu-Phe-Met-Pro-Met-Glu. A sequence corresponding to amino acids 45-417 of human hepsin was engineered using PCR, for subcloning into either an insect cell (Sf9 cells) or mammalian cell expression vector. The

vectors supply an in frame N-terminal signal sequence for secretion followed by a 6 amino acid epitope tag for purification ("EE-tag", EFMPME). 5' - and 3' - cloning sites add ASAA and AGSA sequences to either side of the insert.

5 Purification of recombinant EE-tagged human hepsin from a baculoviral expression system was performed by concentration of the cell supernant and centrifugation at 40,000 rpm (approximately 100,000 x g) for 1 hour at 4°C. The pellet was discarded and the supernatant solution was filtered through a 0.8µm nitrocellulose filter followed by a 0.2µm polyethersulfone filter. The filtered media was pumped onto 50 mL anti-EE antibody-Protein G Sepharose (prepared as described in Stern, 10 A.S. and Podlaski, Techniques in Protein Chemistry 4 (1993) 353-360) contained in a Millipore Vantage Chromatography Column and protected by a 100 ml guard column consisting of Sepharose 4 FF. The two columns linked in tandem had been equilibrated in TBS/0.1mM EDTA. The flow rate was 1.75 ml/min. After loading the media, the column was washed with TBS/0.1mM EDTA until an A280 baseline 15 was achieved. The guard column was disconnected and the anti-EE antibody-Protein G Sepharose was eluted with TBS/0.1 mM EDTA containing 100µg/ml of EE peptide (the sequence for the hexapeptide is Glu-Phe-Met-Pro-Met-Glu). Collected eluant (approximately 110 ml) containing the hepsin zymogen.

20 Purification of recombinant EE-tagged human hepsin from a mammalian expression system was performed by concentration of the cell supernant and filtered through a 0.8µm nitrocellulose filter followed by a 0.2µm polyethersulfone filter. The filtered media was pumped onto 50 mL anti-EE antibody-Protein G Sepharose (prepared as described in Stern, A.S. and Podlaski, F.J., Techniques in Protein Chemistry 4 (1993) 353-36099 contained in a Millipore Vantage 25 Chromatography Column and protected by a 100 ml guard column consisting of Sepharose 4 FF. The two columns linked in tandem had been equilibrated in TBS/0.1mM EDTA. The flow rate was 1.75 ml/min. After loading the media, the column was washed with TBS/0.1mM EDTA until an A280 baseline was achieved. The guard column was disconnected and the anti-EE antibody-Protein G Sepharose 30 was eluted with TBS/0.1 mM EDTA containing 100µg/ml of EE peptide (the sequence for the hexapeptide is Glu-Phe-Met-Pro-Met-Glu).

b) Immunization

35 Balb/c mice were immunized for at least 3 months with the recombinant enzymatically active two-chain form of hepsin protein every 4 weeks for 3 times followed by an i.v. boost on day 4 before fusion. Serum test bleeds were taken and

half-maximal serum titer was determined using Hepsin protein coated ELISA microtiter plates. Mice with a half-maximal titer of 1:12,800 were selected for i.v. boost. Three days following the i.v. boost, splenocytes were harvested, and fused with Ag8 myeloma cells. Screening for Hepsin specific antibodies was started by identifying antibodies binding to Hepsin coated onto microtiter plates. Positive clones binding to immobilized Hepsin were then cultivated in serum free medium (Hyclone ADCF-Mab-Thermo Scientific, Cat. No. SH30349.02) for assessing the inhibitory potential thus avoiding unspecific inhibition by serum derived components. Hybridoma supernatants were diluted in the range 1:4 to 1:32 fold for obtaining the maximal distance of lowest and highest inhibitory values. Inhibition of enzymatic activity was analyzed by using a FRET quenched protease assay. Cleavage of a Gln-Arg-Arg-Lys peptide, coupled to a FRET pair of fluorochromes, causes the dyes to separate and to generate a fluorescence signal. Mab 2.7.35 displays unique strong inhibitory activity in the FRET quenched assay.

Immunization with cells expressing membrane-bound hepsin or with DNA encoding hepsin did not yield suitable titers and/or antibodies neutralizing the enzymatic activity of Hepsin. Screening for specific antibodies on hybridoma supernatants without serum free culture medium and appropriate dilution did not yield suitable antibodies neutralizing the enzymatic activity of Hepsin.

Example 1b

Expression of hepsin antibodies in mammalian cells

Chimeric hepsin antibody (Mab2.7.35) was transiently expressed in HEK293 cells by transfection of the light and heavy chain plasmids via lipofection. Supernatant was collected 7 days after transfection and purified via protein A.

A humanized variant of Mab2.7.35, the hepsin antibody Mab55 was stably expressed in CHO cells. Humanized light and heavy chain constructs were cloned into a mammalian expression vector containing glutamine synthetase as selection marker. Transfected cells were selected with methionine sulfoximine (MSX) for stable expression of the antibody construct. Cells were screened for antibody expression in the supernatant and cloned as single cells by limited dilution. The final clone was expressed by a fed-batch shake flask culture, and the purified product was analyzed to confirm mass identity. Large amounts of antibody were produced by a 20 l fed-batch fermentation.

Example 1c**Humanization and determination of affinity of hepsin antibodies**

5 The murine hepsin antibody Mab2.7.35 was humanized using the CDRs (Complementarity Determining Regions) grafting method, i.e. by keeping the six loops that are recognizing the antigen intact and exchanging the murine framework for a human one. The CDRs were identified according to the Kabat nomenclature whereas several frameworks for both heavy and light chains were chosen from human germline IMGT database. To decide whether back-mutations (maintaining the murine antibody conformation) in the framework were required or forward-
10 mutations (to adopt to the human germline) could be applied in the CDRs, we have created a homology 3D model of the Mab2.7.35 variable regions resulting in the sequences of the humanized variants Mab55 and Mab53 and.

Humanization of mouse anti-hepsin antibody led to an increased affinity

15 The amino acid sequences of light and heavy chain complementary regions were grafted from the mouse Mab 2.7.35 antibody onto human antibody light and heavy chains resulting in Mab55 (Figure 7A). Assuming three frameworks with a couple of back- and forward-mutations for each chains, a matrix comprising 10-20 variants was built by combining the C_H and V_L constructed plasmids for each of them. This approach led to the screening of more than 300 humanized candidates.

20 Regarding the heavy chain, one candidate was obtained by applying CDR grafting without back- or forward-mutation. To avoid potential glycosylation, Asn⁵⁶ was mutated to serine. The hH25 V_H is based on the IMGT germline hVH7-4-1 combined with the j element IGHJ4-03-1 where the free cysteine in position 82A in the V region have been mutated to a serine in order to avoid glutathione
25 conjugation.

For the light chain, the lambda type V_L is extremely close to the mouse germline mVL-1 with 99% identity. Thus, we could not envisage back-mutations based on specific matured amino acids. However, as none of the human germline is really related to this particular mouse germline (highest identity percentage: ~62%), we
30 used a homology 3D model of the Mab 2.7.35 variable region to best preserve the CDR integrity. The back-mutations F36V, A46G, and Y49G restored the original smaller side chain in the CDR-H3 surrounding while maintaining the antigen recognition. The back-mutation P44F led to a strongly improved expression rate.

The humanized variant described here is based on the human IMGT germline hVK7_43 combined with the j element IGLJ6-01.

Our subsequent analysis (Figure 4A) showed complete inhibition of the enzyme activity with mouse Mab 2.7.35, chimeric Mab 2.7.35 and Mab55 under tested conditions. Additionally, we found an unexpected inhibition increase by a factor of 14.7 compared to the murine antibody and by a factor of 6.7 compared to the chimeric antibody. Sequence analysis (data not shown) revealed, that this large improvement relied critically on the maintenance of antibody light chain residue Phe44 during humanization.

Example 2a

In vitro characterization of Mab 2.7.35 - specific and selective inhibition of Hepsin enzymatic activity.

Mab 2.7.35, 2.3.35 and 2.14.5 were characterized by Biacore analysis (Kd), inhibition of enzymatic activity of Hepsin protein (human and murine) and inhibition of enzymatic activity of related serine proteases trypsin and chymotrypsin. Mab 2.7.35 shows specific and selective inhibition of Hepsin enzymatic activity.

- Biacore Kd values: 4.0 nM (2.7.35); 22 nM (2.3.35); 2 nM (2.14.5)
- IC₅₀ in enzymatic activity assay of human hepsin (2.7.35): 14 nM
- IC₅₀ in enzymatic activity assay of human hepsin (2.3.35): 7 nM
- IC₅₀ in enzymatic activity assay of human hepsin (2.14.5): not detectable, therefore assumed as at least 200 nM or more
- IC₅₀ in enzymatic activity assay of murine hepsin (2.7.35): >200 nM
- IC₂₀ in trypsin/ chymotrypsin activity assay (2.7.35): >200 nM (selectivity vs. other related serine proteases)

Example 2b

Surface plasmon resonance analysis

For surface plasmon resonance analyses, about 1600 Relative Units (RU) of Protein A and 700 RU of Protein G (10 µg/ml each) in 10 mM acetate pH 5.5 were immobilized on a sensor chip CM5 using the standard EDC/NHS amine coupling procedure (GE Healthcare T100). About 360 RU of antibody mouse Mab 2.7.35 (on Protein G), chimeric Mab 2.7.35 (on Protein A) and Mab55 (on Protein A) were captured, respectively.

For affinity measurements, hepsin was injected at seven different concentrations ranging from 0 to 200 nM. Measurements were performed at 37°C and at a flow rate of 5 µl/min for about 10 min. Dissociation was measured for about 15 minutes. Each hepsin injection was followed by a pulse (30 sec 30 µl/min) of 0.85% H₃PO₄ for regeneration. The 10 nM curves were measured in quadruplicates (Mab55, chimeric Mab 2.7.35) or triplicates (mouse Mab 2.7.35). Data was evaluated using Biacore T100 evaluation software 2.0.3 and a 1:1 langmuir model for fittings.

We further used surface-plasmon resonance with immobilized Mab 2.7.35, chimeric Mab 2.7.35 and Mab55 antibodies and human hepsin injected at varying concentrations, to analyze binding association and dissociation (Figure 4B). We found, that chimeric chimeric Mab 2.7.35 antibody exhibits medium affinity similar to the mouse Mab 2.7.35 antibody, but the humanized antibody Mab55 has a significantly lower k_D value. Inherent to all variants is a very slow on-rate combined with a very low off-rate (Figure 4C). Thus, once the antibody was bound to hepsin, it hardly came off within usual assay durations. However, in this case, a slow-tight binding kinetic was observed.

Example 2c

Enzymatic assays

Purified hepsin was diluted in assay buffer (50 mM Tris-HCl, 100 mM NaCl, 0.1 mg/ml BSA, 0.02% Tween®-20). The peptides Acetyl-KQLR-AMC (AMC is 7-amino-4-methylcoumarin) was synthesized with >95% purity as determined by HPLC and MS analysis [6].

For measuring amidolytic activities hepsin and other proteases were transferred to a 384 well flat bottom plate (Optiplate PerkinElmer). The KQLR-peptide (5 µM) was added and the enzyme reaction started. Assays contained less than 5% DMSO in a final test volume of 30 µl. Fluorescence increase was monitored with excitation at 530 nm and emission at 572 nm on an Envision Reader at 26°C. For determination of the apparent K_m value and inhibition model, hydrolysis rates of at least six different concentrations of peptide in triplicates were measured. Rates of hydrolysis and apparent K_m values were calculated using XLFit® software (IDBS, Surrey U.K.).

The release of AMC by other serine proteases was tested under the same buffer conditions as described for hepsin. The apparent K_m values of the KQLR peptide for recombinant HAT (human airway trypsin-like protease), human matriptase and

bovine enteropeptidase were calculated as described for hepsin. Recombinant HAT, human matriptase/ST14 catalytic domain and bovine enteropeptidase were from R&D Systems (Minneapolis, MN, U.S.A). Trypsin was purchased from Merck.

5 For evaluation of the inhibition mechanism various concentrations of hH35 (20 – 0.31 nM in 2-fold dilutions in triplicates) were incubated with 1 nM hepsin for 15 min. After simultaneously adding 20, 10, 5 and 2.5 μ M Ac-KQLR-AMC peptide the linear rates of fluorescence increase were measured. The data were fitted to the equations for tight binding inhibition using SigmaPlot® kinetic modul software
10 (Version 8.0, Systat, London U.K.).

Inhibition by Mab55 is specific for human hepsin

In order to test for undesired off-target effects, we tested the specificity of our murine, chimeric and humanized hepsin antibodies on other proteases in the peptide substrate activity assay (Figure 6A).

15 The K_m values for the cleavage of the Ac-KQLR-AMC peptide were evaluated by using standard Michaelis-Menten kinetics (Supplementary Table S1). We detected full neutralization of human hepsin activity by our antibodies. In contrast, none of the tested other proteases (matriptase, HAT, Enteropeptidase or trypsin) was significantly inhibited. Thus we conclude, that our antibodies are indeed selective
20 for hepsin.

Example 2d

Binding of Mab55 to cell surface hepsin

Endogenous levels of hepsin in tumour cell lines are too low to be detected by flow cytometry or immunocytochemistry analysis. To study binding of Mab55 on the
25 cell surface, we established a HEK293 cell line that stably overexpressed full length hepsin with an N-terminal GFP fusion tag.

When these cells were incubated with increasing amounts of Mab55, specific surface staining could be detected both by flow cytometry (Figure 5A) and confocal microscopy analysis (Figure 5B). Untransfected cells did not display any
30 detectable binding (data not shown).

Example 3**In vivo characterization of Mab 2.7.35**

5 Mab 2.7.35 is an anti-hepsin MAb that binds to human hepsin and neutralizes serine protease activity. Mab 2.7.35 was tested in 5 xenograft models: HepG2 (hepatoma), LNCAP (prostate), MCF-7 (breast), 22Rv1 (prostate), T47D (breast). In 4 models Mab 2.7.35 displayed substantial tumor growth inhibition >60%.

Animals used were male or female nude mice (10/group), obtained from Charles River Laboratories (Wilmington, MA) were used when they were approximately 8 - 10 weeks old and weighed approximately 25 grams.

10 Tumor cells used were LNCaP and 22Rv1 human prostatic carcinoma cells, MCF7 human mammary adenocarcinoma cells, T47D human mammary ductal carcinoma cells, and HepG2 human hepatocellular carcinoma cells were obtained from ATCC (Manassas, VA). LNCaP, 22Rv1, MCF7, and T47D cells were cultured in RPMI-1640, whereas HepG2 cells were grown in Modified Essential Medium (MEM).
15 All culture media were supplemented with 10% FBS and 1% 200 nM L-glutamine. Mice were implanted with 1×10^7 cells subcutaneously (sc) in a 0.2 ml volume of a 1:1 mixture of matrigel: phenol red-free, Mg^{2+}/Ca^{2+} -free PBS per mouse in the right hind flank.

20 MCF7 and T47D are estrogen receptor positive cell lines which are estrogen-responsive and require hormone supplementation for survival and growth. Thus for MCF7 and T47D studies, female mice were implanted with subcutaneous 90 day 0.72 mg sustained release 17β -estradiol pellets (Innovative Research, Sarasota, FL) on the nape of the neck, at least one day prior to cell implantation. LNCaP is an androgen receptor positive cell line which is androgen-responsive and requires
25 hormone supplementation for survival and growth. Thus for LNCaP studies, male mice were implanted with subcutaneous 90 day 12.5 mg sustained release testosterone pellets (Innovative Research, Sarasota, FL) on the nape of the neck, at least one day prior to cell implantation. For HepG2 and 22Rv1 studies, female mice were utilized without any hormone supplementation.

30 Mab 2.7.35 was diluted immediately prior to use with Histidine buffer (10 mM Histidine, 140 mM NaCl, pH 6.0) for ip injection.

Mice implanted with human xenografts were randomized according to tumor volume so that all groups had similar starting mean tumor volumes.

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Treatment began between day 3 and day 15 post-cell implant, depending upon the study. Vehicle or anti-hepsin antibodies were dosed using a sterile 1cc syringe and 16-gauge needle (0.5 ml/animal) twice weekly (2x/wk) for 3 to 10 weeks.

5 Mab 2.7.35 was tested at a dose of 20 mg/kg twice weekly ip in five different human xenograft models in nude mice (HepG2 hepatocellular carcinoma, LNCaP and 22Rv1 human prostatic carcinoma, MCF7 human mammary adenocarcinoma, and T47D human mammary ductal carcinoma). Tumor growth inhibition was considered to be biologically significant based on the NCI definition of > 60% TGI. Biologically and statistically significant antitumor efficacy was observed in 10 the LNCaP model, with TGIs of 62% and 72% respectively as compared to vehicle treated controls. Furthermore, statistically and biologically significant efficacy was observed with in the MCF7, 22Rv1, and T47D models, with 77%, 66%, and >100% TGI (regression) observed respectively in the three models. Results for Mab 53 and Mab 55 are shown in table 1 and 2.

15 **Table 1: Efficacy Summary for LNCaP**

Antibody amount mg/kg	Mean Tumor Volume (mm ³) Start Study day 15	SD (±)	Mean Tumor Volume (mm ³) End Study day 42	SD (±)	% inhibition end of study
vehicle	183.33	26.57	898.95	334.24	-
5 (Mab53)	163.69	28.29	458.30	158.04	59
10 (Mab 53)	183.20	19.98	412.68	175.49	68
20 (Mab 53)	165.85	36.68	462.68	160.92	59
5 (Mab55)	162.10	35.34	270.81	76.66	85
10 (Mab55)	167.35	25.39	363.15	107.86	73
20 (Mab55)	177.62	29.48	284.80	107.97	85
Taxotere 15 mg/kg	167.67	25.48	66.81	70.87	regression

Table 2: Efficacy Summary for T47D

Antibody amount mg/kg	Mean Tumor Volume (mm ³) Start Study day 15	SD ($\pm$)	Mean Tumor Volume (mm ³) End Study day 42	SD ($\pm$)	% inhibition end of study
vehicle	168.79	43.97	995.62	622.80	-
5 (Mab53)	157.98	31.15	767.05	70.73	26
10 (Mab 53)	141.10	21.05	499.63	115.41	57
20 (Mab 53)	135.56	34.47	326.47	35.98	77
5 (Mab55)	155.92	18.67	386.23	67.69	72
10 (Mab55)	147.06	35.46	308.13	56.38	81

Example 4**Epitope Mapping by Biacore****5 4.1. General Procedure**

For coupling onto SPR chips the Biacore coupling kit (BR-1002-83; GE Healthcare) was chosen by using appropriate buffers for coupling (Acetate buffer pH 4.5 or pH 5). Runs were performed using PBS buffer (10 mM Na₂HPO₄, 1 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH adjusted to pH 7.4 with conc. HCl or NaOH respectively. Addition of 0.05% Tween20 was performed if appropriate.

4.2. SPR – kinetic experiments

Amine coupling of around 1000-2000 resonance units (RU) of a capturing system (capturing mAB or Protein A) was performed on a CM5 chip at pH 4.5. Antibody was captured at a concentration of 10-50 nM. Different concentrations of Hepsin were passed with a flow rate of 10-30 μ l/min through the flow cells at 298 K for 120-600 sec to record the association phase. The dissociation phase was monitored for up to 480 sec and triggered by switching from the sample solution to running buffer. Bulk refractive index differences were corrected for by subtracting the response obtained from a blank-coupled surface. Blank injections are also subtracted (=double referencing).

Mab 2.7.35 showed a K_D value of 31.5 nM, Mab 55 a monovalent K_D value of 7.1 nM and Mab 53 a K_D value of 36 nM.

4.3. SPR – epitope mapping

For epitope mapping and investigation of inhibiting binding of HAI-2 an anti-hepsin antibody to be investigated was immobilized covalently on a CM5 chip. Next Hepsin was captured onto this surface. As analyte now several mABs or HAI-2 were injected. A rise in signal is due to a different epitope whereas no change in signal shows that the analyte antibody binds to the same epitope as the immobilized antibody. In this setting the result were counterchecked by preincubation of Hepsin with the analyte antibodies and injecting this preformed mixture to the chip. Binding indicates that the epitope is still accessible and chip-bound antibody covers a different epitope.

Figure 1 shows a sensogram of an epitope mapping experiment. Here an anti-hepsin antibody was immobilized on a CM5 chip and 50 nM hepsin was added). Then HAI-2 (50nM) was injected. An increase in signal means that the epitope is still accessible and the antibody binds to a different epitope then HAI-2. If Hepsin is bound to antibody 2.14.5 HAI-2 can still be bound meaning that they cover different epitopes whereas hepsin complexed with antibody 2.7.35 or 2.3.35 do not bind significant levels of HAI-2. Therefore the binding of HAI-2 to hepsin is inhibited by antibodies 2.3.35 and 2.7.35.

Figure 2 shows a further sensogram of an epitope mapping experiment. Here anti-hepsin antibodies 2.7.35, 2.3.35 and 2.14.5 were immobilized on a CM5 chip and 50 nM hepsin was added. Then antibody 2.7.35 was injected with a concentration of 50 nM. An increase in signal means that the epitope is still accessible and the antibody binds to a different epitope. Antibodies 2.3.35 and 2.14.5 give rise to a signal resulting in a different epitope than antibody 2.7.35. Therefore the binding of antibody 2.7.35 does not influence the binding of 2.3.35 and 2.14.5 to hepsin and results in different epitopes than antibody 2.7.35.

Figure 3 shows a further sensogram of an epitope mapping experiment. Here anti-hepsin antibody 2.7.35, 2.3.35 and 2.14.5 were immobilized on a CM5 chip and 50 nM hepsin was added. Then antibody 2.3.35 was injected with a concentration of 50 nM. An increase in signal means that the epitope is still accessible and the antibody binds to a different epitope. Antibodies 2.7.35 and 2.14.5 give rise to a signal resulting in a different epitope than antibody 2.3.35. Therefore the binding of antibody 2.3.35 does not influence the binding of 2.7.35 and 2.14.5 to hepsin and results in different epitopes than antibody 2.3.35.

Example 4b**Native epitope binding of Mab55**

HEK293 cells were transfected with a construct encoding full-length hepsin with an N-terminal GFP to generate stable HEK-HPN-GFP cell lines. Stable cells were selected with 3 µg/ml Geneticin (G418, Roche Applied Science, Cat. No. 04727894001).

Clones were analyzed by flow cytometry for hepsin expression using the intrinsic GFP fluorescence. Cell surface binding of Mab55 was determined by incubating the cells with 0.50-50.0 µg/ml hH35 or isotype control IgG for 45 min on ice. The cells were washed twice with PBS before incubation with ALEXA 647-conjugated goat anti-human IgG (Invitrogen) diluted 1:300 in PBS, 1% fetal bovine serum (v/v). After 30 min on ice the cells were washed with PBS, and cell pellets were resuspended and antibody binding was measured on a FACS Canto (BD Biosciences).

For immunocytochemistry analysis HEK-HPN-GFP cells were plated onto glass coverslips and grown o/n. Cells were then incubated with 5 µg/ml Mab55 for 30 min on ice, washed with PBS, fixed with 4% paraformaldehyde, counterstained with CY3-conjugated goat anti-human IgG (Jackson Immuno Research Laboratories Inc.). Confocal microscopy images were taken on a LEICA TCS SP2/MP confocal laser scanning microscope (at 100x/1.46NA). Selective spectral detector emission band passes for each dye were used in sequential scanning mode.

Example 5**a) Cloning of Full Length Cynomolgus Monkey Hepsin cDNA**

The predicted mRNA sequences of the Rhesus monkey hepsin are available at NCBI based on the genomic sequence. The sequence alignment of the chimp, rhesus and human provided information to design the PCR primers for Cynomolgus Monkey Hepsin cDNA. Total RNA was isolated from cynomolgus pancreas, kidney and liver tissue as described in the protocol for the Qiagen RNeasy kit. 5 µg of total RNA was used for the first strand reverse transcriptase reaction using SuperScript (Invitrogen). PCR primers were designed by the cross species comparison of hepsin mRNA sequences. Sequence alignments from the rhesus monkey, chimp and human were used to define the primer sequences. The forward primer 5'ATTAGGCCATTATGGCCCCATGGCGCAGAAGGAGGGTGCC 3' and the reverse primer 5'TAATGGCCGAGGCGGCCTCAGAGCTGGGTCAACCATGCC 3'

were designed to incorporate SfiI sites (underlined) for directional subcloning and used to amplify the full length hepsin open reading frame (ORF). Amplification of the first strand cDNA was done with Taq polymerase under the following conditions: 95°C 5 minutes, 95°C 1 minute, 55°C 30 seconds and 72°C 90 seconds for 30 cycles and then 72°C 10 minutes. Amplicons from pancreas and liver were gel purified and ligated into the corresponding SfiI sites of a mammalian expression vector using the elongation factor 1 alpha promoter ("pEFBos D" vector). One clone from both the pancreas and two clones from liver mRNA/cDNA preparations were sequenced to obtain a consensus cynomolgus monkey sequence. All three clones had the identical sequence.

b) Subcloning of Secreted and Tagged Cynomolgus Hepsin 45-418 into the Expression vector pTT5.

An artificially secreted form of Hepsin comprising an N-terminal signal peptide and being devoid of the transmembrane region was generated by deleting amino acids no 1-44 and fusing the remaining portion 45-418 in frame to the signal peptide of the human IL-12 p40 subunit, followed by an epitope tag ("EE-tag", EFMPME), in the expression vector pTT5 (based on the CMV promoter).

c) Transient Transfection of FreeStyle™ 293 (FS293) Cells with EE-Cyno Hepsin

The cynomolgus cDNA clone was used for transient transfections in the FreeStyle™ 293 Expression System (Invitrogen Corp., www.invitrogen.com). The 293 cell line is a permanent line established from primary embryonal human kidney transformed with sheared human adenovirus type 5 DNA (Graham, F.L., et al., J. Gen. Virol. 36 (1977) 59-74; Harrison, T., et al., Virology 77 (1977) 319-329).

Transfection of FS293 cells was done to ensure EE-Cynomolgus hepsin was being produced. The transfection protocol is as follows: FS293 cells were seeded at a cell density of 0.5×10^6 cells/ml 48 hours prior to the transfection. The cells were counted again on the day of transfection and if necessary, the cell density was adjusted to 1×10^6 cells/ml. EE-Cynomolgus hepsin plasmid DNA diluted to 1 ug/ml was mixed with PEI solution (3 ug/ml) and incubated at RT for 15 minutes. The DNA/PEI complex was added to the FS293 cells and incubated on a shaker at 37°C. Approximately 4 hours later, 20% peptone was added to a final concentration of 0.5%. Supernatant was harvested 72 hours later for Western analysis.

Example 6**Human Hepsin Inhibition Assay**

Purified human hepsin was diluted in assay buffer (50 mM Tris-HCl, 100 mM NaCl, 0.1 mg/ml BSA, 0.02% Tween®-20) to a concentration of 0.446 ug/ml (0.010 uM). Peptide substrate (1 mM, JA133-Z-Gln-Arg-Arg-Z Lys (TAMRA)-NH₂) was diluted in assay buffer to 300 nM. Testing antibodies starting from 600 nM was diluted 2 fold in series (total 11 concentrations) in assay buffer. Hepsin enzyme solution (10 µl/ well) was added into 384 well microtiter plates followed by diluted antibody solutions (10 µl/well). Samples were incubated at room temperature for 30 min. Peptide substrate solution (10 µl/well) was then added. Samples were mixed and incubated at room temperature for 60 min. Signals were quantitated by reading fluorescence (excitation at 530 nm and emission at 572 nm) on a Victor 2 reader (PerkinElmer). Percent inhibition of hepsin activity by an agent at various concentrations was calculated by the following formula:

% Inhibition = $100 \times [1 - (F_s - F_b) / (F_t - F_b)]$, where F_s is the fluorescence signal of the sample including the agent, F_b is the fluorescence signal in the absence of hepsin and agent, F_t is the fluorescence signal in the presence of hepsin without agent.

Example 7**Cynomolgous Hepsin Assay**

Cynomolgous hepsin assay was carried out under the same conditions as that for human hepsin assay. The monoclonal antibodies were tested in human and cynomolgous hepsin assays. Mab 55 is active against human hepsin with an IC₅₀ of 1.02 nM and against cynomolgous hepsin with an IC₅₀ of 0.99 nM. A control antibody is not active against both enzymes.

Example 8**Inhibition of Cell Proliferation of LNCaP Tumor Cells (MTT assay)**

LNCaP tumor cell lines were tested using Cell Proliferation Kit I (MTT), Roche Diagnostics GmbH, Germany, Cat. No. 11 465 007 001. The cell line was treated with antibody Mab 2.7.35 in concentrations of 100 µg/ml (MW 150.000, 0.67 µM). The assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolic active cells. This cellular reduction involves the pyridine nucleotide cofactors NADH and NADPH. The formazan crystals formed

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are solubilized and the resulting colored solution is quantified using a scanning multiwell spectrophotometer (ELISA reader).

Cells were grown in a 96 well tissue culture plate and incubated with the yellow MTT solution for approx. 4 h. After this incubation period, purple formazan salt crystals are formed. These salt crystals were solubilized by adding the solubilization solution and the plates were incubated overnight in humidified atmosphere (37°C, 6.5% CO₂). The solubilized formazan product is spectrophotometrically quantified using an ELISA reader. An increase in number of living cells results in an increase in the total metabolic activity in the sample. This increase directly correlates to the amount of purple formazan crystals formed, as monitored by the absorbance.

It was found that Mab 2.7.35 reduces in a concentration of 100 µg/ml cell proliferation (cell viability in the MTT assay) for 20%.

Example 9

Inhibition of cell growth in soft agar

Antibody 2.7.35 reduced colony formation of LNCaP in soft agar for 40% at 300 µg/ml.

Example 10

Preparation of Mab55, Mab55-Fab fragment and the Hepsin- Mab55 complex

Harvested cell-culture supernatant was sterile filtrated through a 0.2 µm-pore-size membrane (Millipore) prior to purification. The Mab55 was captured on a MabSelectSure resin (GE Healthcare), washed with 1x PBS and eluted with 20 mM sodium-citrate at pH 3.0. The Mab55 was further purified by size exclusion chromatography using a Superdex 200 26/60 GL (Amersham Bioscience) column equilibrated with 20 mM Histidine, 140 mM NaCl, pH 6.0.

Mab55 was cleaved by papain at 37°C. The cleavage was stopped by iodacetamide addition and the Fab fragment purified by separation of the Fc fragment on a 1 ml MabSelect SuRe column (GE). The hepsin : Mab55-Fab complex ("hHepsin-Mab55 complex") was formed by mixing a 1:1.3 molar ratio and 30 min incubation at 20°C. The complex was concentrated slowly in an Amicon filter device and the buffer exchanged to 1xTBS (50 mM Tris-HCl pH 7.4 and 150 mM NaCl). Complex assembly was analyzed by size exclusion chromatography (Superose 12 column, GE) and by SDS-PAGE.

Mode of inhibition of hepsin by antibody Mab55

To describe the binding behaviour of antibody Mab55 in the activity assay the progress curves of the steady-state reactions were analysed by adding the enzyme to a mixture of peptide substrate and antibody (Figure 6C). While the initial reaction velocity remained unchanged the steady-state velocity was obviously reduced depending on inhibitor concentration. The curved nature of the progress curves indicate a slow-binding inhibition. This behaviour was found to be consistent with allosteric macromolecular inhibitors like antibodies interfering with protease activity in a multistep mechanism.

Due to the slow on-rate of Mab55 antibody found in surface plasmon resonance measurements (Figure 4B), we decided to first form the complex of antibody and enzyme by preincubation and then evaluate the inhibition model. Measurements without prebound antibody did not provide reliable fittings and a definite interpretation of a competitive/noncompetitive inhibition mechanism. We fitted our data in SigmaPlot® using non-linear least-squares regression to determine the best fit values with the corrections for the tight binding inhibition. The assessments indicate a mixed/noncompetitive tight binding inhibition mechanism top-ranked. As illustrated by the Eadie-Hofstee plot in Figure 6D, the presence of increasing concentrations of Mab55 affected significantly the $V_{\max}^{\text{app}}$ values while K_m values remained mostly unchanged. After fitting the data to tight binding inhibitor equations of the mixed type mechanism, a K_i^{app} value of 0.39 ± 0.06 nM was obtained. Second in rank was noncompetitive tight type inhibition, which is also common for allosteric inhibition and thus reduced affinity of the substrate for the active centre.

X-ray structure determination

Crystals of hHepsin- Mab55 complex concentrated to 8 mg/ml were grown at 20°C in hanging drops over reservoirs containing 18% PEG 3350, 0.15 M MgSO₄ and 0.01 M barium chloride. Crystals were harvested by gradually adding glycerol to a final concentration of 14% (v/v) and were flash-cooled in liquid nitrogen. Diffraction data were collected in 0.25° increments and at 100 K on a PILATUS 6M detector at the Swiss Light Source SLS, Villigen, Switzerland. Data of a macroscopically twinned plate-crystal was processed by using XDS and XSCALE. The two major lattices of the crystal were indexed and integrated separately, then scaled together. The hepsin substructure was determined by molecular replacement using PHASER with structure PDB 1Z8G as an initial search model. HHpred

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identified structure PDB 1PKQ as a suitable search model for the Fab fragment, which was then used in PHASER split into individual constant and variable domains to solve the complete complex structure. The resulting electron density map allowed model correction and building in COOT. The structure was refined with BUSTER using non-crystallographic (NCS) restraints and TLS parameterisation and has been deposited in the Protein Data Bank. Molecular graphics figures were prepared using CHIMERA

Structure of the hHepsin-Mab55 complex reveals recognition of hepsin in a deep and hydrophobic pocket

The 2.55 Å hHepsin- Mab55 complex structure (Figure 3-8) reveals that the major epitope of hepsin for the Mab55 antibody is located around hepsin helix $\alpha 3$ and following residues. Generally, hepsin in our structure exhibits a similar secondary structure compared to human hepsin in PDB entry 1Z8G (Figure 7B), but shows a slightly skewed SRCR domain. Strikingly, it shows also a few regions, that could not be build due to weak or missing electron density. As indicated in Figure 7B and by dotted lines in Figure 8B, three of these loops (Gly²⁹⁷-Ala³⁰⁶, Glu³⁴³-Gln³⁵⁰, Trp³⁷⁷-Ala³⁸²) are located in a defined region of the protease domain. This was apparently not due to crystal defects since we detected this in both NCS copies. Thus we interpret these loops as flexible regions, which is a frequent cause for blurred electron density in X-ray crystallography.

While some hepsin loops were missing in the structure model, all antibody CDR loops could be build in the complex (Figure 8C). Most importantly, hepsin residues Phe³²⁷-Tyr³²⁸ are recognized by antibody Mab55 in a deep and hydrophobic pocket, that is formed by both heavy and light chain CDRs and neighbouring residues (Figure 8B). Additionally to the dominant hydrophobic interactions, also some side-chain and backbone hydrogen bonds have significant contributions in the binding interface.

Recognition of hepsin by antibody Mab55 is different from published anti-HGFA antibodies

Due to the generally planar or concave antigen-binding site shape, antibodies were originally thought to be ill suited for inhibition of proteases, which exhibit a concave shaped substrate-binding cleft. Nevertheless, it was shown for HGFA, that complete competitive inhibition of a protease by an antibody is possible (Figure 9C), although not easy to achieve. While very long CDR loops may be an

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alternative to solve this dilemma, allosteric antibody inhibition has been recently explored for the first time for serine-proteases using again HGFA as an example.

Similarly to the HGFA-Fab40 structure (Figure 9A and 9B), the epitope region in structure hHepsin-Mab55 is located far away from the active centre cleft thus suggesting an allosteric inhibition mechanism through the antibody as well. However, this mechanism is distinct from that found for HGFA-Fab40 as very distant regions are bound by the particular antibodies for hepsin and HGFA (Figure 9A and 9B). In fact, the superposition of hepsin and HGFA points out that allosteric inhibition of serine-proteases by antibodies is not limited to certain trigger spots, but can rather exploit diverse enzyme surface regions.

Formation of the hepsin recognition pocket is induced by introduction of Phe⁴⁴ in Mab55 V_L

Since formation of such a deep, hydrophobic recognition pocket is unusual for antibodies, we sought to explain its formation in more detail and analyzed differences of Mab55-Fab to other published Fab fragment structures. Strikingly, when superimposed, Mab55 does not exhibit the conventional double or single hydrogen bond between V_H Gln³⁹ and V_L Gln³⁸ or Glu³⁸ residues. This is illustrated by superposition of structure PDB 1NL0, which was chosen as an example due to very similar Fab fragment elbow angles (Figure 10A). Instead, the distance between these two residues is widened by about 2 Å due to Mab55 V_L residue Phe⁴⁴. The Pro⁴⁴, present in 95% of the human germline, to Phe⁴⁴ mutation has been introduced during humanization and induced not only the widening in the central interface between heavy and light variable regions, but has a leverage effect. These further CDRs movements (Figure 10A) form the very defined recognition pocket as illustrated in Figure 8B.

The hHepsin- Mab55 atomic structure explains antibody specificity

As shown in Figure 6, the Mab55 antibody is both specific for the hepsin protease and species specific for hepsin as well. These findings result from the unique sequence of the Mab55 -specificity determining region in hHepsin (Figure 7B, black bar). Other proteases such as HGFA, matriptase or trypsin do not possess a Phe³²⁷Tyr³²⁸-motif that could insert into the recognition pocket to the same extent (Figure 7B). Second, the overall conformation and flexibility of the loop following α3-helix must be different since the Gly³²⁴Ala³²⁵-motif occurs only in human and monkey hepsin, but not in rodent or other more distant species. Instead, often a

Ser-Pro motif is present, which typically leads to a proline-induced kink in the structure.

Binding of Mab55 distorts the hepsin active centre and substrate binding pockets

5 The most obvious allosteric change in human hepsin upon Mab55 binding is the turn of the Phe³²⁷Tyr³²⁸-motif containing loop following the α 3-helix towards the antibody cleft (Figure 10B). Helix α 3 and sheet β 18 are held still in a similar position as in hepsin:peptide-substrate complex structure (PDB 1Z8G), which may be explained by the disulfide bond between residues Cys³²² and Cys³³⁸. The hepsin
10 loop regions 342-344 and 382-386 would clash with the Mab55 V_L chain in case they adopted the same conformation as in hepsin structure PDB 1Z8G. As a consequence, the affected residues propagated partially to new defined positions and to a larger extend into flexible conformations. This is reflected by missing electron density for the loop residues 343-350 and 377-382, which are connected
15 by a disulfide-bond as well and thus adopt concurrently. Moreover, loop 297-306 was disordered, but with unclear cause. Near the end of the disordered loop 343-350, residue Asp³⁵² changed conformation. While the oxygen O^{δ2} of this residue was previously in contact with the residue Ile¹⁶³ backbone nitrogen, in our structure it flipped to contact residue His¹⁸⁶ nitrogen N^{ε2}. Nevertheless, the distance stayed
20 the same with 2.7 Å, arguing for the interchangeability between both conformations, which may occur possibly in a spring-load mechanism.

Resulting from this latter conformational change, also the backbone bearing the catalytic triad residue Ser³⁵³ is twisted (Figure 10B, zoom window). As for the nucleophilic attack of the carbonyl-carbon in the scissile bond, the catalytic triad
25 serine oxygen O^γ must be oriented at exactly 109° angle, we conclude that proteolytic cleavage cannot take place anymore (Figure 7B, zoom window). Residues Asp²⁵⁷ and His²⁰³ of the catalytic triad are slightly distorted as well, but to a lesser extend compared to Ser³⁵³ (Figure 10B).

Aside from changes in the active centre, catalytic and substrate binding pockets
30 were heavily disordered in our structure, too (Figure 7B). The orientation of the oxi-anion hole residue Gly³⁵¹ is changed, so that stabilization of an tetrahedral intermediate would be hampered. Substrate binding pockets S2 and S4 are slightly distorted in our structure, substrate binding pockets S1 including the specificity pocket and S3 are almost completely disordered. In summary, inhibition by Mab55
35 binding results both from distorted hepsin geometry, especially at the catalytic

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triad, and from disordered binding pockets, which likely reduce the affinity of substrates dramatically.

Patent Claims

1. Antibody binding to human hepsin, characterized in that the heavy chain variable domain comprises a CDR1 region of SEQ ID NO: 33, a CDR2 region of SEQ ID NO:34 and a CDR3 region of SEQ ID NO:35 and in that
5 the light chain variable domain comprises a CDR1 region of SEQ ID NO: 36, a CDR2 region of SEQ ID NO:37 and a CDR3 region of SEQ ID NO:38.
2. Antibody according to claim 1, characterized in comprising
the heavy chain variable domain defined by amino acid sequence of SEQ ID
10 NO: 9 and the light chain variable domain defined by amino acid sequence of SEQ ID NO: 10,
3. Antibody according to claim 1 or 2, characterized in inhibiting serine protease activity of human hepsin with an IC_{50} value of 20nM or lower and reducing in a concentration of 0.67 μ M cell proliferation of LNCaP cells (ATCC CRL-1740) in the MTT assay for 20% or more in relation to cell
15 proliferation without said antibody.
4. Antibody according to claim 1 or 2, characterized in inhibiting binding of human HGF activator inhibitor type 2 of SEQ ID NO:17 to hepsin.
5. Antibody binding to human hepsin, characterized in that the heavy chain variable domain comprises a CDR1 region of SEQ ID NO: 1, a CDR2 region of SEQ ID NO:2 and a CDR3 region of SEQ ID NO:3 and in that the light
20 chain variable domain comprises a CDR1 region of SEQ ID NO: 4, a CDR2 region of SEQ ID NO:5 and a CDR3 region of SEQ ID NO:6 or that the heavy chain variable domain comprises a CDR1 region of SEQ ID NO: 18, a CDR2 region of SEQ ID NO:19 and a CDR3 region of SEQ ID NO:20 and
25 the light chain variable domain comprises a CDR1 region of SEQ ID NO: 21, a CDR2 region of SEQ ID NO:22 and a CDR3 region of SEQ ID NO:23;
or a humanized variant thereof
6. Antibody according to claim 5, characterized in that the heavy chain variable domain comprises SEQ ID NO:7 or a humanized variant thereof and the light
30 chain variable domain comprises SEQ ID NO:8 or a humanized variant thereof.

7. Antibody according to claim 5, characterized in that the heavy chain variable domain comprises SEQ ID NO:24 or a humanized variant thereof and the light chain variable domain comprises SEQ ID NO:25 or a humanized variant thereof.
- 5 8. Antibody according to claim 5, characterized in comprising
 - a) the heavy chain variable domain defined by amino acid sequence of SEQ ID NO:7 and the light chain variable domain defined by amino acid sequence of SEQ ID NO:8,
 - 10 b) the heavy chain variable domain defined by amino acid sequence of SEQ ID NO: 9 and the light chain variable domain defined by amino acid sequence of SEQ ID NO: 10,
 - c) the heavy chain variable domain defined by amino acid sequence of SEQ ID NO: 11 and the light chain variable domain defined by amino acid sequence of SEQ ID NO: 12, or
 - 15 d) the heavy chain variable domain defined by amino acid sequence of SEQ ID NO: 24 and the light chain variable domain defined by amino acid sequence of SEQ ID NO: 25.
9. Nucleic acid, characterized in encoding a heavy chain variable domain and a light chain variable domain of an antibody according to claim 1 to 8.
- 20 10. A host cell comprising the nucleic acid of claim 9.
11. Method for the production of a recombinant human or humanized antibody according claims 1 to 8, characterized by expressing a nucleic acid according claim 10 in a prokaryotic or eukaryotic host cell and recovering said antibody from said cell or the cell culture supernatant.
- 25 12. Pharmaceutical composition, characterized in comprising an antibody according to claims 1 to 8.
13. Method for the manufacture of a pharmaceutical composition comprising an antibody according to claims 1 to 8.
- 30 14. Use of an antibody according to claims 1 to 8 for the manufacture of a pharmaceutical composition.

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15. Use of an antibody according to claims 1 to 8 for cancer treatment.
16. Antibody according to claims 1 to 8 for use in cancer treatment.
17. Method for the treatment of a patient suffering from cancer, characterized by administering to the patient an antibody according to claims 1 to 8.
- 5 18. An immunoconjugate comprising the antibody of claim 1 to 8 and a cytotoxic agent.

Fig. 1

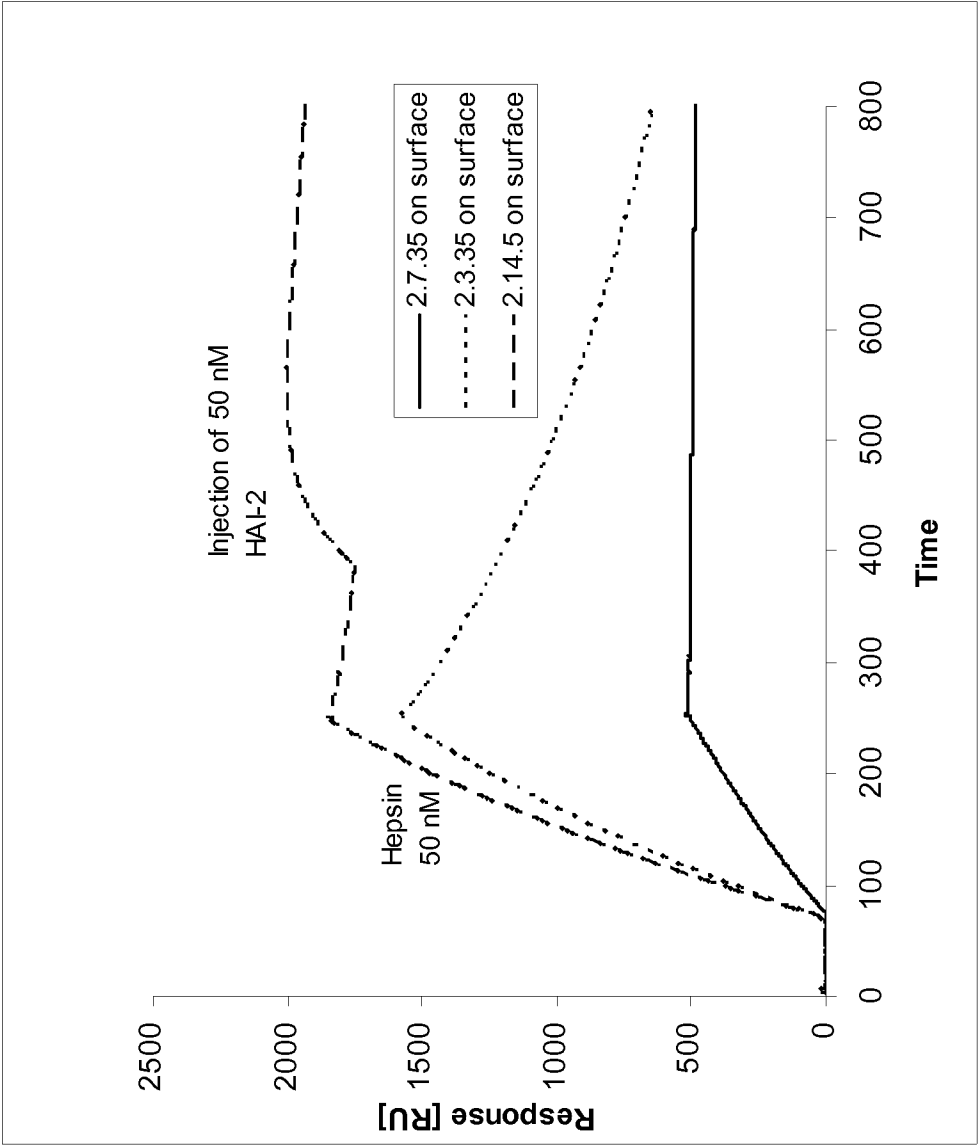


Fig. 2

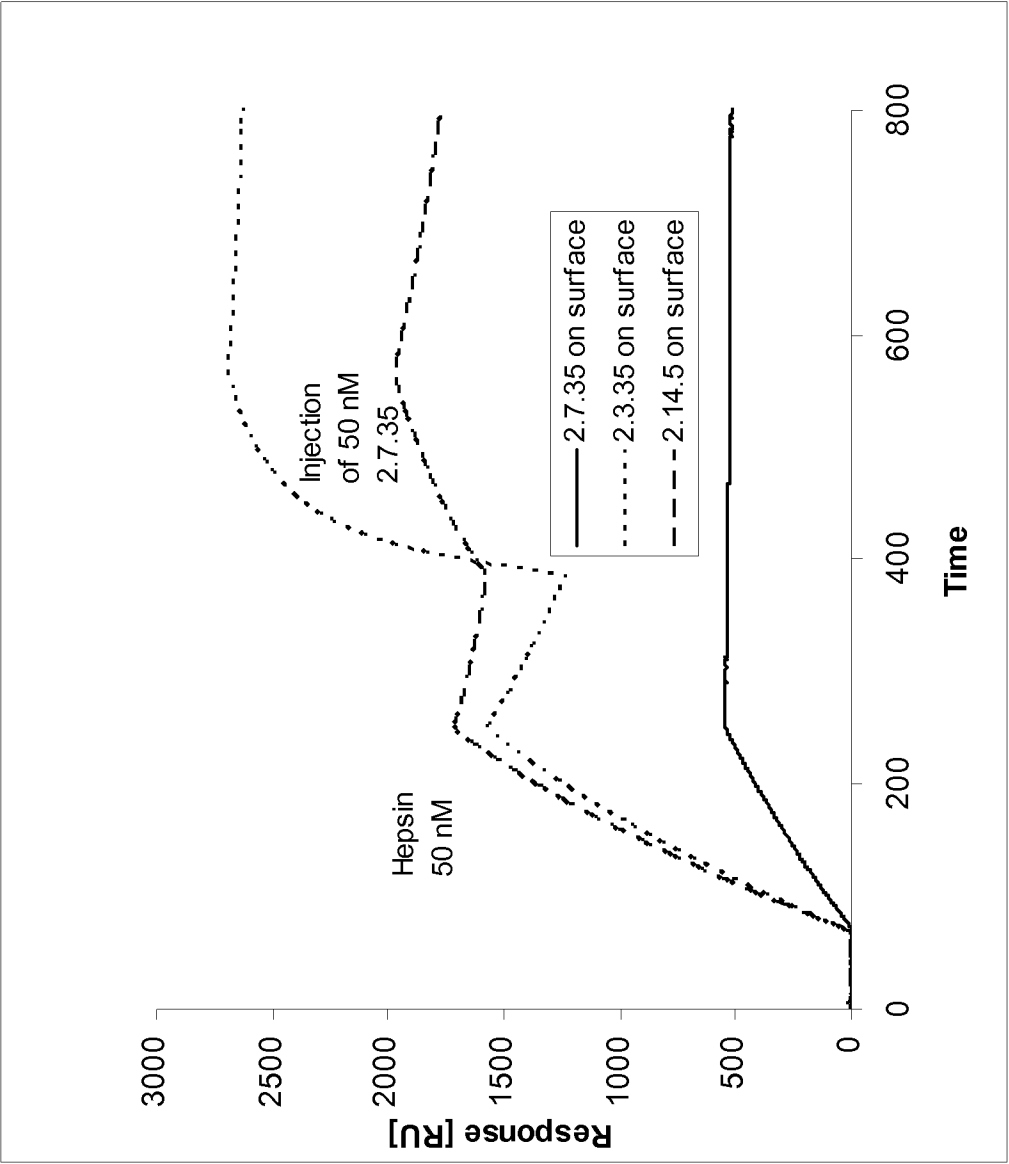


Fig. 3

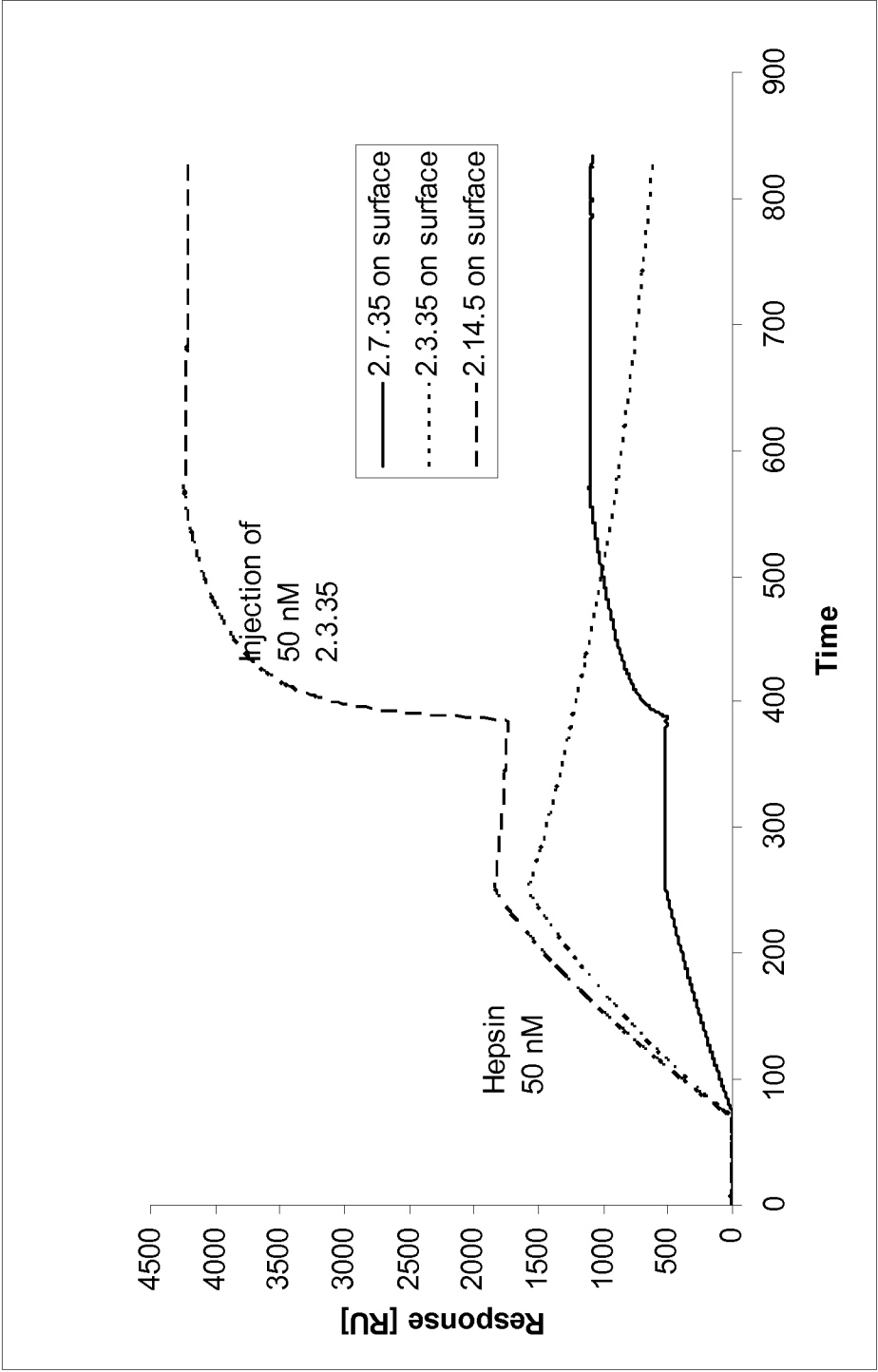


Fig. 4

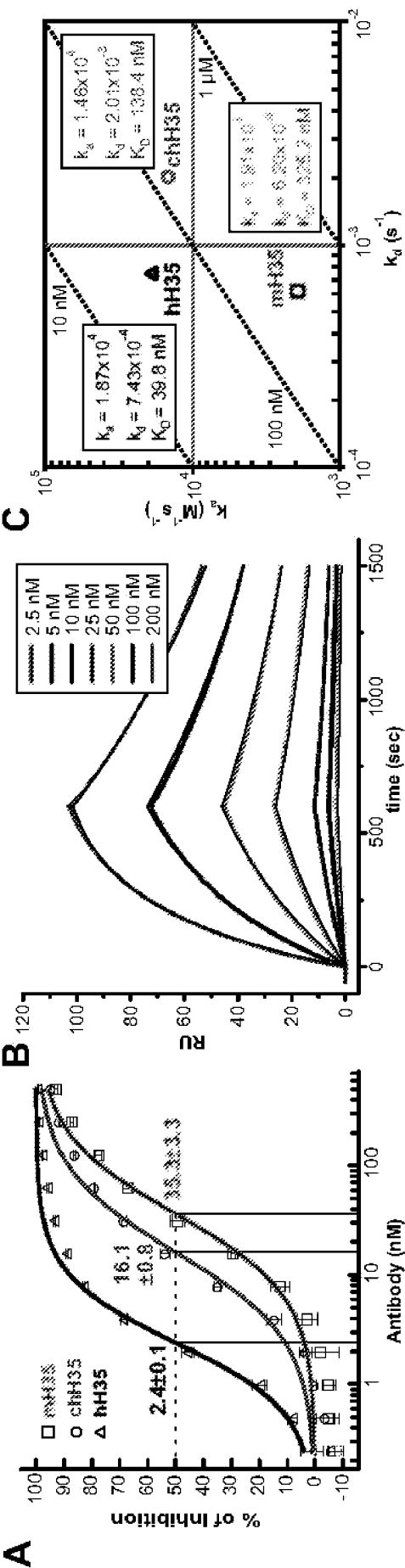


Fig. 5

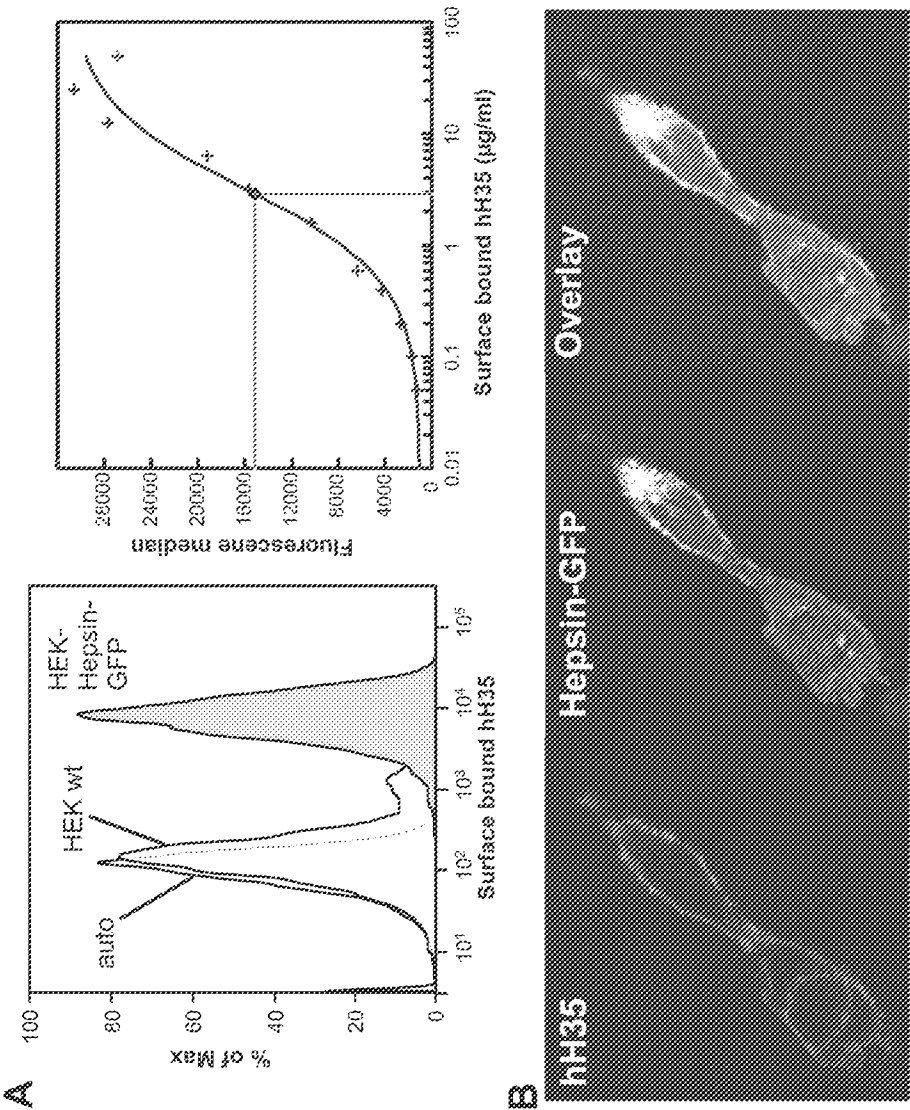
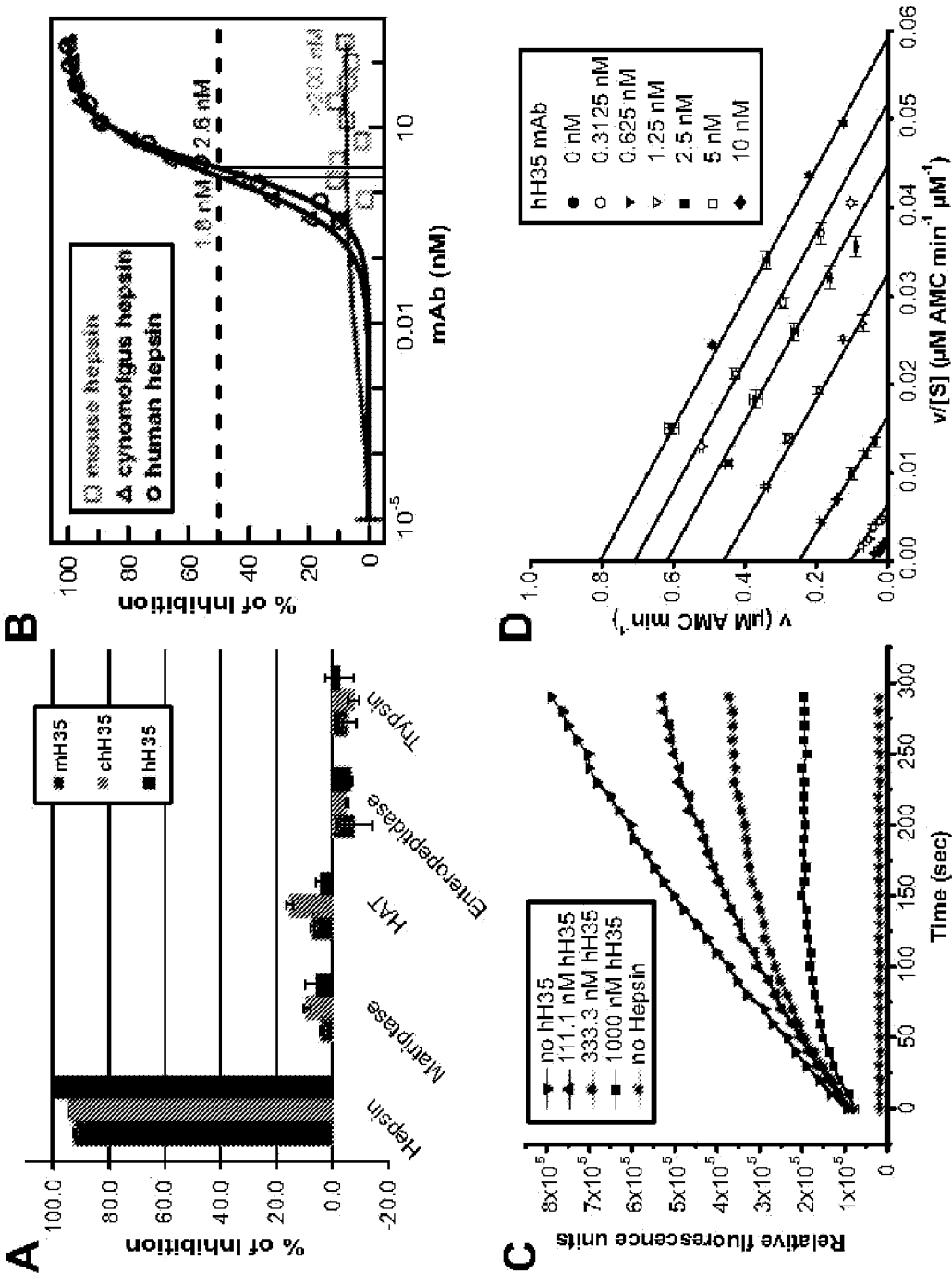


Fig. 6



[illegible]

Fig. 8

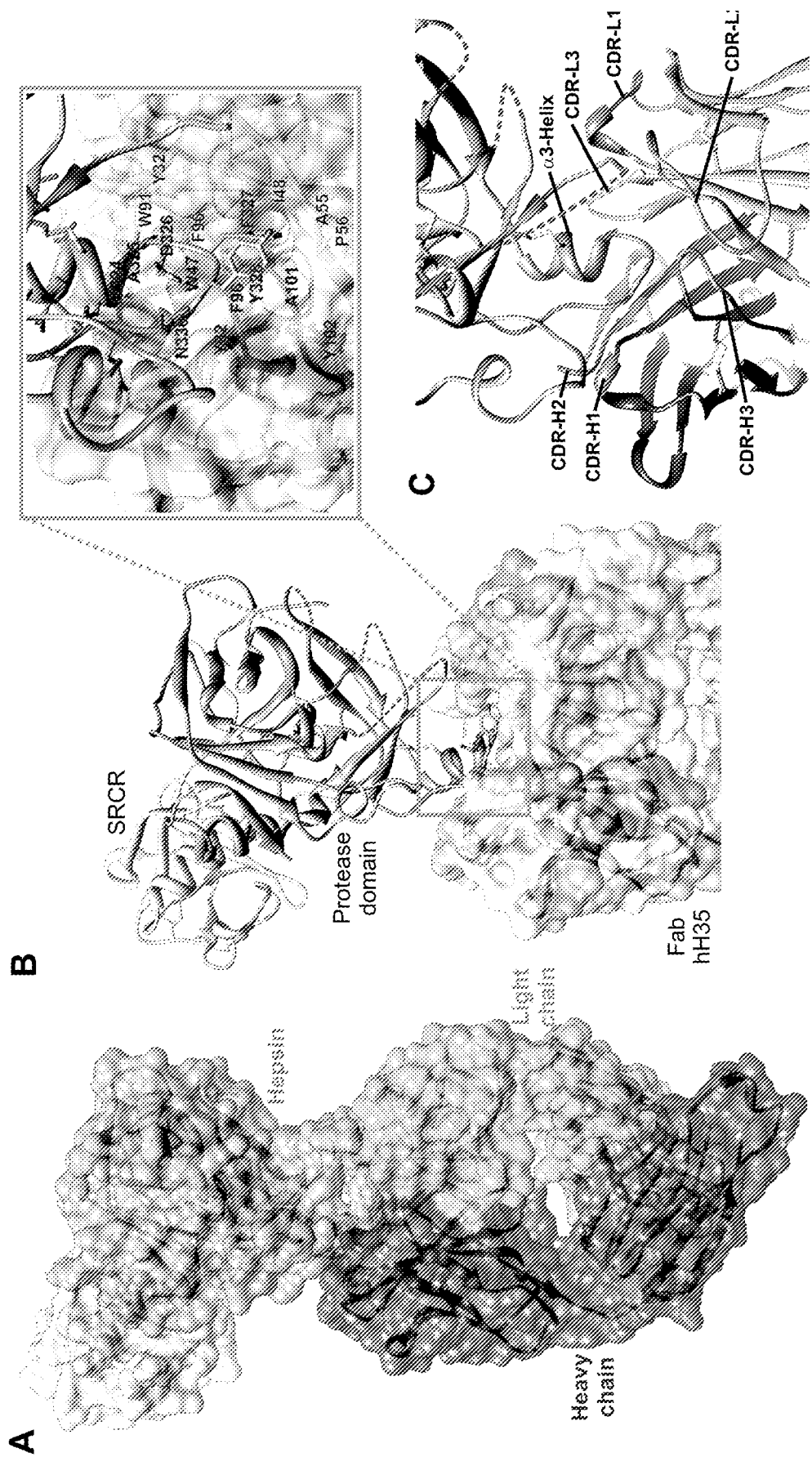


Fig. 9

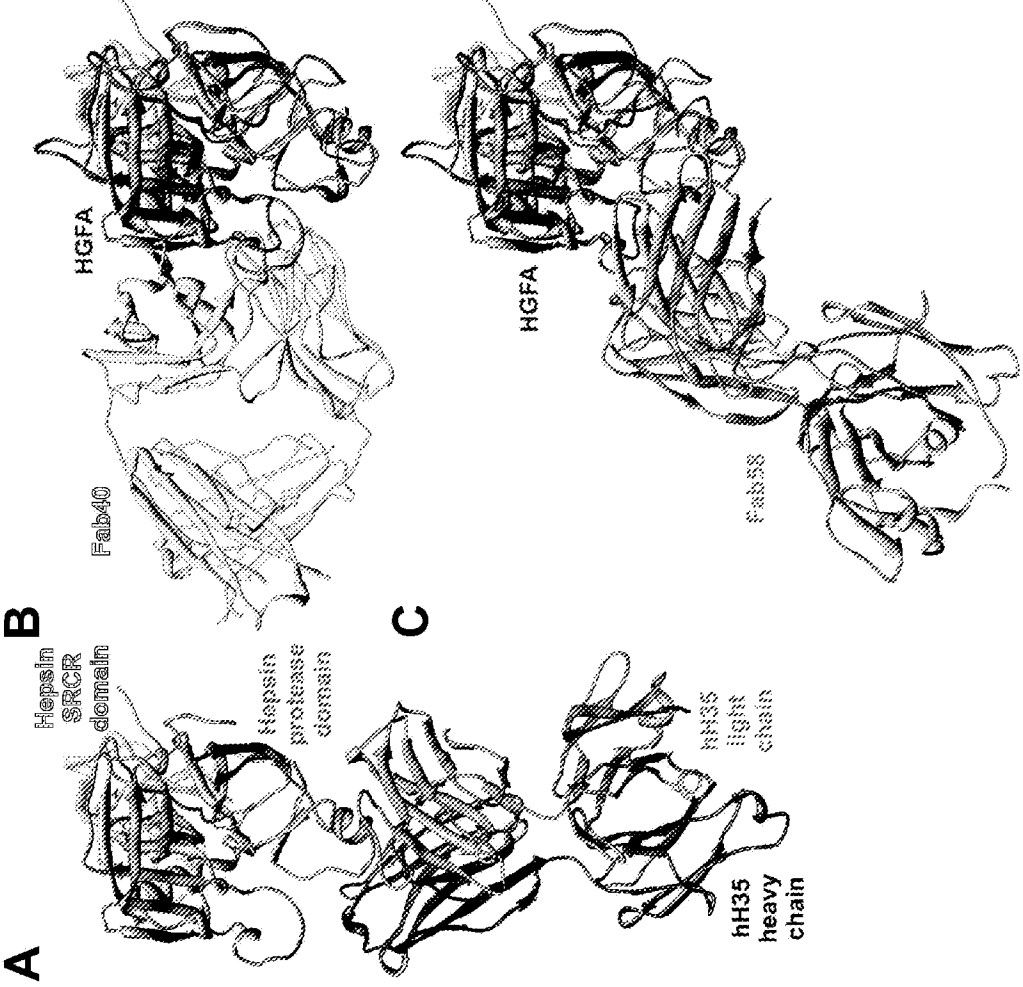
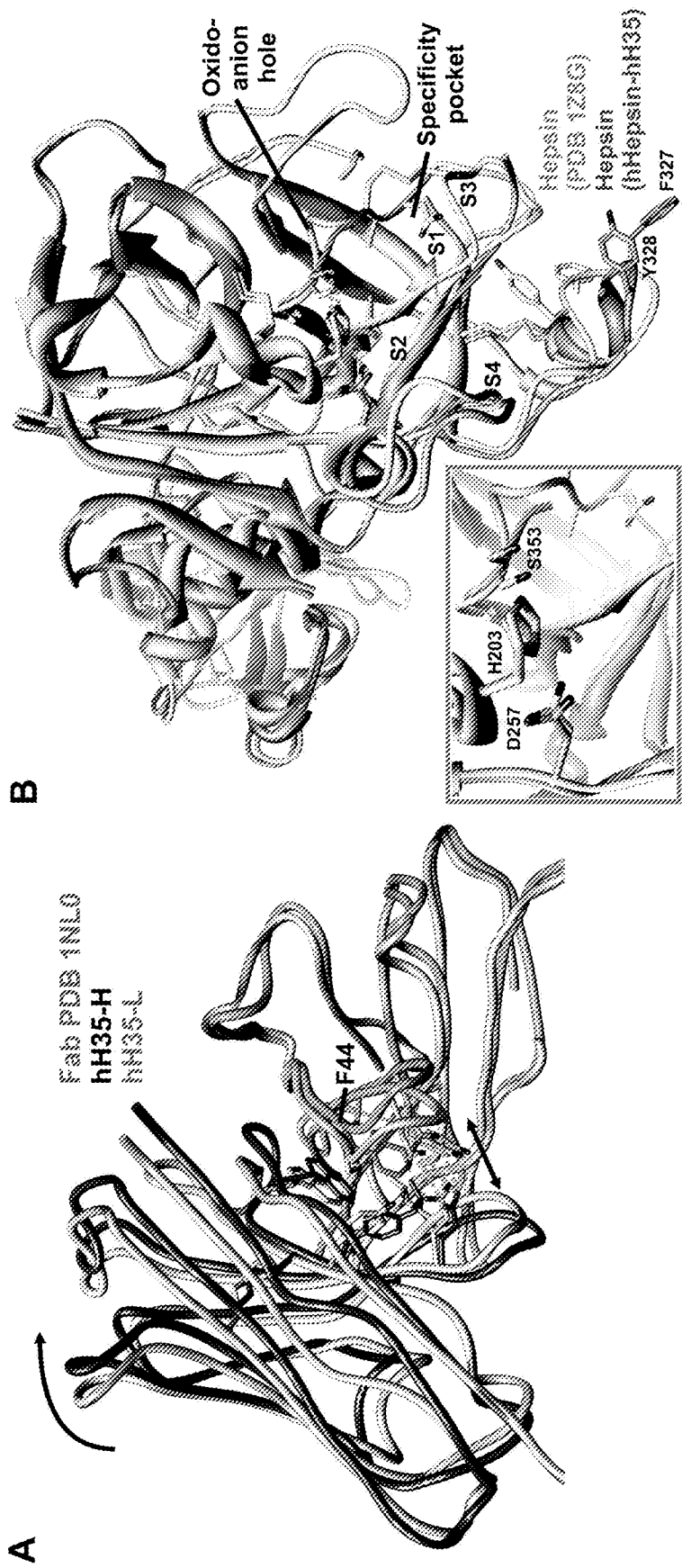


Fig. 10



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/060501

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/30 A61K39/395
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Further documents are listed in the continuation of Box C.



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Date of the actual completion of the international search

1 August 2011

Date of mailing of the international search report

12/08/2011

Name and mailing address of the ISA/

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Fax: (+31-70) 340-3016

Authorized officer

Morawetz, Renate

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PCT/EP2011/060501

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Information on patent family members

International application No

PCT/EP2011/060501

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