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ANTI-HEPSIN ANTIBODIES AND METHODS USING SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 61/253,953, filed October 22, 2009, the contents of which is incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates generally to the field of molecular biology. More specifically, the invention concerns anti-hepsin antibodies, and uses of same.

BACKGROUND OF THE INVENTION

Hepsin is a type II transmembrane serine protease (TTSP) expressed on the surface of epithelial cells. The 417-amino acid protein is composed of a short N-terminal cytoplasmic domain, a transmembrane domain and a single scavenger receptor cysteine-rich domain that packs tightly against the C-terminal protease domain (Somoza et al (2003) *Structure* 11(9), 1123-1131). The physiologic function of hepsin is unclear. Despite its expression in the very early stages of embryogenesis (Vu et al (1997) *J Biol Chem* 272 (50), 31315-31320), hepsin-deficient mice were viable and developed normally (Yu et al (2000) *Thromb Haemost* 84(5), 865-870; Wu et al (1998) *J Clin Invest* 101(2), 321-326). Hepsin was found not to be essential for liver regeneration and for coagulation-related physiological functions (*Id.*). A recent study demonstrated that hepsin knock-out mice are hearing impaired. Guipponi et al. (2007) *Am J Pathol* 171:608-616. However, hepsin has been implicated in ovarian [(Tanimoto et al (1997) *Cancer Res* 57(14), 2884-2887); WO2001/62271] and prostate cancer. Several gene expression studies identified hepsin as one of the most highly induced genes in prostate cancer (Dhanasekaran et al (2001) *Nature* 412, 822-826; Luo et al (2001) *Cancer Res* 61(12), 4683-4688; Magee et al (2001) *Cancer Res* 61(15), 5692-5696; Stamey et al (2001) *J Urol* 166(6), 2171-2177; Stephan et al (2004) *J Urol* 171(1), 187-191; Welsh et al (20010) *Cancer Res* 61(16), 5974-5978). Hepsin RNA levels were found to be low in normal prostate and benign hyperplasia, but strongly increased in prostate carcinoma, particularly in advanced stages ((Dhanasekaran et al (2001) *Nature* 412, 822-826; Luo et al (2001) *Cancer Res* 61(12), 4683-4688; Magee et al (2001) *Cancer Res* 61(15), 5692-5696; Stamey et al (2001) *J Urol* 166(6), 2171-2177; Stephan et al (2004) *J Urol* 171(1), 187-191; Welsh et al (20010) *Cancer Res* 61(16), 5974-5978). Hepsin protein staining with a monoclonal anti-hepsin antibody showed that hepsin expression was highest at sites of bone

metastasis and in late stage primary tumors (Xuan et al (2006) *Cancer Res* 66(7), 3611-3619), which is consistent with the finding that increased hepsin RNA levels correlated with higher Gleason grades and tumor progression ((Luo et al (2001) *Cancer Res* 61(12), 4683-4688; Magee et al (2001) *Cancer Res* 61(15), 5692-5696; Stamey et al (2001) *J Urol* 166(6), 2171-2177; Stephan et al (2004) *J Urol* 171(1), 187-191; Chen et al (2003) *J Urol* 169(4), 1316-1319).

Experimental evidence for a role of hepsin in prostate cancer came from a study by Klezovitch et al. (Klezovitch et al (2004) *Cancer Cell* 6(2), 185-195) demonstrating that in a mouse model of non-metastasizing prostate cancer, overexpression of hepsin led to primary tumor progression and metastasis. Intriguingly, hepsin overexpression was associated with basement membrane disruption (*Id.*) pointing towards the possibility that hepsin activity is somehow linked to the degradation of basement membrane components. In-vitro, hepsin is able to convert the latent growth factor pro-hepatocyte growth factor (pro-HGF) into its active two-chain form (HGF), which induced Met receptor signaling (Herter et al (2005) *Biochem J* 390 (Pt 1), 125-136; Kirchhofer et al (2005) *FEBS Lett* 579(9), 1945-1950; WO2006/014928). Hepsin is also able to convert pro-uPA to its active form (Moran et al, (2006) *J Biol Chem.* 281(41):30439-46), and to cleave lamin in vitro (Tripathi et al. (2008) *J Biol Chem.* 283:30576). Because the HGF/Met pathway has been implicated in invasive tumor growth and metastasis, it is possible that overexpression of hepsin activates the HGF/Met axis in prostate cancer (Herter et al (2005) *Biochem J* 390 (Pt 1), 125-136; Kirchhofer et al (2005) *FEBS Lett* 579(9), 1945-1950; WO2006/014928). Hepsin was also shown to cleave other substrates in-vitro, mainly coagulation-related proteins (Herter et al, *id*; Kazama et al (1995) *J Biol Chem* 270(1), 66-72). However, their role in tumorigenesis is not known.

It is clear that there continues to be a need for agents that have clinical attributes that are optimal for development as therapeutic agents. The invention described herein meets this need and provides other benefits.

All references cited herein, including patent applications and publications, are incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

The invention is based in part on the identification of a variety of hepsin binding agents (such as antibodies, and fragments thereof). Hepsin presents an important and advantageous therapeutic target, and the invention provides compositions and methods based on binding of the agents to hepsin. Hepsin binding agents of the invention, as described

herein, provide important therapeutic and diagnostic agents for use in targeting pathological conditions associated with expression and/or activity of the hepsin signaling pathways. Accordingly, the invention provides methods, compositions, kits, and articles of manufacture related to hepsin binding.

The active site of trypsin-like serine proteases, such as hepsin, is formed by several intrinsically mobile loops (the 'activation domain') (Huber and Bode, 1978). In particular, the 220-loop forms part of the S1 pocket and can adopt various conformational states in some serine proteases (Johnson et al., 2005; Shia et al., 2005; Spraggon et al., 2009; Wilken et al., 2004). However, the co-crystal structures of serine proteases with an active site inhibitor showed properly formed active sites, most likely due to stabilizing forces applied by the inhibitor (Arni et al., 1994; Shia et al., 2005; Spraggon et al., 2009). It was reasoned that occupancy of the S1 pocket by a serine protease inhibitor may apply stabilizing forces on the serine protease active site loop flexibility, facilitating antibody recognition of the serine protease active site. Thus, to identify anti-hepsin antibodies that block hepsin enzymatic activity, antibodies that bound hepsin in complex with a serine protease inhibitor that occupies the S1 pocket, 3,4-dichloro-isocoumarin (DCI) were obtained.

The present invention provides antibodies that bind to hepsin. In one aspect, the invention features an isolated antibody that binds a hepsin.

In one aspect, the invention provides an isolated anti-hepsin antibody, wherein a monovalent form (such as a Fab form) of the antibody specifically binds human hepsin with a binding affinity of about 10 nM or better. In some embodiments, the antibody specifically binds human hepsin with a binding affinity of about 6 nM or better. As is well-established in the art, binding affinity of a ligand to its receptor can be determined using any of a variety of assays, and expressed in terms of a variety of quantitative values. Accordingly, in one embodiment, the binding affinity is expressed as Kd values and reflects intrinsic binding affinity (e.g., with minimized avidity effects). Generally and preferably, binding affinity is measured in vitro, whether in a cell-free or cell-associated setting. Any of a number of assays known in the art, including those described herein, can be used to obtain binding affinity measurements, including, for example, Biacore, radioimmunoassay (RIA) and ELISA.

In another aspect, the invention provides anti-hepsin antibodies that bind a Kunitz domain binding region of hepsin. In one aspect, the invention provides an isolated anti-hepsin antibody that competes with a Kunitz domain for binding to hepsin. In one embodiment, said Kunitz domain sequence is Kunitz domain 1 (KD1) of HAI-1 or HAI-1B. In one embodiment, the Kunitz domain sequence is a variant KD1 sequence having at least

about 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99% sequence identity with wild type KD1 of human HAI-1, wherein said variant sequence has at least comparable ability as wild type KD1 in inhibiting hepsin activity. In one embodiment, said Kunitz domain sequence is one or both of the Kunitz domains of HAI-2. In one embodiment, a variant HAI-2 Kunitz domain sequence has between about 70% and 99%, about 75% and 98%, about 80% and 97%, 85% and 95% sequence identity with the corresponding Kunitz domain(s) of wild type human HAI-2, wherein said sequence has at least comparable ability as wild type HAI-2 in inhibiting hepsin activity.

In one aspect, the invention provides anti-hepsin antibodies that bind hepsin catalytic site.

In one aspect, the invention provides anti-hepsin antibodies that bind hepsin outside of the s1 subsite. In some embodiments, the antibodies bind hepsin s2 and/or s3 subsite.

In one aspect, the invention provides anti-hepsin antibodies that bind catalytically inactivated hepsin.

In one aspect, the invention provides anti-hepsin antibodies that are resistant to hepsin proteolysis. In some embodiments, the antibodies are exposed to hepsin for 24 hours under conditions permitting hepsin cleavage of hepsin substrate.

In one aspect, the invention provides anti-hepsin antibodies that bind hepsin present in a complex comprising hepsin and a serine protease inhibitor that binds the hepsin S1 subsite. In some embodiments, hepsin present in the complex is inactivated. In some embodiments, the serine protease inhibitor is 3,4-dichloro-isocoumarin (DCI). In some embodiments, the serine protease inhibitor binds hepsin catalytic amino acid residues Ser195 and His 57, whereby hepsin is inactivated.

In one aspect, the invention provides anti-hepsin antibodies that bind specifically to human hepsin and substantially inhibit in vivo and/or in vitro hepsin enzymatic activity. In one embodiment, the enzymatic activity comprises cleavage of polypeptide substrate of hepsin. In one embodiment, the polypeptide substrate of hepsin is one or more of pro-macrophage stimulating protein (pro-MSP), pro-uPA, Factor VII and pro-HGF. Hepsin activation of pro-MSP is described in co-pending, co-owned US provisional patent application no. 61/253,990, filed October 22, 2009. In one embodiment, the enzymatic activity comprises cleavage of a synthetic substrate of hepsin. In some embodiments, the hepsin synthetic substrate is a substrate shown in Table 1.

In one aspect, the invention provides anti-hepsin antibodies, wherein the antibodies substantially inhibit human and/or mouse hepsin catalytic activity. In some embodiments, a

monovalent form of an anti-hepsin antibody inhibits human hepsin catalytic activity with a Ki of about (in some embodiments, less than or equal to) 4 nM. In some embodiments, a monovalent form of an anti-hepsin antibody inhibits mouse hepsin catalytic activity with a Ki of about (in some embodiments, less than or equal to) 330 nM.

In one aspect, the invention provides anti-hepsin antibodies, wherein the antibodies substantially inhibit hepsin cleavage of pro-uPA. In some embodiments, the anti-hepsin antibodies inhibit hepsin cleavage of pro-uPA with an IC50 of about 3 nM or stronger.

In one aspect, the invention provides anti-hepsin antibodies, wherein the antibodies substantially inhibit laminin-dependent cell migration.

In one aspect, the invention provides anti-hepsin antibodies, wherein the antibodies were generated by a method comprising selecting antibodies that bind a complex comprising (a) hepsin and (b) a serine protease inhibitor that binds hepsin S1 subsite. In some embodiments, hepsin present in the complex is inactivated. In some embodiments, the serine protease inhibitor is 3,4-dichloro-isocoumarin (DCI). In some embodiments, the serine protease inhibitor binds hepsin catalytic amino acid residues Ser195 and His 57, whereby hepsin is inactivated. In some embodiments, prior to selecting the antibody, the antibody is incubated with hepsin and the serine protease inhibitor. In some embodiments, the method further comprises the step of selecting antibodies that compete for hepsin binding with a Kunitz domain. In some embodiments, the Kunitz domain is KD1.

In one aspect, the invention provides anti-hepsin antibodies, wherein the antibodies were generated by a method comprising selecting (identifying) antibodies that compete with a Kunitz domain for hepsin binding. In some embodiments, the Kunitz domain is KD1.

In one aspect, the invention provides anti-hepsin antibodies that are not substantially cleaved by hepsin. In some embodiments, the anti-hepsin antibodies are substantiall resistance to hepsin cleavage.

Generally, the anti-hepsin antibodies of the present invention are antagonist antibodies.

In one aspect, the invention provides an anti-hepsin antibody comprising: at least one, two, three, four, five, and/or six hypervariable region (HVR) sequences selected from the group consisting of:

(a) HVR-L1 comprising sequence RASQSVSSAVA (SEQ ID NO:1),

(b) HVR-L2 comprising sequence SASSLYS (SEQ ID NO:2),

(c) HVR-L3 comprising sequence QQYYSSYYLLT (SEQ ID NO:3),

(d) HVR-H1 comprising sequence GFNFSYSYMH (SEQ ID NO:4),

(e) HVR-H2 comprising sequence ASIYSYYGSTYYADSVKG (SEQ ID NO:5), and

(f) HVR-H3 comprising sequence ARSDSWSYKSGYTQKIYSKGLDY (SEQ ID NO:6).

In one aspect, the invention provides an anti-hepsin antibody comprising (a) a light chain comprising (i) HVR-L1 comprising sequence RASQSVSSAVA (SEQ ID NO:1); (ii) HVR-L2 comprising sequence SASSLYS (SEQ ID NO:2); and (iii) HVR-L3 comprising sequence QQYYSSYYLLT (SEQ ID NO:3) ; and/or (b) a heavy chain comprising (i) HVR-H1 comprising sequence GFNFSYSYMH (SEQ ID NO:4); (ii) HVR-H2 comprising sequence ASIYSYYGSTYYADSVKG (SEQ ID NO:5); and (iii) HVR-H3 comprising sequence ARSDSWSYKSGYTQKIYSKGLDY (SEQ ID NO:6).

In one aspect, the invention provides an anti-hepsin antibody comprising a HVR-L1 comprising the sequence of SEQ ID NO:1. In one aspect, the invention provides an anti-hepsin antibody comprising a HVR-L2 comprising the sequence of SEQ ID NO:2. In one aspect, the invention provides an anti-hepsin antibody comprising a HVR-L3 comprising the sequence of SEQ ID NO:3. In one aspect, the invention provides an anti-hepsin antibody comprising a HVR-H1 region comprising the sequence of SEQ ID NO:4. In one aspect, the invention provides an anti-hepsin antibody comprising a HVR-H2 region comprising the sequence of SEQ ID NO:5. In one aspect, the invention provides an anti-hepsin antibody comprising a HVR-H3 region comprising the sequence of SEQ ID NO:6.

In one aspect, an anti-hepsin antibody comprises a light chain variable region comprising HVR-L1, HVR-L2, HVR-L3, wherein each, in order, comprises sequence RASQDVN/STAVA (SEQ ID NO:7), SEQ ID NO: 2, 3, and/or a heavy chain variable region comprising HVR-H1, HVR-H2, and HVR-H3, where each, in order, contains SEQ ID NO: 4, 5, 6.

In one aspect, an anti-hepsin antibody comprises a light chain variable region comprising HVR-L1, HVR-L2, HVR-L3, wherein each, in order, comprises sequence RASQDVN/STAVA (SEQ ID NO:7), SEQ ID NO: 1, sequence SASFLYS (SEQ ID NO:8), SEQ ID NO:3, and/or a heavy chain variable region comprising HVR-H1, HVR-H2, and HVR-H3, where each, in order, contains SEQ ID NO: 4, 5, 6.

In one aspect, an anti-hepsin antibody comprises a light chain variable region comprising HVR-L1, HVR-L2, HVR-L3, wherein each, in order, comprises SEQ ID NO:7, 8, 3, and/or a heavy chain variable region comprising HVR-H1, HVR-H2, and HVR-H3, where each, in order, contains SEQ ID NO: 4, 5, 6

The amino acid sequences of SEQ ID NOs:1-6 are numbered with respect to individual HVR (i.e., H1, H2 or H3) as indicated in Figure 1, the numbering being consistent with the Kabat numbering system as described below.

Antibodies of the invention can comprise any suitable framework variable domain sequence, provided binding activity to hepsin is substantially retained. For example, in some embodiments, antibodies of the invention comprise a human subgroup III heavy chain framework consensus sequence. In one embodiment of these antibodies, the framework consensus sequence comprises a substitution at position 71, 73, and/or 78. In some embodiments of these antibodies, position 71 is A, 73 is T and/or 78 is A. In one embodiment, these antibodies comprise heavy chain variable domain framework sequences of huMAb4D5-8 (HERCEPTIN®, Genentech, Inc., South San Francisco, CA, USA) (also referred to in U.S. Patent Nos. 6,407,213 & 5,821,337, and Lee et al., J. Mol. Biol. (2004), 340(5):1073-1093). In one embodiment, these antibodies further comprise a human κI light chain framework consensus sequence. In some embodiments, the framework consensus sequence comprises a substitution a position 66. In some embodiments, position 66 is G. In a particular embodiment, these antibodies comprise light chain HVR sequences of huMAb4D5-8 as described in U.S. Patent Nos. 6,407,213 & 5,821,337.) In one embodiment, these antibodies comprise light chain variable domain sequences of huMAb4D5-8 (HERCEPTIN®, Genentech, Inc., South San Francisco, CA, USA) (also referred to in U.S. Patent Nos. 6,407,213 & 5,821,337, and Lee et al., J. Mol. Biol. (2004), 340(5):1073-1093).

In one embodiment, an antibody of the invention comprises a heavy chain variable domain, wherein the framework sequence comprises the sequences disclosed in Figures 2A-B, and HVR H1, H2, and H3 sequences are SEQ ID NOS: 4, 5, and/or 6, respectively.

In one embodiment, an antibody of the invention comprises a heavy chain variable domain, wherein the framework sequence comprises the sequence of SEQ ID NOS 14-15, 48, and/or 16, and HVR H1, H2, and H3 sequences are SEQ ID NOS: 4, 5, and/or 6, respectively. In another embodiment, the framework sequence comprises the sequence of SEQ ID NOS 14-15, 43, and/or 16, and HVR H1, H2, and H3 sequences are SEQ ID NOS: 4, 5, and/or 6 , respectively.

In a particular embodiment, an antibody of the invention comprises a light chain variable domain, wherein the framework sequence comprises the sequence of SEQ ID NOS 17-20; 49-51 & 20; 52-54 & 20; and/or 55-57 & 20, respectively, and HVR L1, L2, and L3 sequences are SEQ ID NOS: 1, 2, and/or 3, respectively. In another embodiment, an

antibody of the invention comprises a light chain variable domain, wherein the framework sequence comprises the sequence of SEQ ID NOS 17-18, 58 and/or 20, and HVR L1, L2, and L3 sequences are SEQ ID NOS: 1, 2, and/or 3, respectively. In another embodiment, an antibody of the invention comprises a light chain variable domain, wherein the framework sequence comprises the sequence of SEQ ID NOS 17, 18, 19 and/or 20, and HVR L1, L2, and L3 sequences are SEQ ID NOS: 1, 2, and/or 3, respectively.

In one embodiment, an antibody of the invention comprises a heavy chain variable domain, wherein the framework sequence comprises the sequence of SEQ ID NOS 14-15, 43 and/or 16, and HVR H1, H2, and H3 sequences are SEQ ID NOS: 4, 5, and/or 6 , respectively, and a light chain variable domain, wherein the framework sequence comprises the sequence of SEQ ID NOS 17-18, 58 and/or 20, and HVR L1, L2, and L3 sequences are SEQ ID NOS: 1, 2, and/or 3, respectively.

In another aspect, an antibody of the invention comprises a heavy chain variable domain comprising the sequence of SEQ ID NO:10 and/or a light chain variable domain comprising the sequence of SEQ ID NO:9.

In another aspect, an antibody of the invention comprises a heavy chain variable domain comprising the sequence of SEQ ID NO:10 and a light chain variable domain.

In another aspect, an antibody of the invention comprises a lightchain variable domain comprising the sequence of SEQ ID NO:9 and a heavy chain variable domain.

Some embodiments of antibodies of the invention comprise a light chain variable domain of humanized 4D5 antibody (huMAb4D5-8) (HERCEPTIN®, Genentech, Inc., South San Francisco, CA, USA) (also referred to in U.S. Pat. No. 6,407,213 and Lee et al., J. Mol. Biol. (2004), 340(5):1073-93) as depicted in SEQ ID NO: 11 below.

1 Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val ***Asn*** Thr Ala
Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Ser Arg Phe Ser Gly
Ser ***Arg*** Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu
Asp Phe Ala Thr Tyr Tyr Cys Gln Gln ***His*** Tyr Thr Thr Pro Pro Thr
Phe Gly Gln Gly Thr Lys Val Glu Ile Lys 107 (SEQ ID NO: 11)
(HVR residues are underlined)

In one embodiment, the huMAb4D5-8 light chain variable domain sequence is modified at one or more of positions 30, 66 and 91 (Asn, Arg and His as indicated in bold/italics above, respectively). In one embodiment, the modified huMAb4D5-8

sequence comprises Ser in position 30, Gly in position 66 and/or Ser in position 91. Accordingly, in one embodiment, an antibody of the invention comprises a light chain variable domain comprising the sequence depicted in SEQ ID NO: 45 below:

1 Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val ***Ser*** Thr Ala
Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu
Asp Phe Ala Thr Tyr Tyr Cys Gln Gln ***Ser*** Tyr Thr Thr Pro Pro Thr
Phe Gly Gln Gly Thr Lys Val Glu Ile Lys 107 (SEQ ID NO: 45)
(HVR residues are underlined).

In one aspect, the invention provides an anti- hepsin antibody that competes with any of the above-mentioned antibodies for binding to hepsin. In one aspect, the invention provides an anti- hepsin antibody that binds to the same or a similar epitope on hepsin as any of the above-mentioned antibodies.

As is known in the art, and as described in greater detail hereinbelow, the amino acid position/boundary delineating a hypervariable region of an antibody can vary, depending on the context and the various definitions known in the art (as described below). Some positions within a variable domain may be viewed as hybrid hypervariable positions in that these positions can be deemed to be within a hypervariable region under one set of criteria while being deemed to be outside a hypervariable region under a different set of criteria. One or more of these positions can also be found in extended hypervariable regions (as further defined below).

In some embodiments, the antibody is a monoclonal antibody. In other embodiments, the antibody is a polyclonal antibody. In some embodiments, the antibody is selected from the group consisting of a chimeric antibody, an affinity matured antibody, a humanized antibody, and a human antibody. In certain embodiments, the antibody is an antibody fragment. In some embodiments, the antibody is a Fab, Fab', Fab'-SH, $F(ab')_2$, or scFv.

In one embodiment, the antibody is a chimeric antibody, for example, an antibody comprising antigen binding sequences from a non-human donor grafted to a heterologous non-human, human, or humanized sequence (e.g., framework and/or constant domain sequences). In one embodiment, the non-human donor is a mouse. In a further embodiment, an antigen binding sequence is synthetic, e.g., obtained by mutagenesis (e.g., phage display screening, etc.). In a particular embodiment, a chimeric antibody of the invention has murine

V regions and a human C region. In one embodiment, the murine light chain V region is fused to a human kappa light chain. In another embodiment, the murine heavy chain V region is fused to a human IgG1 C region.

Humanized antibodies of the invention include those that have amino acid substitutions in the framework region (FR) and affinity maturation variants with changes in the grafted CDRs. The substituted amino acids in the CDR or FR are not limited to those present in the donor or recipient antibody. In other embodiments, the antibodies of the invention further comprise changes in amino acid residues in the Fc region that lead to improved effector function including enhanced CDC and/or ADCC function and B-cell killing. Other antibodies of the invention include those having specific changes that improve stability. In other embodiments, the antibodies of the invention comprise changes in amino acid residues in the Fc region that lead to decreased effector function, e.g., decreased CDC and/or ADCC function and/or decreased B-cell killing. In some embodiments, the antibodies of the invention are characterized by decreased binding (such as absence of binding) to human complement factor C1q and/or human Fc receptor on natural killer (NK) cells. In some embodiments, the antibodies of the invention are characterized by decreased binding (such as the absence of binding) to human FcγRI, FcγRIIA, and/or FcγRIIIA. In some embodiments, the antibodies of the invention are of the IgG class (e.g., IgG1 or IgG4) and comprise at least one mutation in E233, L234, G236, D265, D270, N297, E318, K320, K322, A327, A330, P331, and/or P329 (numbering according to the EU index). In some embodiments, the antibodies comprise the mutations L234A/L235A or D265A/N297A.

In one aspect, the invention provides hepsin binding polypeptides comprising any of the antigen binding sequences provided herein, wherein the hepsin binding polypeptides specifically bind to a hepsin, e.g., a human and/or cyno and/or mouse hepsin.

The antibodies of the invention bind (such as specifically bind) hepsin, and in some embodiments, may modulate (e.g. inhibit) one or more aspects of hepsin activity (such as hepsin enzymatic activity) and/or disruption of any biologically relevant hepsin and/or hepsin polypeptide substrate biological pathway, and/or treatment and/or prevention of a tumor, cell proliferative disorder or a cancer; and/or treatment or prevention of a disorder associated with hepsin expression and/or activity (such as increased hepsin expression and/or activity). In some embodiments, the hepsin antibody specifically binds to a polypeptide consisting of or consisting essentially of a hepsin (e.g., a human and/or mouse hepsin). In one embodiment, the hepsin enzymatic activity comprises cleavage of polypeptide substrate of hepsin. In one

embodiment, the polypeptide substrate of hepsin is one or more of pro-macrophage stimulating protein (pro-MSP), pro-uPA, Factor VII and pro-HGF.

In one embodiment, an antibody of the invention is not an anti-hepsin antibody described in Cancer Research, Volume 66, pages 3611-3619 published in 2006 (e.g., antibody 1A12, 85B11, 94A7, A6, A174, A21 and/or A24 as exemplified in Figure 4), or an isolated hepsin antibody disclosed in PCT Publications WO2004/033630 (e.g., antibody 47A5, 14C7, 46D12, 38E2, 37G10, 31C1, 11C1 and/or 72H6 referred to on page 93 and in Figures 15A-D) or an isolated hepsin antibody disclosed in Xuan et al (2006) *Cancer Res* 66(7), 3611, or an hepsin antibody disclosed in WO2007/149932.

In one embodiment, an antibody of the invention does not compete for binding to human hepsin with an anti-hepsin antibody described in Cancer Research, Volume 66, pages 3611-3619 published in 2006 (e.g., antibody 1A12, 85B11, 94A7, A6, A174, A21 and/or A24 as exemplified in Figure 4), or an isolated hepsin antibody disclosed in PCT Publications WO2004/033630 (e.g., antibody 47A5, 14C7, 46D12, 38E2, 37G10, 31C1, 11C1 and/or 72H6 referred to on page 93 and in Figures 15A-D) or an isolated hepsin antibody disclosed in Xuan et al (2006) *Cancer Res* 66(7), 3611, or an hepsin antibody disclosed in WO2007/149932.

In one embodiment, an antibody of the invention does not bind to the same epitope on human hepsin as an anti-hepsin antibody described in Cancer Research, Volume 66, pages 3611-3619 published in 2006 (e.g., antibody 1A12, 85B11, 94A7, A6, A174, A21 and/or A24 as exemplified in Figure 4), or an isolated hepsin antibody disclosed in PCT Publications WO2004/033630 (e.g., antibody 47A5, 14C7, 46D12, 38E2, 37G10, 31C1, 11C1 and/or 72H6 referred to on page 93 and in Figures 15A-D) or an isolated hepsin antibody disclosed in Xuan et al (2006) *Cancer Res* 66(7), 3611, or an hepsin antibody disclosed in WO2007/149932.

In one aspect, the invention provides compositions comprising one or more antibodies of the invention and a carrier. In one embodiment, the carrier is pharmaceutically acceptable.

In another aspect, the invention provides nucleic acids encoding a hepsin antibody of the invention.

In yet another aspect, the invention provides vectors comprising a nucleic acid of the invention.

In a further aspect, the invention provides compositions comprising one or more nucleic acids of the invention and a carrier. In one embodiment, the carrier is pharmaceutically acceptable.

In one aspect, the invention provides host cells comprising a nucleic acid or a vector of the invention. A vector can be of any type, for example, a recombinant vector such as an expression vector. Any of a variety of host cells can be used. In one embodiment, a host cell is a prokaryotic cell, for example, *E. coli*. In another embodiment, a host cell is a eukaryotic cell, for example a mammalian cell such as Chinese Hamster Ovary (CHO) cell.

In a further aspect, the invention provides methods of making an antibody of the invention. For example, the invention provides methods of making an anti-hepsin antibody (which, as defined herein includes full length antibody and fragments thereof), said method comprising culturing a host cell comprising nucleic acid encoding the humanized antibody so that the nucleic acid is expressed. In some embodiments, the method further comprises recovering the antibody from the host cell culture. In some embodiments, the antibody is recovered from the host cell culture medium. In some embodiments, the method further comprises combining the recovered antibody with a pharmaceutically acceptable carrier, excipient, or carrier to prepare a pharmaceutical formulation comprising the humanized antibody. In some embodiments, the invention provides methods of making an anti-hepsin antibody, said method comprising selecting antibodies that bind a complex comprising (a) hepsin and (b) a serine protease inhibitor that binds hepsin S1 subsite. In some embodiments, hepsin present in the complex is inactivated. In some embodiments, the serine protease inhibitor is 3,4-dichloro-isocoumarin (DCI). In some embodiments, the serine protease inhibitor binds hepsin catalytic amino acid residues Ser195 and His 57, whereby hepsin is inactivated. In some embodiments, prior to selecting the antibody, the antibody is incubated with hepsin and the serine protease inhibitor. In some embodiments, the method further comprises the step of selecting antibodies that compete for hepsin binding with a Kunitz domain. In some embodiments, the Kunitz domain is KD1.

In one aspect, the invention provides an article of manufacture comprising a container; and a composition contained within the container, wherein the composition comprises one or more hepsin antibodies of the invention. In one embodiment, the composition comprises a nucleic acid of the invention. In another embodiment, a composition comprising an antibody further comprises a carrier, which in some embodiments is pharmaceutically acceptable. In one embodiment, an article of manufacture of the invention further comprises instructions for administering the composition (e.g., the antibody) to an individual (such as instructions for any of the methods described herein).

In another aspect, the invention provides a kit comprising a first container comprising a composition comprising one or more anti-hepsin antibodies of the invention; and a second

container comprising a buffer. In one embodiment, the buffer is pharmaceutically acceptable. In one embodiment, a composition comprising an antibody further comprises a carrier, which in some embodiments is pharmaceutically acceptable. In another embodiment, a kit further comprises instructions for administering the composition (e.g., the antibody) to an individual.

The hepsin pathway is involved in multiple biological and physiological functions, including, e.g., activation of HGF/c-met pathway, activation of MSP/Ron pathway, disruption/degradation of basement membrane, matrix degradation, etc. These functions are in turn often dysregulated in disorders such as cancer. Thus, in another aspect, the invention provides a method of inhibiting disruption/degradation of basement membrane and/or matrix degradation, said method comprising contacting a cell or tissue with an antagonist of the invention, whereby disruption/degradation of basement membrane and/or matrix degradation is inhibited. In yet another aspect, the invention provides a method of inhibiting disruption/degradation of basement membrane and/or matrix degradation, said method comprising administering to a cell, tissue, and/or subject with a condition associated with abnormal disruption/degradation of basement membrane and/or matrix degradation an antagonist of the invention, whereby disruption/degradation of basement membrane and/or matrix degradation is inhibited.

In one aspect, the invention provides a method for treating or preventing a disorder associated with increased hepsin activity, said method comprising administering to a subject in need of such treatment an effective amount of an antagonist of the invention, thereby effectively treating or preventing said disorder. In one embodiment, said disorder is cancer.

In a further aspect, the invention provides use of an anti-hepsin antibody of the invention in the preparation of a medicament for the therapeutic and/or prophylactic treatment of a disorder, such as a cancer, a tumor, and/or a cell proliferative disorder. In some embodiments, the cancer, a tumor, and/or a cell proliferative disorder is prostate cancer. In some embodiments, the cancer, a tumor, and/or a cell proliferative disorder is ovarian or renal cancer.

In one aspect, the invention provides use of a nucleic acid of the invention in the preparation of a medicament for the therapeutic and/or prophylactic treatment of a disorder, such as a cancer, a tumor, and/or a cell proliferative disorder. In some embodiments, the cancer, a tumor, and/or a cell proliferative disorder is prostate cancer. In some embodiments, the cancer, a tumor, and/or a cell proliferative disorder is ovarian or renal cancer.

In another aspect, the invention provides use of an expression vector of the invention in the preparation of a medicament for the therapeutic and/or prophylactic treatment of a

disorder, such as a cancer, a tumor, and/or a cell proliferative disorder. In some embodiments, the cancer, a tumor, and/or a cell proliferative disorder is prostate cancer. In some embodiments, the cancer, a tumor, and/or a cell proliferative disorder is ovarian or renal cancer.

In yet another aspect, the invention provides use of a host cell of the invention in the preparation of a medicament for the therapeutic and/or prophylactic treatment of a disorder, such as a cancer, a tumor, and/or a cell proliferative disorder. In some embodiments, the cancer, a tumor, and/or a cell proliferative disorder is prostate cancer. In some embodiments, the cancer, a tumor, and/or a cell proliferative disorder is ovarian or renal cancer.

In a further aspect, the invention provides use of an article of manufacture of the invention in the preparation of a medicament for the therapeutic and/or prophylactic treatment of a disorder, such as a cancer, a tumor, and/or a cell proliferative disorder. In some embodiments, the cancer, a tumor, and/or a cell proliferative disorder is prostate cancer. In some embodiments, the cancer, a tumor, and/or a cell proliferative disorder is ovarian or renal cancer.

In one aspect, the invention also provides use of a kit of the invention in the preparation of a medicament for the therapeutic and/or prophylactic treatment of a disorder, such as a cancer, a tumor, and/or a cell proliferative disorder. In some embodiments, the cancer, a tumor, and/or a cell proliferative disorder is prostate cancer. In some embodiments, the cancer, a tumor, and/or a cell proliferative disorder is ovarian or renal cancer.

The invention provides methods and compositions useful for modulating disorders associated with expression and/or signaling of hepsin, such as increased expression and/or signaling or undesired expression and/or signaling.

Methods of the invention can be used to affect any suitable pathological state. Exemplary disorders are described herein, and include a cancer selected from the group consisting of non-small cell lung cancer, ovarian cancer, thyroid cancer, testicular cancer, endometrial cancer, head and neck cancer (e.g., head and neck squamous cell carcinoma), brain cancer (e.g., neuroblastoma or meningioma), skin cancer (e.g., melanoma, basal cell carcinoma, or squamous cell carcinoma), bladder cancer (e.g., transitional cell carcinoma), breast carcinoma, gastric cancer, colorectal cancer (CRC), hepatocellular carcinoma, cervical cancer, lung cancer, pancreatic cancer, prostate cancer, and renal cancer, and endometrial cancer.

In one embodiment, a cell that is targeted in a method of the invention is a cancer cell. For example, a cancer cell can be one selected from the group consisting of a breast cancer

cell, a colorectal cancer cell, a lung cancer cell (e.g., a non-small cell lung cancer cell), a thyroid cancer cell, a multiple myeloma cell, a testicular cancer cell, a papillary carcinoma cell, a colon cancer cell, a pancreatic cancer cell, an ovarian cancer cell, a cervical cancer cell, a central nervous system cancer cell, an osteogenic sarcoma cell, a renal carcinoma cell, a hepatocellular carcinoma cell, a bladder cancer cell (e.g., a transitional cell carcinoma cell), a gastric carcinoma cell, a head and neck squamous carcinoma cell, a melanoma cell, a leukemia cell, an endometrial cancer cell, and a colon adenoma cell. In one embodiment, a cell that is targeted in a method of the invention is a hyperproliferative and/or hyperplastic cell. In another embodiment, a cell that is targeted in a method of the invention is a dysplastic cell. In yet another embodiment, a cell that is targeted in a method of the invention is a metastatic cell.

Methods of the invention can further comprise additional treatment steps. For example, in one embodiment, a method further comprises a step wherein a targeted cell and/or tissue (e.g., a cancer cell) is exposed to radiation treatment or a chemotherapeutic agent.

In one aspect, the invention provides methods comprising administration of an effective amount of an anti-hepsin antibody in combination with an effective amount of another therapeutic agent (such as an anti-angiogenesis agent, another antibody, a chemotherapeutic agent, a cytotoxic agent, an immunosuppressive agent, a prodrug, a cytokine, cytotoxic radiotherapy, a corticosteroid, an anti-emetic, a cancer vaccine, an analgesic, or a growth inhibitory agent). For example, anti-hepsin antibodies are used in combinations with an anti-cancer agent or an anti-angiogenic agent to treat various neoplastic or non-neoplastic conditions.

Depending on the specific cancer indication to be treated, the combination therapy of the invention can be combined with additional therapeutic agents, such as chemotherapeutic agents, or additional therapies such as radiotherapy or surgery. Many known chemotherapeutic agents can be used in the combination therapy of the invention. Preferably those chemotherapeutic agents that are standard for the treatment of the specific indications will be used. Dosage or frequency of each therapeutic agent to be used in the combination is preferably the same as, or less than, the dosage or frequency of the corresponding agent when used without the other agent(s).

In another aspect, the invention provides any of the anti-hepsin antibodies described herein, wherein the anti-hepsin antibody comprises a detectable label.

In another aspect, the invention provides a complex of any of the anti-hepsin antibodies described herein and hepsin. In some embodiments, the complex is in vivo or in vitro. In some embodiments, the complex comprises a cancer cell. In some embodiments, the anti-hepsin antibody is detectably labeled.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURES 1: Heavy chain and light chain HVR loop sequences of anti-hepsin antibody. The figure shows the heavy chain HVR sequences, H1, H2, and H3, and light chain HVR sequences, L1, L2, and L3. Sequence numbering is as follows:

Clone 25 (HVR-H1 is SEQ ID NO:4; HVR-H2 is SEQ ID NO:5; HVR-H3 is SEQ ID NO:6; HVR-L1 is SEQ ID NO:1; HVR-L2 is SEQ ID NO:2; HVR-L3 is SEQ ID NO:3);

Amino acid positions are numbered according to the Kabat numbering system as described below.

FIGURES 2A, 2B, and 3: depict exemplary acceptor human consensus framework sequences for use in practicing the instant invention with sequence identifiers as follows:

Variable heavy (VH) consensus frameworks (FIG. 2A, 2B)

human VH subgroup I consensus framework minus Kabat CDRs (SEQ ID NOS 21-23 & 16, respectively)

human VH subgroup I consensus framework minus extended hypervariable regions (SEQ ID NOS 24-25, 23 & 16; 24-26 & 16; and 24-25, 27 & 16, respectively)

human VH subgroup II consensus framework minus Kabat CDRs (SEQ ID NOS 28-30 & 16, respectively)

human VH subgroup II consensus framework minus extended hypervariable regions (SEQ ID NOS 31-32, 30 & 16; 31-33 & 16; and 31-32, 34 & 16, respectively)

human VH subgroup II consensus framework minus extended

human VH subgroup III consensus framework minus Kabat CDRs (SEQ ID NOS 35-37 & 16, respectively)

human VH subgroup III consensus framework minus extended hypervariable regions (SEQ ID NOS 14-15, 37 & 16; 14-15, 38 & 16; and 14-15, 43 & 16, respectively)

human VH acceptor framework minus Kabat CDRs (SEQ ID NOS 39, 36, 40 & 16, respectively)

human VH acceptor framework minus extended hypervariable regions (SEQ ID NOS 14-15, 40 & 16; and 14-15, 41 & 16, respectively)

human VH acceptor 2 framework minus Kabat CDRs (SEQ ID NOS 39, 36, 42 & 16, respectively)

human VH acceptor 2 framework minus extended hypervariable regions (SEQ ID NOS 14-15, 42 & 16; 14-15, 44 & 16; and 14-15, 48 & 16, respectively)

Variable light (VL) consensus frameworks (FIG. 3)

human VL kappa subgroup I consensus framework (SEQ ID NOS 17-20, respectively)

human VL kappa subgroup II consensus framework (SEQ ID NOS 49-51 & 20, respectively)

human VL kappa subgroup III consensus framework (SEQ ID NOS 52-54 & 20, respectively)

human VL kappa subgroup IV consensus framework (SEQ ID NOS 55-57 & 20, respectively)

FIGURE 4: depicts framework region sequences of huMAb4D5-8 light (SEQ ID NOS 17-18, 58 & 20, respectively, in order of appearance) and heavy chains (SEQ ID NOS 14-15, 48 & 16, respectively, in order of appearance). Numbers in superscript/bold indicate amino acid positions according to Kabat.

FIGURE 5: depicts modified/variant framework region sequences of huMAb4D5-8 light (SEQ ID NOS 17-20, respectively, in order of appearance) and heavy chains (SEQ ID NOS 14-15, 43 & 16, respectively, in order of appearance). Numbers in superscript/bold indicate amino acid positions according to Kabat.

FIGURE 6: depicts anti-hepsin antibody mab 25 heavy chain variable region (SEQ ID NO: 10 and light chain variable region (SEQ ID NO:9).

FIGURE 7: One embodiment of an amino acid sequence of native human hepsin.

FIGURE 8A & B: Another embodiment of an amino acid sequence of native human hepsin.

FIGURE 9: Inactivation of hepsin by 3,4-dichloro-isocoumarin (DCI). A. Concentration-dependent inhibition of enzyme activity towards S2765 substrate after a 40 min incubation of hepsin with increasing DCI concentrations. B. Chemical structure of DCI and its potential adducts with hepsin according to (Powers et al., 1989).

FIGURE 10: KD1 competes with anti-hepsin Fab-phage for binding to hepsin. Binding of Fab25-phage to hepsin in the presence of increasing concentrations of KD1.

FIGURE 11: Inhibition of human and murine hepsin enzymatic activity by Fab25. Human (hu) and murine (mu) hepsin were incubated with Fab25 or a control Fab (ctrl Fab) for 40 min before addition of S2765 substrate. The initial linear velocities were measured on a kinetic microplate reader and enzyme activity was expressed as fractional activity (v_i/v_o).

FIGURE 12: Specificity of Fab25. Fab25 (1 μM) was incubated with hepsin and 9 trypsin-like serine proteases for 40 min before addition of synthetic para-nitroanilide (pNA)

substrates. The initial linear velocities were measured on a kinetic microplate reader and enzyme activity was expressed as fractional activity (v_i/v_o).

FIGURE 13: Inhibition by Fab25 of macromolecular substrate processing by hepsin. A. Inhibition of pro-uPA activation by Fab25. The effect of Fab25 on hepsin-mediated processing of pro-uPA was determined in a two-stage enzymatic assay. The initial linear velocities were measured on a kinetic microplate reader and enzyme activity was expressed as fractional activity (v_i/v_o). By use of an uPA standard curve the uninhibited rate of uPA formation by hepsin was determined to be 0.81 ± 0.22 μM uPA/min (n=3). Inhibition of hepsin substrate processing by Fab25 was performed for 3 known substrates: B. factor VII (FVII); C. pro-HGF; and D. pro-MSP. Factor VII cleavage by hepsin was carried out for 0.5 h and 2.0 h in the presence or absence of Fab25 and a control Fab (ctrl Fab), while pro-HGF and pro-MSP cleavage experiments were performed for 30 min. Reaction aliquots were analyzed by SDS-PAGE (reducing conditions) and gels stained.

FIGURE 14: Effects of Fab25 integrity after prolonged exposure to hepsin. The CDR-H3 loop in Fab25 contains three lysine and one arginine residue which could be potentially cleaved by hepsin, but no proteolytic processing of Fab25 by hepsin was observed when incubated for 24 h either at pH 6.0 or pH 8.0. Proteolysis was monitored by gel mobility-shift on a 4-20% (w/v) polyacrylamide gradient gel and stained with Coomassie brilliant blue.

FIGURE 15: Inhibition by Fab25 of laminin-dependent migration of DU145 cells. A. DU145 cells ($2x10^4$) in serum-free Dulbecco's modified Eagle's medium were added to pretreated upper chambers of fluoroblok inserts and allowed to migrate for 5 h at 37 °C. After incubation, non-migratory cells and media were washed and those cells that migrated to the bottom of the filters were fixed, stained with the YO-PRO-I, and imaged using an inverted microscope. Representative images were taken of pretreated filters (laminin, laminin co-incubated with hepsin, laminin co-incubated with hepsin:Fab25 complex or PBS control) with fixed cells. B. Measure of relative fluorescence units (R.F.U) from cells stained with the YO-PRO substrates which are reference subtracted for PBS-wells. DU145 cells treated with hepsin processed laminin had marked increase in migration compared to cells which are treated with laminin alone. Lack of laminin processing by hepsin in presence of Fab25 renders DU145 to be less migratory.

FIGURE 16: A. Activation of pro-MSP by cell surface expressed hepsin in LnCap-34 cells. LnCap-34 cells which stably overexpress hepsin were serum starved and treated with 125I-pro-MSP alone or in combination with different inhibitors for 3 h. Recombinant hepsin

(10 nM) was used as a positive control. Significant increase in pro-MSP processing was observed after 3h compared to the start of the experiment. Inhibitors KQLR (SEQ ID NO: 12), KD1 and anti-hepsin antibody Fab25 effectively blocked the activation of pro-MSP. B. Activation of pro-HGF by cell surface expressed hepsin in LnCap-34 cells. LnCap-34 cells were treated with ^{125}I-pro-HGF in presence of varied Fab25 concentration (20 nM – 0.15 nM) and incubated for 3 hours. Recombinant hepsin (10 nM) was used as a positive control. Significant increase in pro-HGF processing was observed after 3h compared to the start of the experiment. Anti-hepsin antibody Fab25 effectively blocked the activation of pro-HGF in a concentration dependent manner.

FIGURE 17: A. Surface plasmon resonance was used to measure the binding affinity of Ab25 with active human hepsin. Serial dilutions of hepsin (0.39 nM to 200 nM) were injected to a CM5 biosensor chip with captured Ab25 for 3 minutes and the dissociation was monitored for 15 minutes. Fitting of the experimental data gave the equilibrium dissociation rate (K_D) of 10.6 nM. B. As binding of Ab25 to pro-hepsin displayed rapid kinetics, binding affinity was measured by steady state affinity measurements. Serial dilutions of pro-hepsin (195 nM to 80 μM) were injected to a CM5 biosensor chip with captured Ab25 for 2 minutes. The equilibrium dissociation rate (K_D = 5.52 μM) was determined by steady state analysis from a plot of R_{eq} against the concentration of pro-hepsin.

FIGURE 18: Isothermal titration calorimetric experiment of Fab25 binding to active hepsin. The association reaction is exothermic and the stoichiometry of binding is 1:1 as expected. The dissociation constant (K_D) from ITC is 6.1 nM that correlates excellently with the previous data from BIAcore. The enthalpy (ΔH) and entropy ($T\Delta S$) of binding are -27.5 kcal/mol and -16.3 kcal/mol showing that the binding is enthalpically driven with unfavorable entropy.

DETAILED DESCRIPTION OF THE INVENTION

The invention herein provides anti-hepsin antibodies that are useful for, e.g., treatment or prevention of disease states associated with expression and/or activity of hepsin, such as increased expression and/or activity or undesired expression and/or activity. In some embodiments, the antibodies of the invention are used to treat a tumor, a cancer, and/or a cell proliferative disorder.

In another aspect, the anti-hepsin antibodies of the invention find utility as reagents for detection and/or isolation of hepsin, such as detection of hepsin in various tissues and cell type.

The invention further provides methods of making and using anti-hepsin antibodies, and polynucleotides encoding anti-hepsin antibodies.

General techniques

The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized methodologies described in Sambrook et al., Molecular Cloning: A Laboratory Manual 3rd. edition (2001) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel, et al. eds., (2003)); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.): PCR 2: A PRACTICAL APPROACH (M. J. MacPherson, B. D. Hames and G. R. Taylor eds. (1995)), Harlow and Lane, eds. (1988) ANTIBODIES, A LABORATORY MANUAL, and ANIMAL CELL CULTURE (R. I. Freshney, ed. (1987)).

Definitions

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody *in situ* within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

An "isolated" nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the nucleic acid. An isolated nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated nucleic acid molecules therefore are distinguished from the nucleic acid molecule as it exists in natural cells. However, an isolated nucleic acid molecule includes a nucleic acid molecule contained in cells that ordinarily express the nucleic acid (for example, an antibody encoding nucleic

acid) where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The term "variable domain residue numbering as in Kabat" or "amino acid position numbering as in Kabat," and variations thereof, refers to the numbering system used for heavy chain variable domains or light chain variable domains of the compilation of antibodies in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991). Using this numbering system, the actual linear amino acid sequence may contain fewer or additional amino acids corresponding to a shortening of, or insertion into, a FR or CDR of the variable domain. For example, a heavy chain variable domain may include a single amino acid insert (residue 52a according to Kabat) after residue 52 of H2 and inserted residues (e.g. residues 82a, 82b, and 82c, etc according to Kabat) after heavy chain FR residue 82. The Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence.

The phrase "substantially similar," or "substantially the same," as used herein, denotes a sufficiently high degree of similarity between two numeric values (generally one associated with an antibody of the invention and the other associated with a reference/comparator antibody) such that one of skill in the art would consider the difference between the two values to be of little or no biological and/or statistical significance within the context of the biological characteristic measured by said values (e.g., Kd values). The difference between said two values is preferably less than about 50%, preferably less than about 40%, preferably less than about 30%, preferably less than about 20%, preferably less than about 10% as a function of the value for the reference/comparator antibody.

"Binding affinity" generally 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 (Kd). Desirably the Kd is 1×10^{-7}, 1×10^{-8}, 5×10^{-8}, 1×10^{-9}, 3×10^{-9}, 5×10^{-9}, or even 1×10^{-10} or stronger. Affinity can be measured by common methods known in the art, including those described herein. Low-affinity antibodies generally bind antigen slowly and tend to dissociate readily, whereas high-affinity antibodies generally bind antigen faster and tend to remain bound longer. A variety of methods of measuring binding affinity are known in the art, any of which can be used for

purposes of the present invention. Specific illustrative embodiments are described in the following.

In one embodiment, the "Kd" or "Kd value" according to this invention is measured by a radiolabeled antigen binding assay (RIA) performed with the Fab version of an antibody of interest and its antigen as described by the following assay that measures solution binding affinity of Fabs for antigen by equilibrating Fab with a minimal concentration of (^{125}I)-labeled antigen in the presence of a titration series of unlabeled antigen, then capturing bound antigen with an anti-Fab antibody-coated plate (Chen, et al., (1999) J. Mol. Biol. 293:865-881). To establish conditions for the assay, microtiter plates (Dynex) are coated overnight with 5 µg/ml of a capturing anti-Fab antibody (Cappel Labs) in 50 mM sodium carbonate (pH 9.6), and subsequently blocked with 2% (w/v) bovine serum albumin in PBS for two to five hours at room temperature (approximately 23°C). In a non-adsorbant plate (Nunc #269620), 100 pM or 26 pM [^{125}I]-antigen are mixed with serial dilutions of a Fab of interest (*e.g.*, consistent with assessment of an anti-VEGF antibody, Fab-12, in Presta et al., (1997) Cancer Res. 57:4593-4599). The Fab of interest is then incubated overnight; however, the incubation may continue for a longer period (*e.g.*, 65 hours) to insure that equilibrium is reached. Thereafter, the mixtures are transferred to the capture plate for incubation at room temperature (*e.g.*, for one hour). The solution is then removed and the plate washed eight times with 0.1% Tween-20 in PBS. When the plates have dried, 150 µl/well of scintillant (MicroScint-20; Packard) is added, and the plates are counted on a Topcount gamma counter (Packard) for ten minutes. Concentrations of each Fab that give less than or equal to 20% of maximal binding are chosen for use in competitive binding assays. According to another embodiment the Kd or Kd value is measured by using surface plasmon resonance assays using a BIAcore™-2000 or a BIAcore™-3000 (BIAcore, Inc., Piscataway, NJ) at 25°C with immobilized antigen CM5 chips at ~10 response units (RU). Briefly, carboxymethylated dextran biosensor chips (CM5, BIAcore Inc.) are activated with N-ethyl-N'- (3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) according to the supplier's instructions. Antigen is diluted with 10mM sodium acetate, pH 4.8, into 5µg/ml (~0.2µM) before injection at a flow rate of 5µl/minute to achieve approximately 10 response units (RU) of coupled protein. Following the injection of antigen, 1M ethanolamine is injected to block unreacted groups. For kinetics measurements, two-fold serial dilutions of Fab (0.78 nM to 500 nM) are injected in PBS with 0.05% Tween 20 (PBST) at 25°C at a flow rate of approximately 25µl/min. In some embodiments, the following modifications are used for the surface Plasmon resonance assay method: antibody

is immobilized to CM5 biosensor chips to achieve approximately 400 RU, and for kinetic measurements, two-fold serial dilutions of target protein (e.g., hepsin-IIIb or –IIIc) (starting from 67 nM) are injected in PBST buffer at 25°C with a flow rate of about 30 ul/minute. Association rates (k_{on}) and dissociation rates (k_{off}) are calculated using a simple one-to-one Langmuir binding model (BIAcore Evaluation Software version 3.2) by simultaneous fitting the association and dissociation sensorgram. The equilibrium dissociation constant (Kd) is calculated as the ratio k_{off}/k_{on}. See, e.g., Chen, Y., et al., (1999) J. Mol. Biol. 293:865-881. If the on-rate exceeds 10^6 M^{-1} S^{-1} by the surface plasmon resonance assay above, then the on-rate can be determined by using a fluorescent quenching technique that measures the increase or decrease in fluorescence emission intensity (excitation = 295 nm; emission = 340 nm, 16 nm band-pass) at 25°C of a 20nM anti-antigen antibody (Fab form) in PBS, pH 7.2, in the presence of increasing concentrations of antigen as measured in a spectrometer, such as a stop-flow equipped spectrophometer (Aviv Instruments) or a 8000-series SLM-Aminco spectrophotometer (ThermoSpectronic) with a stir red cuvette.

An "on-rate" or "rate of association" or "association rate" or "k_{on}" according to this invention can also be determined with the same surface plasmon resonance technique described above using a BIAcore™-2000 or a BIAcore™-3000 (BIAcore, Inc., Piscataway, NJ) at 25°C with immobilized antigen CM5 chips at ~10 response units (RU). Briefly, carboxymethylated dextran biosensor chips (CM5, BIAcore Inc.) are activated with N-ethyl-N'- (3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) according to the supplier's instructions. Antigen is diluted with 10mM sodium acetate, pH 4.8, into 5µg/ml (~0.2uM) before injection at a flow rate of 5µl/minute to achieve approximately 10 response units (RU) of coupled protein. Following the injection of antigen, 1M ethanolamine is injected to block unreacted groups. For kinetics measurements, two-fold serial dilutions of Fab (0.78 nM to 500 nM) are injected in PBS with 0.05% Tween 20 (PBST) at 25°C at a flow rate of approximately 25µl/min. In some embodiments, the following modifications are used for the surface Plasmon resonance assay method: antibody is immobilized to CM5 biosensor chips to achieve approximately 400 RU, and for kinetic measurements, two-fold serial dilutions of target protein (e.g., hepsin-IIIb or –IIIc) (starting from 67 nM) are injected in PBST buffer at 25°C with a flow rate of about 30 ul/minute. Association rates (k_{on}) and dissociation rates (k_{off}) are calculated using a simple one-to-one Langmuir binding model (BIAcore Evaluation Software version 3.2) by simultaneous fitting the association and dissociation sensorgram. The equilibrium dissociation constant (Kd) was calculated as the ratio k_{off}/k_{on}. See, e.g., Chen, Y., et al., (1999) J. Mol. Biol. 293:865-881.

However, if the on-rate exceeds 10^6 M^{-1} S^{-1} by the surface plasmon resonance assay above, then the on-rate is preferably determined by using a fluorescent quenching technique that measures the increase or decrease in fluorescence emission intensity (excitation = 295 nm; emission = 340 nm, 16 nm band-pass) at 25°C of a 20nM anti-antigen antibody (Fab form) in PBS, pH 7.2, in the presence of increasing concentrations of antigen as measured in a spectrometer, such as a stop-flow equipped spectrophometer (Aviv Instruments) or a 8000-series SLM-Aminco spectrophotometer (ThermoSpectronic) with a stir red cuvette.

The term "vector," as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid," which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a phage vector. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "recombinant vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector.

"Polynucleotide," or "nucleic acid," as used interchangeably herein, refer to polymers of nucleotides of any length, and include DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase, or by a synthetic reaction. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and their analogs. If present, modification to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after synthesis, such as by conjugation with a label. Other types of modifications include, for example, "caps," substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (*e.g.*, methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.) and with

charged linkages (*e.g.*, phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, ply-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide(s). Further, any of the hydroxyl groups ordinarily present in the sugars may be replaced, for example, by phosphonate groups, phosphate groups, protected by standard protecting groups, or activated to prepare additional linkages to additional nucleotides, or may be conjugated to solid or semi-solid supports. The 5' and 3' terminal OH can be phosphorylated or substituted with amines or organic capping group moieties of from 1 to 20 carbon atoms. Other hydroxyls may also be derivatized to standard protecting groups. Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, for example, 2'-O-methyl-, 2'-O-allyl, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, alpha-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and a basic nucleoside analogs such as methyl riboside. One or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S ("thioate"), P(S)S ("dithioate"), (O)NR_2 ("amidate"), P(O)R, P(O)OR', CO or CH_2 ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (-O-) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.

"Oligonucleotide," as used herein, generally refers to short, generally single stranded, generally synthetic polynucleotides that are generally, but not necessarily, less than about 200 nucleotides in length. The terms "oligonucleotide" and "polynucleotide" are not mutually exclusive. The description above for polynucleotides is equally and fully applicable to oligonucleotides.

"Percent (%) amino acid sequence identity" with respect to a peptide or 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 specific peptide or 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 measuring alignment, 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, wherein the complete source code for the ALIGN-2 program is provided in Table A below. 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 through Genentech, Inc., South San Francisco, California or may be compiled from the source code provided in, e.g., WO2007/001851. The ALIGN-2 program should be compiled for use on a UNIX operating system, preferably 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 follows:

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.

In some embodiments, two or more amino acid sequences are at least 50%, 60%, 70%, 80%, or 90% identical. In some embodiments, two or more amino acid sequences are at least 95%, 97%, 98%, 99%, or even 100% identical. 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 "hepsin," as used herein, refers, unless specifically or contextually indicated otherwise, to any native or variant hepsin polypeptide. The term "native sequence" specifically encompasses naturally occurring truncated forms (e.g., an extracellular domain sequence or a transmembrane subunit sequence), naturally occurring variant forms (e.g., alternatively spliced forms) and naturally-occurring allelic variants. The term "wild-type hepsin" generally refers to a polypeptide comprising an amino acid sequence of a naturally occurring hepsin protein. The term "wild type hepsin sequence" generally refers to an amino acid sequence found in a naturally occurring hepsin. In one embodiment, a native sequence hepsin polypeptide comprises the amino acid sequence of SEQ ID NO:46 (see Figure 7). In one embodiment, a native sequence hepsin polypeptide comprises the amino acid sequence of SEQ ID NO:47 (see Figure 8)

"Hepsin polypeptide variant", or variations thereof, means a hepsin polypeptide, generally an active hepsin polypeptide, as defined herein having at least about 80% amino acid sequence identity with any of the native sequence hepsin polypeptide sequences as disclosed herein. Such hepsin polypeptide variants include, for instance, hepsin polypeptides wherein one or more amino acid residues are added, or deleted, at the N– or C-terminus of a native amino acid sequence. Ordinarily, a hepsin polypeptide variant will have at least about 80% amino acid sequence identity, alternatively at least about 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% amino acid sequence identity, to a native sequence hepsin polypeptide sequence as disclosed herein. Ordinarily, hepsin variant polypeptides are at least about 10 amino acids in length, alternatively at least about 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600 amino acids in length, or more. Optionally, hepsin variant polypeptides will have no more than one conservative amino acid substitution as compared to a native hepsin polypeptide sequence, alternatively no more than 2, 3, 4, 5, 6, 7, 8, 9, or 10 conservative amino acid substitution as compared to the native hepsin polypeptide sequence.

A "tyrosine kinase inhibitor" is a molecule which inhibits to some extent tyrosine kinase activity of a tyrosine kinase such as a hepsin receptor.

To "inhibit" is to decrease or reduce an activity, function, and/or amount as compared to a reference.

Protein "expression" refers to conversion of the information encoded in a gene into messenger RNA (mRNA) and then to the protein.

Herein, a sample or cell that "expresses" a protein of interest (such as hepsin) is one in which mRNA encoding the protein, or the protein, including fragments thereof, is determined to be present in the sample or cell.

An " immunoconjugate" (interchangeably referred to as "antibody-drug conjugate," or "ADC") means an antibody conjugated to one or more cytotoxic agents, such as a chemotherapeutic agent, a drug, a growth inhibitory agent, a toxin (e.g., a protein toxin, an enzymatically active toxin of bacterial, fungal, plant, or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate).

A "blocking" antibody or an antibody "antagonist" is one which inhibits or reduces biological activity of the antigen it binds. Preferred blocking antibodies or antagonist antibodies completely inhibit the biological activity of the antigen.

A "naked antibody" is an antibody that is not conjugated to a heterologous molecule, such as a cytotoxic moiety or radiolabel.

An antibody having a "biological characteristic" of a designated antibody is one which possesses one or more of the biological characteristics of that antibody which distinguish it from other antibodies that bind to the same antigen.

In order to screen for antibodies which bind to an epitope on an antigen bound by an antibody of interest, a routine cross-blocking assay such as that described in Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed.

To increase the half-life of the antibodies or polypeptide containing the amino acid sequences of this invention, one can attach a salvage receptor binding epitope to the antibody (especially an antibody fragment), as described, e.g., in US Patent 5,739,277. For example, a nucleic acid molecule encoding the salvage receptor binding epitope can be linked in frame to a nucleic acid encoding a polypeptide sequence of this invention so that the fusion protein expressed by the engineered nucleic acid molecule comprises the salvage receptor binding epitope and a polypeptide sequence of this invention. As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (*e.g.*, IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the *in vivo* serum half-life of the IgG molecule (e.g., Ghetie et al., *Ann. Rev. Immunol.* 18:739-766 (2000), Table 1). Antibodies with substitutions in an Fc region thereof and increased serum half-lives are also described in WO00/42072, WO 02/060919; Shields et al., *J. Biol. Chem.* 276:6591-6604 (2001); Hinton,

J. Biol. Chem. 279:6213-6216 (2004)). In another embodiment, the serum half-life can also be increased, for example, by attaching other polypeptide sequences. For example, antibodies or other polypeptides useful in the methods of the invention can be attached to serum albumin or a portion of serum albumin that binds to the FcRn receptor or a serum albumin binding peptide so that serum albumin binds to the antibody or polypeptide, e.g., such polypeptide sequences are disclosed in WO01/45746. In one preferred embodiment, the serum albumin peptide to be attached comprises an amino acid sequence of DICLPRWGCLW (SEQ ID NO: 13). In another embodiment, the half-life of a Fab is increased by these methods. *See also*, Dennis et al. *J. Biol. Chem.* 277:35035-35043 (2002) for serum albumin binding peptide sequences.

By "fragment" is meant a portion of a polypeptide or nucleic acid molecule that contains, preferably, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or more of the entire length of the reference nucleic acid molecule or polypeptide. A fragment may contain 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100, 200, 300, 400, 500, 600, or more nucleotides or 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180, 190, 200 amino acids or more.

The terms "antibody" and "immunoglobulin" are used interchangeably in the broadest sense and include monoclonal antibodies (e.g., full length or intact monoclonal antibodies), polyclonal antibodies, multivalent antibodies, multispecific antibodies (e.g., bispecific antibodies so long as they exhibit the desired biological activity) and may also include certain antibody fragments (as described in greater detail herein). An antibody can be human, humanized, and/or affinity matured.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a β-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat *et al.*, *Sequences of Proteins of Immunological Interest*,

Fifth Edition, National Institute of Health, Bethesda, MD (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. In a two-chain Fv species, this region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv species, one heavy- and one light-chain variable domain can be covalently linked by a flexible peptide linker such that the light and heavy chains can associate in a "dimeric" structure analogous to that in a two-chain Fv species. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the VH-VL dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these can be further divided into subclasses (isotypes), e.g., IgG_1, IgG_2, IgG_3, IgG_4, IgA_1, and IgA_2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of

different classes of immunoglobulins are well known. "Antibody fragments" comprise only a portion of an intact antibody, wherein the portion preferably retains at least one, preferably most or all, of the functions normally associated with that portion when present in an intact antibody. Examples of antibody fragments include Fab, Fab', F(ab')2, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. In one embodiment, an antibody fragment comprises an antigen binding site of the intact antibody and thus retains the ability to bind antigen. In another embodiment, an antibody fragment, for example one that comprises the Fc region, retains at least one of the biological functions normally associated with the Fc region when present in an intact antibody, such as FcRn binding, antibody half life modulation, ADCC function and complement binding. In one embodiment, an antibody fragment is a monovalent antibody that has an in vivo half life substantially similar to an intact antibody. For e.g., such an antibody fragment may comprise on antigen binding arm linked to an Fc sequence capable of conferring in vivo stability to the fragment.

The term "hypervariable region," "HVR," or "HV," when used herein refers to the regions of an antibody variable domain which are hypervariable in sequence and/or form structurally defined loops. Generally, antibodies comprise six hypervariable regions; three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). A number of hypervariable region delineations are in use and are encompassed herein. The Kabat Complementarity Determining Regions (CDRs) are based on sequence variability and are the most commonly used (Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). Chothia refers instead to the location of the structural loops (Chothia and Lesk, J. Mol. Biol. 196:901-917 (1987)). The AbM hypervariable regions represent a compromise between the Kabat CDRs and Chothia structural loops, and are used by Oxford Molecular's AbM antibody modeling software. The "contact" hypervariable regions are based on an analysis of the available complex crystal structures. The residues from each of these hypervariable regions are noted below.

Loop	Kabat	AbM	Chothia	Contact
L1	L24-L34	L24-L34	L26-L32	L30-L36
L2	L50-L56	L50-L56	L50-L52	L46-L55
L3	L89-L97	L89-L97	L91-L96	L89-L96
H1	H31-H35B	H26-H35B	H26-H32	H30-H35B

(Kabat Numbering)

H1	H31-H35	H26-H35	H26-H32	H30-H35
		(Chothia Numbering)		
H2	H50-H65	H50-H58	H53-H55	H47-H58
H3	H95-H102	H95-H102	H96-H101	H93-H101

Hypervariable regions may comprise "extended hypervariable regions" as follows: 24-36 or 24-34 (L1), 46-56 or 50-56 (L2) and 89-97 (L3) in the VL and 26-35 (H1), 50-65 or 49-65 (H2) and 93-102, 94-102 or 95-102 (H3) in the VH. The variable domain residues are numbered according to Kabat *et al.*, *supra* for each of these definitions.

"Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally will also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., Nature 321:522-525 (1986); Riechmann et al., Nature 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol. 2:593-596 (1992). See also the following review articles and references cited therein: Vaswani and Hamilton, Ann. Allergy, Asthma & Immunol. 1:105-115 (1998); Harris, Biochem. Soc. Transactions 23:1035-1038 (1995); Hurle and Gross, Curr. Op. Biotech. 5:428-433 (1994).

"Chimeric" antibodies (immunoglobulins) have a portion of the heavy and/or light chain identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder

of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; and Morrison *et al., Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)). Humanized antibody as used herein is a subset of chimeric antibodies.

"Single-chain Fv" or "scFv" antibody fragments comprise the VH and VL domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the scFv polypeptide further comprises a polypeptide linker between the VH and VL domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv see Pluckthun, in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

An "antigen" is a predetermined antigen to which an antibody can selectively bind. The target antigen may be polypeptide, carbohydrate, nucleic acid, lipid, hapten or other naturally occurring or synthetic compound. Preferably, the target antigen is a polypeptide.

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) in the same polypeptide chain (VH - VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger *et al., Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993).

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues.

An "affinity matured" antibody is one with one or more alterations in one or more CDRs thereof which result in an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). Preferred affinity matured antibodies will have nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies are produced by procedures known in the art. Marks et al. Bio/Technology 10:779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of CDR and/or framework residues is described by: Barbas et al., Proc Nat. Acad. Sci, USA 91:3809-3813 (1994); Schier et al., Gene 169:147-155

(1995); Yelton et al., J. Immunol. 155:1994-2004 (1995); Jackson et al., J. Immunol. 154(7):3310-9 (1995); and Hawkins et al., J. Mol. Biol. 226:889-896 (1992).

Antibody "effector functions" refer to those biological activities attributable to the Fc region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody, and vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity; 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.

"Antibody-dependent cell-mediated cytotoxicity" or "ADCC" refers to a form of cytotoxicity in which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (e.g., Natural Killer (NK) cells, neutrophils, and macrophages) enable these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins. The antibodies "arm" the cytotoxic cells and are absolutely required for such killing. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an *in vitro* ADCC assay, such as that described in US Patent No. 5,500,362 or 5,821,337 or Presta U.S. Patent No. 6,737,056 may be performed. 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 et al., *PNAS (USA)* 95:652-656 (1998).

"Human effector cells" are leukocytes which express one or more FcRs and perform effector functions. Preferably, the cells express at least FcγRIII and perform ADCC effector function. Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils; with PBMCs and NK cells being preferred. The effector cells may be isolated from a native source, *e.g.*, from blood.

"Fc receptor" or "FcR" describes a receptor that binds to the Fc region of an antibody. The preferred FcR is a native sequence human FcR. Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of these receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an

"inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. (see review M. in Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed in Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991); Capel *et al.*, *Immunomethods* 4:25-34 (1994); and de Haas et al., *J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer *et al.*, *J. Immunol.* 117:587 (1976) and Kim et al., *J. Immunol.* 24:249 (1994)) and regulates homeostasis of immunoglobulins. WO 00/42072 (Presta) describes antibody variants with improved or diminished binding to FcRs. The content of that patent publication is specifically incorporated herein by reference. See, also, Shields et al., *J. Biol. Chem.* 9(2): 6591-6604 (2001).

Methods of measuring binding to FcRn are known (see, e.g., Ghetie 1997, Hinton 2004). Binding to human FcRn in vivo and serum half life of human FcRn high affinity binding polypeptides can be assayed, e.g., in transgenic mice or transfected human cell lines expressing human FcRn, or in primates administered with the Fc variant polypeptides.

"Complement dependent cytotoxicity" or "CDC" refers to the lysis of a target cell in the presence of complement. Activation of the classical complement pathway is initiated by the binding of the first component of the complement system (C1q) to antibodies (of the appropriate subclass) which are bound to their cognate antigen. To assess complement activation, a CDC assay, *e.g.*, as described in Gazzano-Santoro et al., *J. Immunol. Methods* 202:163 (1996), may be performed.

Polypeptide variants with altered Fc region amino acid sequences and increased or decreased C1q binding capability are described in US patent No. 6,194,551B1 and WO 99/51642. The contents of those patent publications are specifically incorporated herein by reference. See, also, Idusogie et al., *J. Immunol.* 164:4178-4184 (2000).

The term "Fc region-comprising polypeptide" refers to a polypeptide, such as an antibody or immunoadhesin, which comprises an Fc region. The C-terminal lysine (residue 447 according to the EU numbering system) of the Fc region may be removed, for example, during purification of the polypeptide or by recombinant engineering the nucleic acid encoding the polypeptide. Accordingly, a composition comprising a polypeptide having an

Fc region according to this invention can comprise polypeptides with K447, with all K447 removed, or a mixture of polypeptides with and without the K447 residue.

An "acceptor human framework" for the purposes herein is a framework comprising the amino acid sequence of a VL or VH framework derived from a human immunoglobulin framework, or from a human consensus framework. An acceptor human framework "derived from" a human immunoglobulin framework or human consensus framework may comprise the same amino acid sequence thereof, or may contain pre-existing amino acid sequence changes. Where pre-existing amino acid changes are present, preferably no more than 5 and preferably 4 or less, or 3 or less, pre-existing amino acid changes are present. Where pre-existing amino acid changes are present in a VH, preferably those changes are only at three, two, or one of positions 71H, 73H, and 78H; for instance, the amino acid residues at those positions may be 71A, 73T, and/or 78A. In one embodiment, the VL acceptor human framework is identical in sequence to the VL human immunoglobulin framework sequence or human consensus framework sequence.

A "human consensus framework" is a framework which represents the most commonly occurring amino acid residue in a selection of human immunoglobulin 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. In one embodiment, for the VL, the subgroup is subgroup kappa I as in Kabat et al. In one embodiment, for the VH, the subgroup is subgroup III as in Kabat et al.

A "VH subgroup III consensus framework" comprises the consensus sequence obtained from the amino acid sequences in variable heavy subgroup III of Kabat et al. In one embodiment, the VH subgroup III consensus framework amino acid sequence comprises at least a portion or all of each of the following sequences: EVQLVESGGGLVQPGGSLRLSCAAS (SEQ ID NO: 14)-H1-WVRQAPGKGLEWV (SEQ ID NO: 15)-H2-RFTISRDNSKNTLYLQMNSLRAEDTAVYYC (SEQ ID NO: 43)-H3-WGQGTLVTVSS (SEQ ID NO: 16).

A "VL subgroup I consensus framework" comprises the consensus sequence obtained from the amino acid sequences in variable light kappa subgroup I of Kabat et al. In one embodiment, the VH subgroup I consensus framework amino acid sequence comprises at least a portion or all of each of the following sequences:

DIQMTQSPSSLSASVGDRVTITC (SEQ ID NO: 17)-L1-WYQQKPGKAPKLLIY (SEQ ID NO: 18)-L2-GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC (SEQ ID NO: 19)-L3-FGQGTKVEIK (SEQ ID NO: 20).

As used herein, "antibody mutant" or "antibody variant" refers to an amino acid sequence variant of an antibody wherein one or more of the amino acid residues of the species-dependent antibody have been modified. Such mutants necessarily have less than 100% sequence identity or similarity with the species-dependent antibody. In one embodiment, the antibody mutant will have an amino acid sequence having at least 75% amino acid sequence identity or similarity with the amino acid sequence of either the heavy or light chain variable domain of the species-dependent antibody, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, and most preferably at least 95%. Identity or similarity with respect to this sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical (i.e. same residue) or similar (i.e. amino acid residue from the same group based on common side-chain properties, see below) with the species-dependent antibody residues, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. None of N-terminal, C-terminal, or internal extensions, deletions, or insertions into the antibody sequence outside of the variable domain shall be construed as affecting sequence identity or similarity

A "disorder" or "disease" is any condition that would benefit from treatment with a substance/molecule or method of the invention. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include malignant and benign tumors; carcinoma, blastoma, and sarcoma.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already having a benign, pre-cancerous, or non-metastatic tumor as well as those in which the occurrence or recurrence of cancer is to be prevented.

The term "therapeutically effective amount" refers to an amount of a therapeutic agent to treat or prevent a disease or disorder in a mammal. In the case of cancers, the therapeutically effective amount of the therapeutic agent may reduce the number of cancer cells; reduce the primary tumor size; inhibit (i.e., slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (i.e., slow to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent

one or more of the symptoms associated with the disorder. To the extent the drug may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. For cancer therapy, efficacy in vivo can, for example, be measured by assessing the duration of survival, time to disease progression (TTP), the response rates (RR), duration of response, and/or quality of life.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Included in this definition are benign and malignant cancers. By "early stage cancer" or "early stage tumor" is meant a cancer that is not invasive or metastatic or is classified as a Stage 0, I, or II cancer. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma (including medulloblastoma and retinoblastoma), sarcoma (including liposarcoma and synovial cell sarcoma), neuroendocrine tumors (including carcinoid tumors, gastrinoma, and islet cell cancer), mesothelioma, schwannoma (including acoustic neuroma), meningioma, adenocarcinoma, melanoma, and leukemia or lymphoid malignancies. More particular examples of such cancers include squamous cell cancer (*e.g.* epithelial squamous cell cancer), lung cancer including small-cell lung cancer (SCLC), non-small cell lung cancer (NSCLC), adenocarcinoma of the lung and squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer (including metastatic breast cancer), colon cancer, rectal cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, testicular cancer, esophageal cancer, tumors of the biliary tract, as well as head and neck cancer and multiple myeloma.

The term "pre-cancerous" refers to a condition or a growth that typically precedes or develops into a cancer. A "pre-cancerous" growth will have cells that are characterized by abnormal cell cycle regulation, proliferation, or differentiation, which can be determined by markers of cell cycle regulation, cellular proliferation, or differentiation.

By "dysplasia" is meant any abnormal growth or development of tissue, organ, or cells. Preferably, the dysplasia is high grade or precancerous.

By "metastasis" is meant the spread of cancer from its primary site to other places in the body. Cancer cells can break away from a primary tumor, penetrate into lymphatic and blood vessels, circulate through the bloodstream, and grow in a distant focus (metastasize) in normal tissues elsewhere in the body. Metastasis can be local or distant. Metastasis is a

sequential process, contingent on tumor cells breaking off from the primary tumor, traveling through the bloodstream, and stopping at a distant site. At the new site, the cells establish a blood supply and can grow to form a life-threatening mass.

Both stimulatory and inhibitory molecular pathways within the tumor cell regulate this behavior, and interactions between the tumor cell and host cells in the distant site are also significant.

By "non-metastatic" is meant a cancer that is benign or that remains at the primary site and has not penetrated into the lymphatic or blood vessel system or to tissues other than the primary site. Generally, a non-metastatic cancer is any cancer that is a Stage 0, I, or II cancer, and occasionally a Stage III cancer.

By "primary tumor" or "primary cancer" is meant the original cancer and not a metastatic lesion located in another tissue, organ, or location in the subject's body.

By "benign tumor" or "benign cancer" is meant a tumor that remains localized at the site of origin and does not have the capacity to infiltrate, invade, or metastasize to a distant site.

By "tumor burden" is meant the number of cancer cells, the size of a tumor, or the amount of cancer in the body. Tumor burden is also referred to as tumor load.

By "tumor number" is meant the number of tumors.

By "subject" is meant a mammal, including, but not limited to, a human or non-human mammal, such as a bovine, equine, canine, ovine, or feline. Preferably, the subject is a human.

The term "anti-cancer therapy" refers to a therapy useful in treating cancer. Examples of anti-cancer therapeutic agents include, but are limited to, e.g., chemotherapeutic agents, growth inhibitory agents, cytotoxic agents, agents used in radiation therapy, anti-angiogenesis agents, apoptotic agents, anti-tubulin agents, and other agents to treat cancer , anti-CD20 antibodies, platelet derived growth factor inhibitors (e.g., Gleevec™ (Imatinib Mesylate)), a COX-2 inhibitor (e.g., celecoxib), interferons, cytokines, antagonists (e.g., neutralizing antibodies) that bind to one or more of the following targets ErbB2, ErbB3, ErbB4, PDGFR-beta, BlyS, APRIL, BCMA or VEGF receptor(s), TRAIL/Apo2, and other bioactive and organic chemical agents, etc. Combinations thereof are also included in the invention.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (*e.g.*, I^{131}, I^{125}, Y^{90} and Re^{186}), chemotherapeutic agents, and

toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and CYTOXAN® cyclosphosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethiylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; antibiotics such as the enediyne antibiotics (e. g., calicheamicin, especially calicheamicin gammaII and calicheamicin omegaII (see, e.g., Agnew, *Chem Intl. Ed. Engl.*, 33: 183-186 (1994)); dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, carminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN® doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate,

epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2- ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, OR); razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g., TAXOL® paclitaxel (Bristol- Myers Squibb Oncology, Princeton, N.J.), ABRAXANE™ Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel (American Pharmaceutical Partners, Schaumberg, Illinois), and TAXOTERE® doxetaxel (Rhône-Poulenc Rorer, Antony, France); chloranbucil; GEMZAR® gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; NAVELBINE® vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; irinotecan (Camptosar, CPT-11) (including the treatment regimen of irinotecan with 5-FU and leucovorin); topoisomerase inhibitor RFS 2000; difluorometlhylornithine (DMFO); retinoids such as retinoic acid; capecitabine; combretastatin; VELCADE bortezomib; REVLIMID lenalidomide; leucovorin (LV); oxaliplatin, including the oxaliplatin treatment regimen (FOLFOX); inhibitors of PKC-alpha, Raf, H-Ras, EGFR (e.g., erlotinib (Tarceva™)) and VEGF-A that reduce cell proliferation and pharmaceutically acceptable salts, acids or derivatives of any of the above.

Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens and selective estrogen receptor modulators (SERMs), including, for example, tamoxifen (including NOLVADEX® tamoxifen), raloxifene, droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and FARESTON· toremifene; aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, such as, for example, 4(5)-imidazoles, aminoglutethimide, MEGASE® megestrol acetate, AROMASIN® exemestane, formestanie, fadrozole, RIVISOR® vorozole, FEMARA® letrozole, and ARIMIDEX® anastrozole; and

anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; as well as troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); antisense oligonucleotides, particularly those which inhibit expression of genes in signaling pathways implicated in abherant cell proliferation, such as, for example, PKC-alpha, Raf and H-Ras; ribozymes such as a VEGF expression inhibitor (e.g., ANGIOZYME® ribozyme) and a HER2 expression inhibitor; vaccines such as gene therapy vaccines, for example, ALLOVECTIN® vaccine, LEUVECTIN® vaccine, and VAXID® vaccine; PROLEUKIN® rIL-2; LURTOTECAN® topoisomerase 1 inhibitor; ABARELIX® rmRH; Vinorelbine and Esperamicins (see U.S. Pat. No. 4,675,187), and pharmaceutically acceptable salts, acids or derivatives of any of the above.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions*, 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella et al., "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt et al., (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

By "radiation therapy" is meant the use of directed gamma rays or beta rays to induce sufficient damage to a cell so as to limit its ability to function normally or to destroy the cell altogether. It will be appreciated that there will be many ways known in the art to determine the dosage and duration of treatment. Typical treatments are given as a one time administration and typical dosages range from 10 to 200 units (Grays) per day.

A "biological sample" (interchangeably termed "sample" or "tissue or cell sample") encompasses a variety of sample types obtained from an individual and can be used in a diagnostic or monitoring assay. The definition encompasses blood and other liquid samples of biological origin, solid tissue samples such as a biopsy specimen or tissue cultures or cells

derived therefrom, and the progeny thereof. The definition also includes samples that have been manipulated in any way after their procurement, such as by treatment with reagents, solubilization, or enrichment for certain components, such as proteins or polynucleotides, or embedding in a semi-solid or solid matrix for sectioning purposes. The term "biological sample" encompasses a clinical sample, and also includes cells in culture, cell supernatants, cell lysates, serum, plasma, biological fluid, and tissue samples. The source of the biological sample may be solid tissue as from a fresh, frozen and/or preserved organ or tissue sample or biopsy or aspirate; blood or any blood constituents; bodily fluids such as cerebral spinal fluid, amniotic fluid, peritoneal fluid, or interstitial fluid; cells from any time in gestation or development of the individual. In some embodiments, the biological sample is obtained from a primary or metastatic tumor. The biological sample may contain compounds which are not naturally intermixed with the tissue in nature such as preservatives, anticoagulants, buffers, fixatives, nutrients, antibiotics, or the like.

For the purposes herein a "section" of a tissue sample is meant a single part or piece of a tissue sample, e.g., a thin slice of tissue or cells cut from a tissue sample. It is understood that multiple sections of tissue samples may be taken and subjected to analysis according to the present invention. In some embodiments, the same section of tissue sample is analyzed at both morphological and molecular levels, or is analyzed with respect to both protein and nucleic acid.

The word "label" when used herein refers to a compound or composition which is conjugated or fused directly or indirectly to a reagent such as a nucleic acid probe or an antibody and facilitates detection of the reagent to which it is conjugated or fused. The label may itself be detectable (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

Compositions of the invention and methods of making and using same

This invention encompasses compositions, including pharmaceutical compositions, comprising an anti-hepsin antibody; and polynucleotides comprising sequences encoding an anti-hepsin antibody. As used herein, compositions comprise one or more antibodies that bind to hepsin, and/or one or more polynucleotides comprising sequences encoding one or more antibodies that bind to hepsin. These compositions may further comprise suitable carriers, such as pharmaceutically acceptable excipients including buffers, which are well known in the art.

The invention also encompasses isolated antibody and polynucleotide embodiments. The invention also encompasses substantially pure antibody and polynucleotide embodiments.

The invention also encompasses method of treating a disorder, e.g. prostate cancer, using an anti-hepsin antibody (as described herein or as known in the art).

Compositions

The anti-hepsin antibodies of the invention are preferably monoclonal. Also encompassed within the scope of the invention are Fab, Fab', Fab'-SH and $F(ab')_2$ fragments of the anti-hepsin antibodies provided herein. These antibody fragments can be created by traditional means, such as enzymatic digestion, or may be generated by recombinant techniques. Such antibody fragments may be chimeric or humanized. These fragments are useful for the diagnostic and therapeutic purposes set forth below.

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

The anti-hepsin monoclonal antibodies of the invention can be made using the hybridoma method first described by Kohler *et al., Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Antibodies to hepsin may be raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of hepsin and an adjuvant. Hepsin may be prepared using methods well-known in the art, some of which are further described herein. For example, recombinant production of human and mouse hepsin is described below. In one embodiment, animals are immunized with a hepsin fused to the Fc portion of an immunoglobulin heavy chain. In a preferred embodiment, animals are immunized with a hepsin-IgG1 fusion protein. Animals ordinarily are immunized against immunogenic conjugates or derivatives of hepsin with monophosphoryl lipid A (MPL)/trehalose dicrynomycolate (TDM) (Ribi Immunochem. Research, Inc., Hamilton, MT) and the solution is injected intradermally at multiple sites. Two weeks later the animals are boosted. 7 to 14 days later animals are bled and the serum is assayed for anti-hepsin titer. Animals are boosted until titer plateaus.

Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against hepsin. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoadsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson *et al.*, *Anal. Biochem.*, 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

The anti-hepsin antibodies of the invention can be made by using combinatorial libraries to screen for synthetic antibody clones with the desired activity or activities. In principle, synthetic antibody clones are selected by screening phage libraries containing phage that display various fragments of antibody variable region (Fv) fused to phage coat protein. Such phage libraries are panned by affinity chromatography against the desired antigen. Clones expressing Fv fragments capable of binding to the desired antigen are adsorbed to the antigen and thus separated from the non-binding clones in the library. The binding clones are then eluted from the antigen, and can be further enriched by additional cycles of antigen adsorption/elution. Any of the anti-hepsin antibodies of the invention can be obtained by designing a suitable antigen screening procedure to select for the phage clone of interest followed by construction of a full length anti-hepsin antibody clone using the Fv sequences from the phage clone of interest and suitable constant region (Fc) sequences described in Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, Fifth Edition, NIH Publication 91-3242, Bethesda MD (1991), vols. 1-3. An exemplary method for generating anti-hepsin antibodies is disclosed in the Examples.

The antigen-binding domain of an antibody is formed from two variable (V) regions of about 110 amino acids, one each from the light (VL) and heavy (VH) chains, that both present three hypervariable loops or complementarity-determining regions (CDRs). Variable domains can be displayed functionally on phage, either as single-chain Fv (scFv) fragments, in which VH and VL are covalently linked through a short, flexible peptide, or as Fab fragments, in which they are each fused to a constant domain and interact non-covalently, as described in Winter *et al.*, *Ann. Rev. Immunol.*, 12: 433-455 (1994). As used herein, scFv encoding phage clones and Fab encoding phage clones are collectively referred to as "Fv phage clones" or "Fv clones".

Repertoires of VH and VL genes can be separately cloned by polymerase chain reaction (PCR) and recombined randomly in phage libraries, which can then be searched for antigen-binding clones as described in Winter *et al.*, *Ann. Rev. Immunol.*, 12: 433-455 (1994). Libraries from immunized sources provide high-affinity antibodies to the immunogen without the requirement of constructing hybridomas. Alternatively, the naive repertoire can be cloned to provide a single source of human antibodies to a wide range of non-self and also self

antigens without any immunization as described by Griffiths *et al.*, *EMBO J*, 12: 725-734 (1993). Finally, naive libraries can also be made synthetically by cloning the unrearranged V-gene segments from stem cells, and using PCR primers containing random sequence to encode the highly variable CDR3 regions and to accomplish rearrangement in vitro as described by Hoogenboom and Winter, *J. Mol. Biol.*, 227: 381-388 (1992).

Filamentous phage is used to display antibody fragments by fusion to the minor coat protein pIII. The antibody fragments can be displayed as single chain Fv fragments, in which VH and VL domains are connected on the same polypeptide chain by a flexible polypeptide spacer, e.g., as described by Marks *et al.*, *J. Mol. Biol.*, 222: 581-597 (1991), or as Fab fragments, in which one chain is fused to pIII and the other is secreted into the bacterial host cell periplasm where assembly of a Fab-coat protein structure which becomes displayed on the phage surface by displacing some of the wild type coat proteins, e.g., as described in Hoogenboom *et al.*, *Nucl. Acids Res.*, 19: 4133-4137 (1991).

In general, nucleic acids encoding antibody gene fragments are obtained from immune cells harvested from humans or animals. If a library biased in favor of anti-hepsin clones is desired, the individual is immunized with hepsin to generate an antibody response, and spleen cells and/or circulating B cells other peripheral blood lymphocytes (PBLs) are recovered for library construction. In a preferred embodiment, a human antibody gene fragment library biased in favor of anti-hepsin clones is obtained by generating an anti-hepsin antibody response in transgenic mice carrying a functional human immunoglobulin gene array (and lacking a functional endogenous antibody production system) such that hepsin immunization gives rise to B cells producing human antibodies against hepsin. The generation of human antibody-producing transgenic mice is described below.

Additional enrichment for anti-hepsin reactive cell populations can be obtained by using a suitable screening procedure to isolate B cells expressing hepsin-specific membrane bound antibody, e.g., by cell separation with hepsin affinity chromatography or adsorption of cells to fluorochrome-labeled hepsin followed by flow-activated cell sorting (FACS).

Alternatively, the use of spleen cells and/or B cells or other PBLs from an unimmunized donor provides a better representation of the possible antibody repertoire, and also permits the construction of an antibody library using any animal (human or non-human) species in which hepsin is not antigenic. For libraries incorporating in vitro antibody gene construction, stem cells are harvested from the individual to provide nucleic acids encoding unrearranged antibody gene segments. The immune cells of interest can be obtained from a

variety of animal species, such as human, mouse, rat, lagomorpha, luprine, canine, feline, porcine, bovine, equine, and avian species, etc.

Nucleic acid encoding antibody variable gene segments (including VH and VL segments) are recovered from the cells of interest and amplified. In the case of rearranged VH and VL gene libraries, the desired DNA can be obtained by isolating genomic DNA or mRNA from lymphocytes followed by polymerase chain reaction (PCR) with primers matching the 5' and 3' ends of rearranged VH and VL genes as described in Orlandi *et al.*, *Proc. Natl. Acad. Sci. (USA)*, 86: 3833-3837 (1989), thereby making diverse V gene repertoires for expression. The V genes can be amplified from cDNA and genomic DNA, with back primers at the 5' end of the exon encoding the mature V-domain and forward primers based within the J-segment as described in Orlandi *et al.* (1989) and in Ward *et al.*, *Nature*, 341: 544-546 (1989). However, for amplifying from cDNA, back primers can also be based in the leader exon as described in Jones *et al.*, *Biotechnol.*, 9: 88-89 (1991), and forward primers within the constant region as described in Sastry *et al.*, *Proc. Natl. Acad. Sci. (USA)*, 86: 5728-5732 (1989). To maximize complementarity, degeneracy can be incorporated in the primers as described in Orlandi *et al.* (1989) or Sastry *et al.* (1989). Preferably, the library diversity is maximized by using PCR primers targeted to each V-gene family in order to amplify all available VH and VL arrangements present in the immune cell nucleic acid sample, e.g. as described in the method of Marks *et al.*, *J. Mol. Biol.*, 222: 581-597 (1991) or as described in the method of Orum *et al.*, *Nucleic Acids Res.*, 21: 4491-4498 (1993). For cloning of the amplified DNA into expression vectors, rare restriction sites can be introduced within the PCR primer as a tag at one end as described in Orlandi *et al.* (1989), or by further PCR amplification with a tagged primer as described in Clackson *et al.*, *Nature*, 352: 624-628 (1991).

Repertoires of synthetically rearranged V genes can be derived in vitro from V gene segments. Most of the human VH-gene segments have been cloned and sequenced (reported in Tomlinson *et al.*, *J. Mol. Biol.*, 227: 776-798 (1992)), and mapped (reported in Matsuda *et al.*, *Nature Genet.*, 3: 88-94 (1993); these cloned segments (including all the major conformations of the H1 and H2 loop) can be used to generate diverse VH gene repertoires with PCR primers encoding H3 loops of diverse sequence and length as described in Hoogenboom and Winter, *J. Mol. Biol.*, 227: 381-388 (1992). VH repertoires can also be made with all the sequence diversity focused in a long H3 loop of a single length as described in Barbas *et al.*, *Proc. Natl. Acad. Sci. USA*, 89: 4457-4461 (1992). Human Vκ and Vλ segments have been cloned and sequenced (reported in Williams and Winter, *Eur. J.*

Immunol., 23: 1456-1461 (1993)) and can be used to make synthetic light chain repertoires. Synthetic V gene repertoires, based on a range of VH and VL folds, and L3 and H3 lengths, will encode antibodies of considerable structural diversity. Following amplification of V-gene encoding DNAs, germline V-gene segments can be rearranged in vitro according to the methods of Hoogenboom and Winter, *J. Mol. Biol.*, 227: 381-388 (1992).

Repertoires of antibody fragments can be constructed by combining VH and VL gene repertoires together in several ways. Each repertoire can be created in different vectors, and the vectors recombined in vitro, e.g., as described in Hogrefe *et al.*, *Gene*, 128:119-126 (1993), or in vivo by combinatorial infection, e.g., the loxP system described in Waterhouse *et al.*, *Nucl. Acids Res.*, 21:2265-2266 (1993). The *in vivo* recombination approach exploits the two-chain nature of Fab fragments to overcome the limit on library size imposed by *E. coli* transformation efficiency. Naive VH and VL repertoires are cloned separately, one into a phagemid and the other into a phage vector. The two libraries are then combined by phage infection of phagemid-containing bacteria so that each cell contains a different combination and the library size is limited only by the number of cells present (about 10^{12} clones). Both vectors contain in vivo recombination signals so that the VH and VL genes are recombined onto a single replicon and are co-packaged into phage virions. These huge libraries provide large numbers of diverse antibodies of good affinity (K_d^{-1} of about 10^{-8} M).

Alternatively, the repertoires may be cloned sequentially into the same vector, e.g., as described in Barbas *et al.*, *Proc. Natl. Acad. Sci. USA*, 88:7978-7982 (1991), or assembled together by PCR and then cloned, e.g. as described in Clackson *et al.*, *Nature*, 352: 624-628 (1991). PCR assembly can also be used to join VH and VL DNAs with DNA encoding a flexible peptide spacer to form single chain Fv (scFv) repertoires. In yet another technique, "in cell PCR assembly" is used to combine VH and VL genes within lymphocytes by PCR and then clone repertoires of linked genes as described in Embleton *et al.*, *Nucl. Acids Res.*, 20:3831-3837 (1992).

The antibodies produced by naive libraries (either natural or synthetic) can be of moderate affinity (K_d^{-1} of about 10^6 to 10^7 M^{-1}), but affinity maturation can also be mimicked *in vitro* by constructing and reselecting from secondary libraries as described in Winter *et al.* (1994), *supra*. For example, mutations can be introduced at random in vitro by using error-prone polymerase (reported in Leung *et al.*, *Technique*, 1:11-15 (1989)) in the method of Hawkins *et al.*, *J. Mol. Biol.*, 226: 889-896 (1992) or in the method of Gram *et al.*, *Proc. Natl. Acad. Sci USA*, 89: 3576-3580 (1992). Additionally, affinity maturation can be performed by randomly mutating one or more CDRs, e.g. using PCR with primers carrying

random sequence spanning the CDR of interest, in selected individual Fv clones and screening for higher affinity clones. WO 96/07754 (published 14 March 1996) described a method for inducing mutagenesis in a complementarity determining region of an immunoglobulin light chain to create a library of light chain genes. Another effective approach is to recombine the VH or VL domains selected by phage display with repertoires of naturally occurring V domain variants obtained from unimmunized donors and screen for higher affinity in several rounds of chain reshuffling as described in Marks *et al.*, *Biotechnol.*, 10:779-783 (1992). This technique allows the production of antibodies and antibody fragments with affinities in the 10^{-9} M range.

Hepsin nucleic acid and amino acid sequences are known in the art. Nucleic acid sequence encoding the hepsin can be designed using the amino acid sequence of the desired region of hepsin. As is well-known in the art, there are two major splice isoforms of hepsin, hepsin IIIb and hepsin IIIc. Hepsin sequences are well-known in the art and may include the sequences shown in Figures 7 and 8.

Nucleic acids encoding hepsin can be prepared by a variety of methods known in the art. These methods include, but are not limited to, chemical synthesis by any of the methods described in Engels *et al.*, *Agnew. Chem. Int. Ed. Engl.*, 28: 716-734 (1989), such as the triester, phosphite, phosphoramidite and H-phosphonate methods. In one embodiment, codons preferred by the expression host cell are used in the design of the hepsin encoding DNA. Alternatively, DNA encoding the hepsin can be isolated from a genomic or cDNA library.

Following construction of the DNA molecule encoding the hepsin, the DNA molecule is operably linked to an expression control sequence in an expression vector, such as a plasmid, wherein the control sequence is recognized by a host cell transformed with the vector. In general, plasmid vectors contain replication and control sequences which are derived from species compatible with the host cell. The vector ordinarily carries a replication site, as well as sequences which encode proteins that are capable of providing phenotypic selection in transformed cells. Suitable vectors for expression in prokaryotic and eukaryotic host cells are known in the art and some are further described herein. Eukaryotic organisms, such as yeasts, or cells derived from multicellular organisms, such as mammals, may be used.

Optionally, the DNA encoding the hepsin is operably linked to a secretory leader sequence resulting in secretion of the expression product by the host cell into the culture medium. Examples of secretory leader sequences include stII, ecotin, lamB, herpes GD, lpp,

alkaline phosphatase, invertase, and alpha factor. Also suitable for use herein is the 36 amino acid leader sequence of protein A (Abrahmsen *et al.*, *EMBO J.*, 4: 3901 (1985)).

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ precipitation and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell. Methods for transfection are well known in the art, and some are further described herein.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. Methods for transformation are well known in the art, and some are further described herein.

Prokaryotic host cells used to produce the hepsin can be cultured as described generally in Sambrook *et al.*, *supra.*

The mammalian host cells used to produce the hepsin can be cultured in a variety of media, which is well known in the art and some of which is described herein.

The host cells referred to in this disclosure encompass cells in *in vitro* culture as well as cells that are within a host animal.

Purification of hepsin may be accomplished using art-recognized methods, some of which are described herein.

The purified hepsin can be attached to a suitable matrix such as agarose beads, acrylamide beads, glass beads, cellulose, various acrylic copolymers, hydroxyl methacrylate gels, polyacrylic and polymethacrylic copolymers, nylon, neutral and ionic carriers, and the like, for use in the affinity chromatographic separation of phage display clones. Attachment of the hepsin protein to the matrix can be accomplished by the methods described in *Methods in Enzymology*, vol. 44 (1976). A commonly employed technique for attaching protein ligands to polysaccharide matrices, e.g. agarose, dextran or cellulose, involves activation of the carrier with cyanogen halides and subsequent coupling of the peptide ligand's primary aliphatic or aromatic amines to the activated matrix.

Alternatively, hepsin can be used to coat the wells of adsorption plates, expressed on host cells affixed to adsorption plates or used in cell sorting, or conjugated to biotin for capture with streptavidin-coated beads, or used in any other art-known method for panning phage display libraries.

The phage library samples are contacted with immobilized hepsin under conditions suitable for binding of at least a portion of the phage particles with the adsorbent. Normally, the conditions, including pH, ionic strength, temperature and the like are selected to mimic physiological conditions. The phages bound to the solid phase are washed and then eluted by acid, e.g. as described in Barbas *et al.*, *Proc. Natl. Acad. Sci USA*, 88: 7978-7982 (1991), or by alkali, e.g. as described in Marks *et al.*, *J. Mol. Biol.*, 222: 581-597 (1991), or by hepsin antigen competition, e.g. in a procedure similar to the antigen competition method of Clackson *et al.*, *Nature*, 352: 624-628 (1991). Phages can be enriched 20-1,000-fold in a single round of selection. Moreover, the enriched phages can be grown in bacterial culture and subjected to further rounds of selection.

The efficiency of selection depends on many factors, including the kinetics of dissociation during washing, and whether multiple antibody fragments on a single phage can simultaneously engage with antigen. Antibodies with fast dissociation kinetics (and weak binding affinities) can be retained by use of short washes, multivalent phage display and high coating density of antigen in solid phase. The high density not only stabilizes the phage through multivalent interactions, but favors rebinding of phage that has dissociated. The selection of antibodies with slow dissociation kinetics (and good binding affinities) can be promoted by use of long washes and monovalent phage display as described in Bass *et al.*, *Proteins*, 8: 309-314 (1990) and in WO 92/09690, and a low coating density of antigen as described in Marks *et al.*, *Biotechnol.*, 10: 779-783 (1992).

It is possible to select between phage antibodies of different affinities, even with affinities that differ slightly, for hepsin. However, random mutation of a selected antibody (e.g. as performed in some of the affinity maturation techniques described above) is likely to give rise to many mutants, most binding to antigen, and a few with higher affinity. With limiting hepsin, rare high affinity phage could be competed out. To retain all the higher affinity mutants, phages can be incubated with excess biotinylated hepsin, but with the biotinylated hepsin at a concentration of lower molarity than the target molar affinity constant for hepsin. The high affinity-binding phages can then be captured by streptavidin-coated paramagnetic beads. Such "equilibrium capture" allows the antibodies to be selected according to their affinities of binding, with sensitivity that permits isolation of mutant clones

with as little as two-fold higher affinity from a great excess of phages with lower affinity. Conditions used in washing phages bound to a solid phase can also be manipulated to discriminate on the basis of dissociation kinetics.

Hepsin clones may be activity selected. Fv clones corresponding to such hepsin antibodies can be selected by (1) isolating hepsin clones from a phage library as described above, and optionally amplifying the isolated population of phage clones by growing up the population in a suitable bacterial host; (2) selecting hepsin and a second protein against which blocking and non-blocking activity, respectively, is desired; (3) adsorbing the anti-hepsin phage clones to immobilized hepsin; (4) using an excess of the second protein to elute any undesired clones that recognize hepsin-binding determinants which overlap or are shared with the binding determinants of the second protein; and (5) eluting the clones which remain adsorbed following step (4). Optionally, clones with the desired blocking/non-blocking properties can be further enriched by repeating the selection procedures described herein one or more times.

DNA encoding the hybridoma-derived monoclonal antibodies or phage display Fv clones of the invention is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide primers designed to specifically amplify the heavy and light chain coding regions of interest from hybridoma or phage DNA template). Once isolated, the DNA can be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of the desired monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of antibody-encoding DNA include Skerra *et al.*, *Curr. Opinion in Immunol.*, 5: 256 (1993) and Pluckthun, *Immunol. Revs*, 130:151 (1992).

DNA encoding the Fv clones of the invention can be combined with known DNA sequences encoding heavy chain and/or light chain constant regions (e.g., the appropriate DNA sequences can be obtained from Kabat *et al.*, supra) to form clones encoding full or partial length heavy and/or light chains. It will be appreciated that constant regions of any isotype can be used for this purpose, including IgG, IgM, IgA, IgD, and IgE constant regions, and that such constant regions can be obtained from any human or animal species. A Fv clone derived from the variable domain DNA of one animal (such as human) species and then fused to constant region DNA of another animal species to form coding sequence(s) for "hybrid," full length heavy chain and/or light chain is included in the definition of "chimeric" and "hybrid" antibody as used herein. In a preferred embodiment, a Fv clone derived from

human variable DNA is fused to human constant region DNA to form coding sequence(s) for all human, full or partial length heavy and/or light chains.

DNA encoding anti-hepsin antibody derived from a hybridoma of the invention can also be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of homologous murine sequences derived from the hybridoma clone (e.g., as in the method of Morrison *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984)). DNA encoding a hybridoma or Fv clone-derived antibody or fragment can be further modified by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. In this manner, "chimeric" or "hybrid" antibodies are prepared that have the binding specificity of the Fv clone or hybridoma clone-derived antibodies of the invention.

Antibody Fragments

The present invention encompasses antibody fragments. In certain circumstances there are advantages of using antibody fragments, rather than whole antibodies. The smaller size of the fragments allows for rapid clearance, and may lead to improved access to solid tumors.

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., Journal of Biochemical and Biophysical Methods 24:107-117 (1992); and Brennan et al., Science, 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. Fab, Fv and ScFv antibody fragments can all be expressed in and secreted from E. coli, thus allowing the facile production of large amounts of these fragments. Antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from E. coli and chemically coupled to form $F(ab')_2$ fragments (Carter et al., Bio/Technology 10:163-167 (1992)). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Fab and $F(ab')_2$ fragment with increased in vivo half-life comprising a salvage receptor binding epitope residues are described in U.S. Pat. No. 5,869,046. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv) (see, e.g., WO 93/16185; U.S. Pat. Nos. 5,571,894 and 5,587,458). Fv and sFv are the only species with intact combining sites that are devoid of constant regions; thus, they are suitable for reduced nonspecific binding during in vivo use. sFv fusion proteins may be constructed to yield fusion of an effector protein at either the amino or the carboxy

terminus of an sFv. See Antibody Engineering, ed. Borrebaeck, supra. The antibody fragment may also be a "linear antibody," e.g., as described, for example, in U.S. Pat. No. 5,641,870. Such linear antibody fragments may be monospecific or bispecific.

Humanized Antibodies

The present invention encompasses humanized antibodies. Various methods for humanizing non-human antibodies are known in the art. For example, a humanized antibody can have one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al.* (1986) *Nature* 321:522-525; Riechmann *et al.* (1988) *Nature* 332:323-327; Verhoeyen *et al.* (1988) *Science* 239:1534-1536), by substituting hypervariable region sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some hypervariable region residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework for the humanized antibody (Sims *et al.* (1993) *J. Immunol.* 151:2296; Chothia *et al.* (1987) *J. Mol. Biol.* 196:901. Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter *et al.* (1992) *Proc. Natl. Acad. Sci. USA*, 89:4285; Presta *et al.* (1993) *J. Immunol.*, 151:2623.

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to one method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly

available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the hypervariable region residues are directly and most substantially involved in influencing antigen binding.

Human antibodies

Human anti-hepsin antibodies of the invention can be constructed by combining Fv clone variable domain sequence(s) selected from human-derived phage display libraries with known human constant domain sequences(s) as described above. Alternatively, human monoclonal anti-hepsin antibodies of the invention can be made by the hybridoma method. Human myeloma and mouse-human heteromyeloma cell lines for the production of human monoclonal antibodies have been described, for example, by Kozbor *J. Immunol.*, 133:3001 (1984); Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987); and Boerner *et al.*, *J. Immunol.*, 147:86 (1991).

It is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (JH) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits *et al.*, *Proc. Natl. Acad. Sci USA*, 90: 2551 (1993); Jakobovits *et al.*, *Nature*, 362: 255 (1993); Bruggermann *et al.*, *Year in Immunol.*, 7:33 (1993).

Gene shuffling can also be used to derive human antibodies from non-human, e.g., rodent, antibodies, where the human antibody has similar affinities and specificities to the starting non-human antibody. According to this method, which is also called "epitope imprinting," either the heavy or light chain variable region of a non-human antibody fragment obtained by phage display techniques as described above is replaced with a repertoire of human V domain genes, creating a population of non-human chain/human chain scFv or Fab

chimeras. Selection with antigen results in isolation of a non-human chain/human chain chimeric scFv or Fab wherein the human chain restores the antigen binding site destroyed upon removal of the corresponding non-human chain in the primary phage display clone, i.e. the epitope governs (imprints) the choice of the human chain partner. When the process is repeated in order to replace the remaining non-human chain, a human antibody is obtained (see PCT WO 93/06213 published April 1, 1993). Unlike traditional humanization of non-human antibodies by CDR grafting, this technique provides completely human antibodies, which have no FR or CDR residues of non-human origin.

Bispecific Antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for hepsin and the other is for any other antigen. Exemplary bispecific antibodies may bind to two different epitopes of the hepsin. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express hepsin. These antibodies possess a hepsin-binding arm and an arm which binds the cytotoxic agent (e.g., saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g., $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy chain-light chain pairs, where the two heavy chains have different specificities (Milstein and Cuello, *Nature*, 305: 537 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829 published May 13, 1993, and in Traunecker *et al.*, *EMBO J.*, 10: 3655 (1991).

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1), containing the site necessary for light chain binding, present in at least one of the fusions.

DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh *et al.*, *Methods in Enzymology*, 121:210 (1986).

According to another approach, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (*e.g.*, tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (*e.g.*, alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (US Patent No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/00373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the

art, and are disclosed in US Patent No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan *et al., Science*, 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby *et al., J. Exp. Med.*, 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling *in vitro* to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the HER2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny *et al., J. Immunol.*, 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger *et al., Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the VH and VL domains of one fragment are forced to pair with the complementary VL and VH domains of another fragment, thereby

forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al., J. Immunol.*, 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt *et al. J. Immunol.* 147: 60 (1991).

Multivalent Antibodies

A multivalent antibody may be internalized (and/or catabolized) faster than a bivalent antibody by a cell expressing an antigen to which the antibodies bind. The antibodies of the present invention can be multivalent antibodies (which are other than of the IgM class) with three or more antigen binding sites (e.g. tetravalent antibodies), which can be readily produced by recombinant expression of nucleic acid encoding the polypeptide chains of the antibody. The multivalent antibody can comprise a dimerization domain and three or more antigen binding sites. The preferred dimerization domain comprises (or consists of) an Fc region or a hinge region. In this scenario, the antibody will comprise an Fc region and three or more antigen binding sites amino-terminal to the Fe region. The preferred multivalent antibody herein comprises (or consists of) three to about eight, but preferably four, antigen binding sites. The multivalent antibody comprises at least one polypeptide chain (and preferably two polypeptide chains), wherein the polypeptide chain(s) comprise two or more variable domains. For instance, the polypeptide chain(s) may comprise VD1-(X1)n -VD2-(X2)n -Fc, wherein VD1 is a first variable domain, VD2 is a second variable domain, Fc is one polypeptide chain of an Fc region, X1 and X2 represent an amino acid or polypeptide, and n is 0 or 1. For instance, the polypeptide chain(s) may comprise: VH-CH1-flexible linker-VH-CH1-Fc region chain; or VH-CH1-VH-CH1-Fc region chain. The multivalent antibody herein preferably further comprises at least two (and preferably four) light chain variable domain polypeptides. The multivalent antibody herein may, for instance, comprise from about two to about eight light chain variable domain polypeptides. The light chain variable domain polypeptides contemplated here comprise a light chain variable domain and, optionally, further comprise a CL domain.

Antibody Variants

In some embodiments, amino acid sequence modification(s) of the antibodies described herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. Amino acid sequence variants of the antibody are prepared by introducing appropriate nucleotide changes into the antibody nucleic acid, or by peptide synthesis. Such modifications include, for example, deletions

from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the antibody. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid alterations may be introduced in the subject antibody amino acid sequence at the time that sequence is made.

A useful method for identification of certain residues or regions of the antibody that are preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (1989) *Science*, 244:1081-1085. Here, a residue or group of target residues are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation *per se* need not be predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed immunoglobulins are screened for the desired activity.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antibody with an N-terminal methionyl residue or the antibody fused to a cytotoxic polypeptide. Other insertional variants of the antibody molecule include the fusion to the N- or C-terminus of the antibody to an enzyme (e.g., for ADEPT) or a polypeptide which increases the serum half-life of the antibody.

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites).

Where the antibody comprises an Fc region, the carbohydrate attached thereto may be altered. For example, antibodies with a mature carbohydrate structure that lacks fucose attached to an Fc region of the antibody are described in US Pat Appl No US 2003/0157108 (Presta, L.). See also US 2004/0093621 (Kyowa Hakko Kogyo Co., Ltd). Antibodies with a bisecting N-acetylglucosamine (GlcNAc) in the carbohydrate attached to an Fc region of the antibody are referenced in WO 2003/011878, Jean-Mairet *et al.* and US Patent No. 6,602,684, Umana *et al.* Antibodies with at least one galactose residue in the oligosaccharide attached to an Fc region of the antibody are reported in WO 1997/30087, Patel *et al.* See, also, WO 1998/58964 (Raju, S.) and WO 1999/22764 (Raju, S.) concerning antibodies with altered carbohydrate attached to the Fc region thereof. See also US 2005/0123546 (Umana *et al.*) on antigen-binding molecules with modified glycosylation.

The preferred glycosylation variant herein comprises an Fc region, wherein a carbohydrate structure attached to the Fc region lacks fucose. Such variants have improved ADCC function. Optionally, the Fc region further comprises one or more amino acid substitutions therein which further improve ADCC, for example, substitutions at positions 298, 333, and/or 334 of the Fc region (Eu numbering of residues). Examples of publications related to "defucosylated" or "fucose-deficient" antibodies 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; WO2005/053742; Okazaki *et al. J. Mol. Biol.* 336:1239-1249 (2004); Yamane-Ohnuki *et al. Biotech. Bioeng.* 87: 614 (2004). Examples of cell lines producing defucosylated antibodies include Lec13 CHO cells deficient in protein fucosylation (Ripka *et al. Arch. Biochem. Biophys.* 249:533-545 (1986); US Pat Appl No US 2003/0157108 A1, Presta, L; 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 (Yamane-Ohnuki *et al. Biotech. Bioeng.* 87: 614 (2004)).

Another type of variant is an amino acid substitution variant. These variants have at least one amino acid (at least two, at least three, at least 4 or more) residue in the antibody molecule replaced by a different residue. The sites of greatest interest for substitutional

mutagenesis include the hypervariable regions, but FR alterations are also contemplated. Conservative substitutions are shown in Table A under the heading of "preferred substitutions." If such substitutions result in a change in biological activity, then more substantial changes, denominated "exemplary substitutions" in Table A, or as further described below in reference to amino acid classes, may be introduced and the products screened.

Table A

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys
Asn (N)	Gln; His; Asp, Lys; Arg	Gln
Asp (D)	Glu; Asn	Glu
Cys (C)	Ser; Ala	Ser
Gln (Q)	Asn; Glu	Asn
Glu (E)	Asp; Gln	Asp
Gly (G)	Ala	Ala
His (H)	Asn; Gln; Lys; Arg	Arg
Ile (I)	Leu; Val; Met; Ala; Phe; Norleucine	Leu
Leu (L)	Norleucine; Ile; Val; Met; Ala; Phe	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Trp; Leu; Val; Ile; Ala; Tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Val; Ser	Ser
Trp (W)	Tyr; Phe	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe; Ala; Norleucine	Leu

Substantial modifications in the biological properties of the antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a

sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile;

(2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;

(3) acidic: asp, glu;

(4) basic: his, lys, arg;

(5) residues that influence chain orientation: gly, pro; and

(6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

One type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (e.g., a humanized or human antibody). Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants involves affinity maturation using phage display. Briefly, several hypervariable region sites (e.g., 6-7 sites) are mutated to generate all possible amino acid substitutions at each site. The antibodies thus generated are displayed from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (e.g., binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis can be performed to identify hypervariable region residues contributing significantly to antigen binding. Alternatively, or additionally, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and antigen. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

Nucleic acid molecules encoding amino acid sequence variants of the antibody are prepared by a variety of methods known in the art. These methods 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 the antibody.

It may be desirable to introduce one or more amino acid modifications in an Fc region of the immunoglobulin polypeptides of the invention, thereby generating a 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 including that of a hinge cysteine.

In accordance with this description and the teachings of the art, it is contemplated that in some embodiments, an antibody used in methods of the invention may comprise one or more alterations as compared to the wild type counterpart antibody, e.g., in the Fc region. These antibodies would nonetheless retain substantially the same characteristics required for therapeutic utility as compared to their wild type counterpart. For example, it is thought that certain alterations can be made in the Fc region that would result in altered (i.e., either improved or diminished) C1q binding and/or Complement Dependent Cytotoxicity (CDC), e.g., as described in WO99/51642. See also Duncan & Winter *Nature* 322:738-40 (1988); US Patent No. 5,648,260; US Patent No. 5,624,821; and WO94/29351 concerning other examples of Fc region variants. WO00/42072 (Presta) and WO 2004/056312 (Lowman) describe antibody variants with improved or diminished binding to FcRs. The content of these patent publications are specifically incorporated herein by reference. See, also, Shields *et al. J. Biol. Chem.* 9(2): 6591-6604 (2001). 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 et al., J. Immunol. 117:587 (1976) and Kim et al., J. Immunol. 24:249 (1994)), are described in US2005/0014934A1 (Hinton et al.). These antibodies comprise an Fc region with one or more substitutions therein which improve binding of the Fc region to FcRn. Polypeptide variants with altered Fc region amino acid sequences and increased or decreased C1q binding capability are described in US patent No. 6,194,551B1, WO99/51642. The contents of those patent publications are specifically incorporated herein by reference. See, also, Idusogie *et al., J. Immunol.* 164: 4178-4184 (2000).

Antibody Derivatives

The antibodies of the present invention can be further modified to contain additional nonproteinaceous moieties that are known in the art and readily available. Preferably, the moieties suitable for derivatization of the antibody are 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, propropylene glycol homopolymers, prolypropylene oxide/ethylene oxide co-polymers, 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 polymers 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.

Screening for antibodies with desired properties

The antibodies of the present invention can be characterized for their physical/chemical properties and biological functions by various assays known in the art (some of which are disclosed herein). In some embodiments, antibodies are characterized for any one or more of reduction or blocking of hepsin binding, reduction or blocking of hepsin activity, reduction or blocking of hepsin and/or hepsin substrate (e.g., pro-MSP, pro-uPA, Factor VII, pro-HGF) downstream molecular signaling, and/or treatment and/or prevention of a tumor, cell proliferative disorder or a cancer; and/or treatment or prevention of a disorder associated with hepsin expression and/or activity (such as increased hepsin expression and/or activity). In some embodiments, hepsin activity is hepsin enzymatic activity. In one embodiment, the enzymatic activity comprises cleavage of polypeptide substrate of hepsin. In one embodiment, the polypeptide substrate of hepsin is one or more of pro-macrophage stimulating protein (pro-MSP), pro-uPA, Factor VII and pro-HGF. Hepsin activation of pro-MSP is described in co-pending, co-owned US provisional patent application no. 61/253,990, filed October 22, 2009. In one embodiment, the enzymatic activity comprises cleavage of a synthetic substrate of hepsin. In some embodiments, the hepsin synthetic substrate is a substrate shown in Table 1.

The purified antibodies can be further characterized by a series of assays including, but not limited to, N-terminal sequencing, amino acid analysis, non-denaturing size exclusion

high pressure liquid chromatography (HPLC), mass spectrometry, ion exchange chromatography and papain digestion.

In certain embodiments of the invention, the antibodies produced herein are analyzed for their biological activity. In some embodiments, the antibodies of the present invention are tested for their antigen binding activity. The antigen binding assays that are known in the art and can be used herein include without limitation any direct or competitive binding assays using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, fluorescent immunoassays, and protein A immunoassays. Illustrative antigen binding and other assay are provided below in the Examples section.

If an anti-hepsin antibody that inhibits cell growth is desired, the candidate antibody can be tested in *in vitro* and/or *in vivo* assays that measure inhibition of cell growth. Methods for examining growth and/or proliferation of a cancer cell are well known in the art. Exemplary methods for determining cell growth and/or proliferation and/or apoptosis include, for example, BrdU incorporation assay, MTT, [3H]-thymidine incorporation (e.g., TopCount assay (PerkinElmer)), cell viability assays (e.g., CellTiter-Glo (Promega)), and the like.

In one embodiment, the present invention contemplates an antibody that possesses effector functions. In certain embodiments, the Fc activities of the antibody are measured. 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. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, Annu. Rev. Immunol 9:457-92 (1991). An example of an in vitro assay to assess ADCC activity of a molecule of interest is described in US Patent No. 5,500,362 or 5,821,337. An assay to detect ADCC activity is also exemplified herein. 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 et al. PNAS (USA) 95:652-656 (1998). C1q binding assays may also be carried out to confirm that the antibody is unable to bind C1q and hence lacks CDC activity. To assess complement activation, a

CDC assay, e.g., as described in Gazzano-Santoro et al., J. Immunol. Methods 202:163 (1996), may be performed. FcRn binding and in vivo clearance/half life determinations can also be performed using methods known in the art.

Vectors, Host Cells, and Recombinant Methods

For recombinant production of an antibody of the invention, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the antibody is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The choice of vector depends in part on the host cell to be used. Generally, preferred host cells are of either prokaryotic or eukaryotic (generally mammalian) origin. It will be appreciated that constant regions of any isotype can be used for this purpose, including IgG, IgM, IgA, IgD, and IgE constant regions, and that such constant regions can be obtained from any human or animal species.

a. Generating antibodies using prokaryotic host cells:

i. Vector Construction

Polynucleotide sequences encoding polypeptide components of the antibody of the invention can be obtained using standard recombinant techniques. Desired polynucleotide sequences may be isolated and sequenced from antibody producing cells such as hybridoma cells. Alternatively, polynucleotides can be synthesized using nucleotide synthesizer or PCR techniques. Once obtained, sequences encoding the polypeptides are inserted into a recombinant vector capable of replicating and expressing heterologous polynucleotides in prokaryotic hosts. Many vectors that are available and known in the art can be used for the purpose of the present invention. Selection of an appropriate vector will depend mainly on the size of the nucleic acids to be inserted into the vector and the particular host cell to be transformed with the vector. Each vector contains various components, depending on its function (amplification or expression of heterologous polynucleotide, or both) and its compatibility with the particular host cell in which it resides. The vector components generally include, but are not limited to: an origin of replication, a selection marker gene, a promoter, a ribosome binding site (RBS), a signal sequence, the heterologous nucleic acid insert and a transcription termination sequence.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are

capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species. pBR322 contains genes encoding ampicillin (Amp) and tetracycline (Tet) resistance and thus provides easy means for identifying transformed cells. pBR322, its derivatives, or other microbial plasmids or bacteriophage may also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of endogenous proteins. Examples of pBR322 derivatives used for expression of particular antibodies are described in detail in Carter et al., U.S. Patent No. 5,648,237.

In addition, phage vectors containing replicon and control sequences that are compatible with the host microorganism can be used as transforming vectors in connection with these hosts. For example, bacteriophage such as λGEM.TM.-11 may be utilized in making a recombinant vector which can be used to transform susceptible host cells such as *E. coli* LE392.

The expression vector of the invention may comprise two or more promoter-cistron pairs, encoding each of the polypeptide components. A promoter is an untranslated regulatory sequence located upstream (5') to a cistron that modulates its expression. Prokaryotic promoters typically fall into two classes, inducible and constitutive. Inducible promoter is a promoter that initiates increased levels of transcription of the cistron under its control in response to changes in the culture condition, e.g., the presence or absence of a nutrient or a change in temperature.

A large number of promoters recognized by a variety of potential host cells are well known. The selected promoter can be operably linked to cistron DNA encoding the light or heavy chain by removing the promoter from the source DNA via restriction enzyme digestion and inserting the isolated promoter sequence into the vector of the invention. Both the native promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the target genes. In some embodiments, heterologous promoters are utilized, as they generally permit greater transcription and higher yields of expressed target gene as compared to the native target polypeptide promoter.

Promoters suitable for use with prokaryotic hosts include the PhoA promoter, the β-galactamase and lactose promoter systems, a tryptophan (trp) promoter system and hybrid promoters such as the tac or the trc promoter. However, other promoters that are functional in bacteria (such as other known bacterial or phage promoters) are suitable as well. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to

ligate them to cistrons encoding the target light and heavy chains (Siebenlist et al., (1980) Cell 20: 269) using linkers or adaptors to supply any required restriction sites.

In one aspect of the invention, each cistron within the recombinant vector comprises a secretion signal sequence component that directs translocation of the expressed polypeptides across a membrane. In general, the signal sequence may be a component of the vector, or it may be a part of the target polypeptide DNA that is inserted into the vector. The signal sequence selected for the purpose of this invention should be one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the signal sequences native to the heterologous polypeptides, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group consisting of the alkaline phosphatase, penicillinase, Ipp, or heat-stable enterotoxin II (STII) leaders, LamB, PhoE, PelB, OmpA, and MBP. In one embodiment of the invention, the signal sequences used in both cistrons of the expression system are STII signal sequences or variants thereof.

In another aspect, the production of the immunoglobulins according to the invention can occur in the cytoplasm of the host cell, and therefore does not require the presence of secretion signal sequences within each cistron. In that regard, immunoglobulin light and heavy chains are expressed, folded and assembled to form functional immunoglobulins within the cytoplasm. Certain host strains (e.g., the *E. coli* trxB- strains) provide cytoplasm conditions that are favorable for disulfide bond formation, thereby permitting proper folding and assembly of expressed protein subunits. Proba and Pluckthun Gene, 159:203 (1995).

Prokaryotic host cells suitable for expressing antibodies of the invention include Archaebacteria and Eubacteria, such as Gram-negative or Gram-positive organisms. Examples of useful bacteria include Escherichia (e.g., *E. coli*), Bacilli (e.g., *B. subtilis*), Enterobacteria, Pseudomonas species (e.g., *P. aeruginosa*), *Salmonella typhimurium*, *Serratia marcescans*, Klebsiella, Proteus, Shigella, Rhizobia, Vitreoscilla, or Paracoccus. In one embodiment, gram-negative cells are used. In one embodiment, E. coli cells are used as hosts for the invention. Examples of *E. coli* strains include strain W3110 (Bachmann, Cellular and Molecular Biology, vol. 2 (Washington, D.C.: American Society for Microbiology, 1987), pp. 1190-1219; ATCC Deposit No. 27,325) and derivatives thereof, including strain 33D3 having genotype W3110 ΔfhuA (ΔtonA) ptr3 lac Iq lacL8 ΔompTΔ(nmpc-fepE) degP41 kanR (U.S. Pat. No. 5,639,635). Other strains and derivatives thereof, such as *E. coli* 294 (ATCC 31,446), E. coli B, E. coliλ 1776 (ATCC 31,537) and *E. coli* RV308(ATCC 31,608)

are also suitable. These examples are illustrative rather than limiting. Methods for constructing derivatives of any of the above-mentioned bacteria having defined genotypes are known in the art and described in, for example, Bass et al., Proteins, 8:309-314 (1990). It is generally necessary to select the appropriate bacteria taking into consideration replicability of the replicon in the cells of a bacterium. For example, *E. coli*, Serratia, or Salmonella species can be suitably used as the host when well known plasmids such as pBR322, pBR325, pACYC177, or pKN410 are used to supply the replicon. Typically the host cell should secrete minimal amounts of proteolytic enzymes, and additional protease inhibitors may desirably be incorporated in the cell culture.

ii. Antibody Production

Host cells are transformed with the above-described expression vectors and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transformation means introducing DNA into the prokaryotic host so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride is generally used for bacterial cells that contain substantial cell-wall barriers. Another method for transformation employs polyethylene glycol/DMSO. Yet another technique used is electroporation.

Prokaryotic cells used to produce the polypeptides of the invention are grown in media known in the art and suitable for culture of the selected host cells. Examples of suitable media include Luria broth (LB) plus necessary nutrient supplements. In some embodiments, the media also contains a selection agent, chosen based on the construction of the expression vector, to selectively permit growth of prokaryotic cells containing the expression vector. For example, ampicillin is added to media for growth of cells expressing ampicillin resistant gene.

Any necessary supplements besides carbon, nitrogen, and inorganic phosphate sources may also be included at appropriate concentrations introduced alone or as a mixture with another supplement or medium such as a complex nitrogen source. Optionally the culture medium may contain one or more reducing agents selected from the group consisting of glutathione, cysteine, cystamine, thioglycollate, dithioerythritol and dithiothreitol.

The prokaryotic host cells are cultured at suitable temperatures. For *E. coli* growth, for example, the preferred temperature ranges from about 20°C to about 39°C, more

preferably from about 25°C to about 37°C, even more preferably at about 30°C. The pH of the medium may be any pH ranging from about 5 to about 9, depending mainly on the host organism. For *E. coli*, the pH is preferably from about 6.8 to about 7.4, and more preferably about 7.0.

If an inducible promoter is used in the expression vector of the invention, protein expression is induced under conditions suitable for the activation of the promoter. In one aspect of the invention, PhoA promoters are used for controlling transcription of the polypeptides. Accordingly, the transformed host cells are cultured in a phosphate-limiting medium for induction. Preferably, the phosphate-limiting medium is the C.R.A.P medium (see, e.g., Simmons et al., J. Immunol. Methods (2002), 263:133-147). A variety of other inducers may be used, according to the vector construct employed, as is known in the art.

In one embodiment, the expressed polypeptides of the present invention are secreted into and recovered from the periplasm of the host cells. Protein recovery typically involves disrupting the microorganism, generally by such means as osmotic shock, sonication or lysis. Once cells are disrupted, cell debris or whole cells may be removed by centrifugation or filtration. The proteins may be further purified, for example, by affinity resin chromatography. Alternatively, proteins can be transported into the culture media and isolated therein. Cells may be removed from the culture and the culture supernatant being filtered and concentrated for further purification of the proteins produced. The expressed polypeptides can be further isolated and identified using commonly known methods such as polyacrylamide gel electrophoresis (PAGE) and Western blot assay.

In one aspect of the invention, antibody production is conducted in large quantity by a fermentation process. Various large-scale fed-batch fermentation procedures are available for production of recombinant proteins. Large-scale fermentations have at least 1000 liters of capacity, preferably about 1,000 to 100,000 liters of capacity. These fermentors use agitator impellers to distribute oxygen and nutrients, especially glucose (the preferred carbon/energy source). Small scale fermentation refers generally to fermentation in a fermentor that is no more than approximately 100 liters in volumetric capacity, and can range from about 1 liter to about 100 liters.

In a fermentation process, induction of protein expression is typically initiated after the cells have been grown under suitable conditions to a desired density, e.g., an OD550 of about 180-220, at which stage the cells are in the early stationary phase. A variety of inducers may be used, according to the vector construct employed, as is known in the art and

described above. Cells may be grown for shorter periods prior to induction. Cells are usually induced for about 12-50 hours, although longer or shorter induction time may be used.

To improve the production yield and quality of the polypeptides of the invention, various fermentation conditions can be modified. For example, to improve the proper assembly and folding of the secreted antibody polypeptides, additional vectors overexpressing chaperone proteins, such as Dsb proteins (DsbA, DsbB, DsbC, DsbD, and/or DsbG) or FkpA (a peptidylprolyl cis,trans-isomerase with chaperone activity) can be used to co-transform the host prokaryotic cells. The chaperone proteins have been demonstrated to facilitate the proper folding and solubility of heterologous proteins produced in bacterial host cells. Chen et al., (1999) J. Biol. Chem. 274:19601-19605; Georgiou et al., U.S. Patent No. 6,083,715; Georgiou et al., U.S. Patent No. 6,027,888; Bothmann and Pluckthun (2000) J. Biol. Chem. 275:17100-17105; Ramm and Pluckthun, (2000) J. Biol. Chem. 275:17106-17113; Arie et al., (2001) Mol. Microbiol. 39:199-210.

To minimize proteolysis of expressed heterologous proteins (especially those that are proteolytically sensitive), certain host strains deficient for proteolytic enzymes can be used for the present invention. For example, host cell strains may be modified to effect genetic mutation(s) in the genes encoding known bacterial proteases such as Protease III, OmpT, DegP, Tsp, Protease I, Protease Mi, Protease V, Protease VI, and combinations thereof. Some *E. coli* protease-deficient strains are available and described in, for example, Joly et al., (1998), supra; Georgiou et al., U.S. Patent No. 5,264,365; Georgiou et al., U.S. Patent No. 5,508,192; Hara et al., Microbial Drug Resistance, 2:63-72 (1996).

In one embodiment, *E. coli* strains deficient for proteolytic enzymes and transformed with plasmids overexpressing one or more chaperone proteins are used as host cells in the expression system of the invention.

iii. Antibody Purification

Standard protein purification methods known in the art can be employed. The following procedures are exemplary of suitable purification procedures: fractionation on immunoaffinity or ion-exchange columns, ethanol precipitation, reverse phase HPLC, chromatography on silica or on a cation-exchange resin such as DEAE, chromatofocusing, SDS-PAGE, ammonium sulfate precipitation, and gel filtration using, for example, Sephadex G-75.

In one aspect, Protein A immobilized on a solid phase is used for immunoaffinity purification of the full length antibody products of the invention. Protein A is a 41kD cell wall protein from *Staphylococcus aureas* which binds with a high affinity to the Fc region of

antibodies. Lindmark et al., (1983) J. Immunol. Meth. 62:1-13. The solid phase to which Protein A is immobilized is preferably a column comprising a glass or silica surface, more preferably a controlled pore glass column or a silicic acid column. In some applications, the column has been coated with a reagent, such as glycerol, in an attempt to prevent nonspecific adherence of contaminants.

As the first step of purification, the preparation derived from the cell culture as described above is applied onto the Protein A immobilized solid phase to allow specific binding of the antibody of interest to Protein A. The solid phase is then washed to remove contaminants non-specifically bound to the solid phase. Finally the antibody of interest is recovered from the solid phase by elution.

b. Generating antibodies using eukaryotic host cells:

The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

(i) Signal sequence component

A vector for use in a eukaryotic host cell may also contain a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide of interest. The heterologous signal sequence selected preferably is one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. In mammalian cell expression, mammalian signal sequences as well as viral secretory leaders, for example, the herpes simplex gD signal, are available.

The DNA for such precursor region is ligated in reading frame to DNA encoding the antibody.

(ii) Origin of replication

Generally, an origin of replication component is not needed for mammalian expression vectors. For example, the SV40 origin may typically be used only because it contains the early promoter.

(iii) Selection gene component

Expression and cloning vectors may contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, where relevant, or (c) supply critical nutrients not available from complex media.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin, mycophenolic acid and hygromycin.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the antibody nucleic acid, such as DHFR, thymidine kinase, metallothionein-I and -II, preferably primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, etc.

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity (e.g., ATCC CRL-9096).

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding an antibody, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

(iv) Promoter component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the antibody polypeptide nucleic acid. Promoter sequences are known for eukaryotes. Virtually alleukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CNCAAT region where N may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Antibody polypeptide transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus, and Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin

promoter, from heat-shock promoters, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978. Alternatively, the Rous Sarcoma Virus long terminal repeat can be used as the promoter.

(v) Enhancer element component

Transcription of DNA encoding the antibody polypeptide of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the antibody polypeptide-encoding sequence, but is preferably located at a site 5' from the promoter.

(vi) Transcription termination component

Expression vectors used in eukaryotic host cells will typically also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding an antibody. One useful transcription termination component is the bovine growth hormone polyadenylation region. See WO94/11026 and the expression vector disclosed therein.

(vii) Selection and transformation of host cells

Suitable host cells for cloning or expressing the DNA in the vectors herein include higher eukaryote cells described herein, including vertebrate host cells. Propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7,

ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen. Virol. 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub et al., Proc. Natl. Acad. Sci. USA 77:4216 (1980)) ; mouse sertoli cells (TM4, Mather, Biol. Reprod. 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y. Acad. Sci. 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors for antibody production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

(viii) Culturing the host cells

The host cells used to produce an antibody of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham et al., Meth. Enz. 58:44 (1979), Barnes et al., Anal. Biochem.102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Patent Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

(ix) Purification of antibody

When using recombinant techniques, the antibody can be produced intracellularly, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, are removed, for example, by centrifugation or ultrafiltration. Where the antibody is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark et al., J. Immunol. Meth. 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human γ3 (Guss et al., EMBO J. 5:15671575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a CH3 domain, the Bakerbond ABX™resin (J. T. Baker, Phillipsburg, NJ) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (e.g., from about 0-0.25M salt).

Immunoconjugates

The invention also provides immunoconjugates (interchangeably termed "antibody-drug conjugates" or "ADC"), comprising any of the anti-hepsin antibodies described herein

conjugated to a cytotoxic agent such as a chemotherapeutic agent, a drug, a growth inhibitory agent, a toxin (e.g., an enzymatically active toxin of bacterial, fungal, plant, or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate).

The use of antibody-drug conjugates for the local delivery of cytotoxic or cytostatic agents, i.e., drugs to kill or inhibit tumor cells in the treatment of cancer (Syrigos and Epenetos (1999) Anticancer Research 19:605-614; Niculescu-Duvaz and Springer (1997) Adv. Drg. Del. Rev. 26:151-172; U.S. Patent No. 4,975,278) allows targeted delivery of the drug moiety to tumors, and intracellular accumulation therein, where systemic administration of these unconjugated drug agents may result in unacceptable levels of toxicity to normal cells as well as the tumor cells sought to be eliminated (Baldwin et al., (1986) Lancet pp. (Mar. 15, 1986):603-05; Thorpe, (1985) "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review," in Monoclonal Antibodies '84: Biological And Clinical Applications, A. Pinchera et al. (ed.s), pp. 475-506). Maximal efficacy with minimal toxicity is sought thereby. Both polyclonal antibodies and monoclonal antibodies have been reported as useful in these strategies (Rowland et al., (1986) Cancer Immunol. Immunother., 21:183-87). Drugs used in these methods include daunomycin, doxorubicin, methotrexate, and vindesine (Rowland et al., (1986) supra). Toxins used in antibody-toxin conjugates include bacterial toxins such as diphtheria toxin, plant toxins such as ricin, small molecule toxins such as geldanamycin (Mandler et al (2000) Jour. of the Nat. Cancer Inst. 92(19):1573-1581; Mandler et al., (2000) Bioorganic & Med. Chem. Letters 10:1025-1028; Mandler et al., (2002) Bioconjugate Chem. 13:786-791), maytansinoids (EP 1391213; Liu et al., (1996) Proc. Natl. Acad. Sci. USA 93:8618-8623), and calicheamicin (Lode et al., (1998) Cancer Res. 58:2928; Hinman et al., (1993) Cancer Res. 53:3336-3342). The toxins may effect their cytotoxic and cytostatic effects by mechanisms including tubulin binding, DNA binding, or topoisomerase inhibition. Some cytotoxic drugs tend to be inactive or less active when conjugated to large antibodies or protein receptor ligands.

ZEVALIN® (ibritumomab tiuxetan, Biogen/Idec) is an antibody-radioisotope conjugate composed of a murine IgG1 kappa monoclonal antibody directed against the CD20 antigen found on the surface of normal and malignant B lymphocytes and ^{111}In or ^{90}Y radioisotope bound by a thiourea linker-chelator (Wiseman et al., (2000) Eur. Jour. Nucl. Med. 27(7):766-77; Wiseman et al., (2002) Blood 99(12):4336-42; Witzig et al., (2002) J. Clin. Oncol. 20(10):2453-63; Witzig et al., (2002) J. Clin. Oncol. 20(15):3262-69). Although ZEVALIN has activity against B-cell non-Hodgkin's Lymphoma (NHL), administration

results in severe and prolonged cytopenias in most patients. MYLOTARG™ (gemtuzumab ozogamicin, Wyeth Pharmaceuticals), an antibody drug conjugate composed of a hu CD33 antibody linked to calicheamicin, was approved in 2000 for the treatment of acute myeloid leukemia by injection (Drugs of the Future (2000) 25(7):686; US Patent Nos. 4,970,198; 5,079,233; 5,585,089; 5,606,040; 5,6937,62; 5,739,116; 5,767,285; 5,773,001). Cantuzumab mertansine (Immunogen, Inc.), an antibody drug conjugate composed of the huC242 antibody linked via the disulfide linker SPP to the maytansinoid drug moiety, DM1, is advancing into Phase II trials for the treatment of cancers that express CanAg, such as colon, pancreatic, gastric, and others. MLN-2704 (Millennium Pharm., BZL Biologics, Immunogen Inc.), an antibody drug conjugate composed of the anti-prostate specific membrane antigen (PSMA) monoclonal antibody linked to the maytansinoid drug moiety, DM1, is under development for the potential treatment of prostate tumors. The auristatin peptides, auristatin E (AE) and monomethylauristatin (MMAE), synthetic analogs of dolastatin, were conjugated to chimeric monoclonal antibodies cBR96 (specific to Lewis Y on carcinomas) and cAC10 (specific to CD30 on hematological malignancies) (Doronina et al., (2003) Nature Biotechnology 21(7):778-784) and are under therapeutic development.

Chemotherapeutic agents useful in the generation of immunoconjugates are described herein (e.g., above). Enzymatically active toxins and fragments thereof that can be used include 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, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothecenes. See, e.g., WO 93/21232 published October 28, 1993. A variety of radionuclides are available for the production of radioconjugated antibodies. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y, and ^{186}Re. Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein-coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), 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 et al., Science, 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

Conjugates of an antibody and one or more small molecule toxins, such as a calicheamicin, maytansinoids, dolastatins, aurostatins, a trichothecene, and CC1065, and the derivatives of these toxins that have toxin activity, are also contemplated herein.

i. Maytansine and maytansinoids

In some embodiments, the immunoconjugate comprises an antibody (full length or fragments) of the invention conjugated to one or more maytansinoid molecules.

Maytansinoids are mitototic inhibitors which act by inhibiting tubulin polymerization. Maytansine was first isolated from the east African shrub *Maytenus serrata* (U.S. Patent No. 3,896,111). Subsequently, it was discovered that certain microbes also produce maytansinoids, such as maytansinol and C-3 maytansinol esters (U.S. Patent No. 4,151,042). Synthetic maytansinol and derivatives and analogues thereof are disclosed, for example, in U.S. Patent Nos. 4,137,230; 4,248,870; 4,256,746; 4,260,608; 4,265,814; 4,294,757; 4,307,016; 4,308,268; 4,308,269; 4,309,428; 4,313,946; 4,315,929; 4,317,821; 4,322,348; 4,331,598; 4,361,650; 4,364,866; 4,424,219; 4,450,254; 4,362,663; and 4,371,533.

Maytansinoid drug moieties are attractive drug moieties in antibody drug conjugates because they are: (i) relatively accessible to prepare by fermentation or chemical modification, derivatization of fermentation products, (ii) amenable to derivatization with functional groups suitable for conjugation through the non-disulfide linkers to antibodies, (iii) stable in plasma, and (iv) effective against a variety of tumor cell lines.

Immunoconjugates containing maytansinoids, methods of making same, and their therapeutic use are disclosed, for example, in U.S. Patent Nos. 5,208,020, 5,416,064 and European Patent EP 0 425 235 B1, the disclosures of which are hereby expressly incorporated by reference. Liu et al., Proc. Natl. Acad. Sci. USA 93:8618-8623 (1996) described immunoconjugates comprising a maytansinoid designated DM1 linked to the monoclonal antibody C242 directed against human colorectal cancer. The conjugate was found to be highly cytotoxic towards cultured colon cancer cells, and showed antitumor activity in an in vivo tumor growth assay. Chari et al., Cancer Research 52:127-131 (1992) describe immunoconjugates in which a maytansinoid was conjugated via a disulfide linker to the murine antibody A7 binding to an antigen on human colon cancer cell lines, or to another murine monoclonal antibody TA.1 that binds the HER-2/neu oncogene. The cytotoxicity of

the TA.1-maytansinoid conjugate was tested in vitro on the human breast cancer cell line SK-BR-3, which expresses 3 x 10^5 HER-2 surface antigens per cell. The drug conjugate achieved a degree of cytotoxicity similar to the free maytansinoid drug, which could be increased by increasing the number of maytansinoid molecules per antibody molecule. The A7-maytansinoid conjugate showed low systemic cytotoxicity in mice.

Antibody-maytansinoid conjugates are prepared by chemically linking an antibody to a maytansinoid molecule without significantly diminishing the biological activity of either the antibody or the maytansinoid molecule. See, e.g., U.S. Patent No. 5,208,020 (the disclosure of which is hereby expressly incorporated by reference). An average of 3-4 maytansinoid molecules conjugated per antibody molecule has shown efficacy in enhancing cytotoxicity of target cells without negatively affecting the function or solubility of the antibody, although even one molecule of toxin/antibody would be expected to enhance cytotoxicity over the use of naked antibody. Maytansinoids are well known in the art and can be synthesized by known techniques or isolated from natural sources. Suitable maytansinoids are disclosed, for example, in U.S. Patent No. 5,208,020 and in the other patents and nonpatent publications referred to hereinabove. Preferred maytansinoids are maytansinol and maytansinol analogues modified in the aromatic ring or at other positions of the maytansinol molecule, such as various maytansinol esters.

There are many linking groups known in the art for making antibody-maytansinoid conjugates, including, for example, those disclosed in U.S. Patent No. 5,208,020 or EP Patent 0 425 235 B1, Chari et al., Cancer Research 52:127-131 (1992), and U.S. Patent Application No. 10/960,602, filed Oct. 8, 2004, the disclosures of which are hereby expressly incorporated by reference. Antibody-maytansinoid conjugates comprising the linker component SMCC may be prepared as disclosed in U.S. Patent Application No. 10/960,602, filed Oct. 8, 2004. The linking groups include disulfide groups, thioether groups, acid labile groups, photolabile groups, peptidase labile groups, or esterase labile groups, as disclosed in the above-identified patents, disulfide and thioether groups being preferred. Additional linking groups are described and exemplified herein.

Conjugates of the antibody and maytansinoid 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). Particularly preferred coupling agents include N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) (Carlsson et al., Biochem. J. 173:723-737 (1978)) and N-succinimidyl-4-(2-pyridylthio)pentanoate (SPP) to provide for a disulfide linkage.

The linker may be attached to the maytansinoid molecule at various positions, depending on the type of the link. For example, an ester linkage may be formed by reaction with a hydroxyl group using conventional coupling techniques. The reaction may occur at the C-3 position having a hydroxyl group, the C-14 position modified with hydroxymethyl, the C-15 position modified with a hydroxyl group, and the C-20 position having a hydroxyl group. In a preferred embodiment, the linkage is formed at the C-3 position of maytansinol or a maytansinol analogue.

ii. Auristatins and dolastatins

In some embodiments, the immunoconjugate comprises an antibody of the invention conjugated to dolastatins or dolostatin peptidic analogs and derivatives, the auristatins (U.S. Patent Nos. 5,635,483 and 5,780,588). Dolastatins and auristatins have been shown to interfere with microtubule dynamics, GTP hydrolysis, and nuclear and cellular division (Woyke et al (2001) Antimicrob. Agents and Chemother. 45(12):3580-3584) and have anticancer (U.S. Patent No. 5,663,149) and antifungal activity (Pettit et al., (1998) Antimicrob. Agents Chemother. 42:2961-2965). The dolastatin or auristatin drug moiety may be attached to the antibody through the N (amino) terminus or the C (carboxyl) terminus of the peptidic drug moiety (WO 02/088172).

Exemplary auristatin embodiments include the N-terminus linked monomethylauristatin drug moieties DE and DF, disclosed in "Monomethylvaline Compounds Capable of Conjugation to Ligands," U.S. Ser. No. 10/983,340, filed Nov. 5, 2004, the disclosure of which is expressly incorporated by reference in its entirety.

Typically, peptide-based drug moieties can be prepared by forming a peptide bond between two or more amino acids and/or peptide fragments. Such peptide bonds can be prepared, for example, according to the liquid phase synthesis method (see E. Schröder and K. Lübke, "The Peptides," volume 1, pp. 76-136, 1965, Academic Press) that is well known in the field of peptide chemistry. The auristatin/dolastatin drug moieties may be prepared according to the methods of: U.S. Patent Nos. 5,635,483 and 5,780,588; Pettit et al., (1989) J. Am. Chem. Soc. 111:5463-5465; Pettit et al., (1998) Anti-Cancer Drug Design 13:243-277; Pettit, G.R., et al., Synthesis, 1996, 719-725; and Pettit et al., (1996) J. Chem. Soc. Perkin

Trans. 1 5:859-863. See also Doronina (2003) Nat. Biotechnol. 21(7):778-784; "Monomethylvaline Compounds Capable of Conjugation to Ligands," US Ser. No. 10/983,340, filed Nov. 5, 2004, hereby incorporated by reference in its entirety (disclosing, e.g., linkers and methods of preparing monomethylvaline compounds such as MMAE and MMAF conjugated to linkers).

iii. Calicheamicin

In other embodiments, the immunoconjugate comprises an antibody of the invention conjugated to one or more calicheamicin molecules. The calicheamicin family of antibiotics are capable of producing double-stranded DNA breaks at sub-picomolar concentrations. For the preparation of conjugates of the calicheamicin family, 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 (all to American Cyanamid Company). Structural analogues of calicheamicin which may be used include, but are not limited to, γ_1^I, α_2^I, α_3^I, N-acetyl-γ_1^I, PSAG and θ^I_1 (Hinman et al., Cancer Research 53:3336-3342 (1993), Lode et al., Cancer Research 58:2925-2928 (1998) and the aforementioned U.S. patents to American Cyanamid). Another anti-tumor drug that the antibody can be conjugated is QFA which is an antifolate. Both calicheamicin and QFA have intracellular sites of action and do not readily cross the plasma membrane. Therefore, cellular uptake of these agents through antibody mediated internalization greatly enhances their cytotoxic effects.

iv. Other cytotoxic agents

Other antitumor agents that can be conjugated to the antibodies of the invention include BCNU, streptozoicin, vincristine and 5-fluorouracil, the family of agents known collectively LL-E33288 complex described in U.S. Patent Nos. 5,053,394 and 5,770,710, as well as esperamicins (U.S. Patent No. 5,877,296).

Enzymatically active toxins and fragments thereof which can be used include 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, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. See, for example, WO 93/21232 published October 28, 1993.

The present invention further contemplates an immunoconjugate formed between an antibody and a compound with nucleolytic activity (e.g., a ribonuclease or a DNA endonuclease such as a deoxyribonuclease; DNase).

For selective destruction of the tumor, the antibody may comprise a highly radioactive atom. A variety of radioactive isotopes are available for the production of radioconjugated antibodies. Examples include At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32}, Pb^{212} and radioactive isotopes of Lu. When the conjugate is used for detection, it may comprise a radioactive atom for scintigraphic studies, for example tc^{99m} or I^{123}, 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.

The radio- or other labels may be incorporated in the conjugate in known ways. For example, the peptide may be biosynthesized or may be synthesized by chemical amino acid synthesis using suitable amino acid precursors involving, for example, fluorine-19 in place of hydrogen. Labels such as tc^{99m} or I^{123}, Re^{186}, Re^{188} and In^{111} can be attached via a cysteine residue in the peptide. Yttrium-90 can be attached via a lysine residue. The IODOGEN method (Fraker et al (1978) Biochem. Biophys. Res. Commun. 80: 49-57 can be used to incorporate iodine-123. "Monoclonal Antibodies in Immunoscintigraphy" (Chatal, CRC Press 1989) describes other methods in detail.

Conjugates of the 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 et al., Science 238:1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026. The linker may be a "cleavable linker" facilitating release of the cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, photolabile linker, dimethyl linker or disulfide-containing linker (Chari et al., Cancer Research 52:127-131 (1992); U.S. Patent No. 5,208,020) may be used.

The compounds of the invention expressly contemplate, but are not limited to, ADC prepared with cross-linker reagents: 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). See pages 467-498, 2003-2004 Applications Handbook and Catalog.

v. Preparation of antibody drug conjugates

In the antibody drug conjugates (ADC) of the invention, an antibody (Ab) is conjugated to one or more drug moieties (D), e.g. about 1 to about 20 drug moieties per antibody, through a linker (L). The ADC of Formula I may be prepared by several routes, employing organic chemistry reactions, conditions, and reagents known to those skilled in the art, including: (1) reaction of a nucleophilic group of an antibody with a bivalent linker reagent, to form Ab-L, via a covalent bond, followed by reaction with a drug moiety D; and (2) reaction of a nucleophilic group of a drug moiety with a bivalent linker reagent, to form D-L, via a covalent bond, followed by reaction with the nucleophilic group of an antibody. Additional methods for preparing ADC are described herein.

$$Ab–(L–D)_p \qquad I$$

The linker may be composed of one or more linker components. Exemplary linker components include 6-maleimidocaproyl ("MC"), maleimidopropanoyl ("MP"), valine-citrulline ("val-cit"), alanine-phenylalanine ("ala-phe"), p-aminobenzyloxycarbonyl ("PAB"), N-Succinimidyl 4-(2-pyridylthio) pentanoate ("SPP"), N-Succinimidyl 4-(N-maleimidomethyl) cyclohexane-1 carboxylate ("SMCC'), and N-Succinimidyl (4-iodo-acetyl) aminobenzoate ("SIAB"). Additional linker components are known in the art and some are described herein. See also "Monomethylvaline Compounds Capable of Conjugation to Ligands," U.S. Ser. No. 10/983,340, filed Nov. 5, 2004, the contents of which are hereby incorporated by reference in its entirety.

In some embodiments, the linker may comprise amino acid residues. Exemplary amino acid linker components include a dipeptide, a tripeptide, a tetrapeptide or a pentapeptide. Exemplary dipeptides include: valine-citrulline (vc or val-cit), alanine-phenylalanine (af or ala-phe). Exemplary tripeptides include: glycine-valine-citrulline (gly-val-cit) and glycine-glycine-glycine (gly-gly-gly). Amino acid residues which comprise an amino acid linker component include those occurring naturally, as well as minor amino acids and non-naturally occurring amino acid analogs, such as citrulline. Amino acid linker components can be designed and optimized in their selectivity for enzymatic cleavage by a

particular enzymes, for example, a tumor-associated protease, cathepsin B, C and D, or a plasmin protease.

Nucleophilic groups on antibodies include, but are not limited to: (i) N-terminal amine groups, (ii) side chain amine groups, e.g. lysine, (iii) side chain thiol groups, e.g. cysteine, and (iv) sugar hydroxyl or amino groups where the antibody is glycosylated. Amine, thiol, and hydroxyl groups are nucleophilic and capable of reacting to form covalent bonds with electrophilic groups on linker moieties and linker reagents including: (i) active esters such as NHS esters, HOBt esters, haloformates, and acid halides; (ii) alkyl and benzyl halides such as haloacetamides; (iii) aldehydes, ketones, carboxyl, and maleimide groups. Certain antibodies have reducible interchain disulfides, i.e. cysteine bridges. Antibodies may be made reactive for conjugation with linker reagents by treatment with a reducing agent such as DTT (dithiothreitol). Each cysteine bridge will thus form, theoretically, two reactive thiol nucleophiles. Additional nucleophilic groups can be introduced into antibodies through the reaction of lysines with 2-iminothiolane (Traut's reagent) resulting in conversion of an amine into a thiol. Reactive thiol groups may be introduced into the antibody by introducing one, two, three, four, or more cysteine residues (e.g., preparing mutant antibodies comprising one or more non-native cysteine amino acid residues).

Antibody drug conjugates of the invention may also be produced by modification of the antibody to introduce electrophilic moieties, which can react with nucleophilic substituents on the linker reagent or drug. The sugars of glycosylated antibodies may be oxidized, e.g., with periodate oxidizing reagents, to form aldehyde or ketone groups which may react with the amine group of linker reagents or drug moieties. The resulting imine Schiff base groups may form a stable linkage, or may be reduced, e.g., by borohydride reagents to form stable amine linkages. In one embodiment, reaction of the carbohydrate portion of a glycosylated antibody with either glactose oxidase or sodium meta-periodate may yield carbonyl (aldehyde and ketone) groups in the protein that can react with appropriate groups on the drug (Hermanson, Bioconjugate Techniques). In another embodiment, proteins containing N-terminal serine or threonine residues can react with sodium meta-periodate, resulting in production of an aldehyde in place of the first amino acid (Geoghegan & Stroh, (1992) Bioconjugate Chem. 3:138-146; U.S. Patent No. 5,362,852). Such aldehyde can be reacted with a drug moiety or linker nucleophile.

Likewise, nucleophilic groups on a drug moiety include, but are not limited to: amine, thiol, hydroxyl, hydrazide, oxime, hydrazine, thiosemicarbazone, hydrazine carboxylate, and arylhydrazide groups capable of reacting to form covalent bonds with electrophilic groups on

linker moieties and linker reagents including: (i) active esters such as NHS esters, HOBt esters, haloformates, and acid halides; (ii) alkyl and benzyl halides such as haloacetamides; (iii) aldehydes, ketones, carboxyl, and maleimide groups.

Alternatively, a fusion protein comprising the antibody and cytotoxic agent may be made, e.g., by recombinant techniques or peptide synthesis. The length of DNA may comprise respective regions encoding the two portions of the conjugate either adjacent one another or separated by a region encoding a linker peptide which does not destroy the desired properties of the conjugate.

In yet another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pre-targeting wherein the antibody-receptor conjugate is administered to the individual, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (e.g., avidin) which is conjugated to a cytotoxic agent (e.g., a radionucleotide).

Methods using anti-hepsin antibodies

The present invention features the use of a hepsin antibody as part of a specific treatment regimen intended to provide a beneficial effect from the activity of this therapeutic agent. The present invention is particularly useful in treating cancers of various types at various stages.

The term cancer embraces a collection of proliferative disorders, including but not limited to pre-cancerous growths, benign tumors, and malignant tumors. Benign tumors remain localized at the site of origin and do not have the capacity to infiltrate, invade, or metastasize to distant sites. Malignant tumors will invade and damage other tissues around them. They can also gain the ability to break off from the original site and spread to other parts of the body (metastasize), usually through the bloodstream or through the lymphatic system where the lymph nodes are located. Primary tumors are classified by the type of tissue from which they arise; metastatic tumors are classified by the tissue type from which the cancer cells are derived. Over time, the cells of a malignant tumor become more abnormal and appear less like normal cells. This change in the appearance of cancer cells is called the tumor grade, and cancer cells are described as being well-differentiated (low grade), moderately-differentiated, poorly-differentiated, or undifferentiated (high grade). Well-differentiated cells are quite normal appearing and resemble the normal cells from which they originated. Undifferentiated cells are cells that have become so abnormal that it is no longer possible to determine the origin of the cells.

Cancer staging systems describe how far the cancer has spread anatomically and attempt to put patients with similar prognosis and treatment in the same staging group. Several tests may be performed to help stage cancer including biopsy and certain imaging tests such as a chest x-ray, mammogram, bone scan, CT scan, and MRI scan. Blood tests and a clinical evaluation are also used to evaluate a patient's overall health and detect whether the cancer has spread to certain organs.

To stage cancer, the American Joint Committee on Cancer first places the cancer, particularly solid tumors, in a letter category using the TNM classification system. Cancers are designated the letter T (tumor size), N (palpable nodes), and/or M (metastases). T1, T2, T3, and T4 describe the increasing size of the primary lesion; N0, N1, N2, N3 indicates progressively advancing node involvement; and M0 and M1 reflect the absence or presence of distant metastases.

In the second staging method, also known as the Overall Stage Grouping or Roman Numeral Staging, cancers are divided into stages 0 to IV, incorporating the size of primary lesions as well as the presence of nodal spread and of distant metastases. In this system, cases are grouped into four stages denoted by Roman numerals I through IV, or are classified as "recurrent." For some cancers, stage 0 is referred to as "in situ" or "Tis," such as ductal carcinoma in situ or lobular carcinoma in situ for breast cancers. High grade adenomas can also be classified as stage 0. In general, stage I cancers are small localized cancers that are usually curable, while stage IV usually represents inoperable or metastatic cancer. Stage II and III cancers are usually locally advanced and/or exhibit involvement of local lymph nodes. In general, the higher stage numbers indicate more extensive disease, including greater tumor size and/or spread of the cancer to nearby lymph nodes and/or organs adjacent to the primary tumor. These stages are defined precisely, but the definition is different for each kind of cancer and is known to the skilled artisan.

Many cancer registries, such as the NCI's Surveillance, Epidemiology, and End Results Program (SEER), use summary staging. This system is used for all types of cancer. It groups cancer cases into five main categories:

In situ is early cancer that is present only in the layer of cells in which it began.

Localized is cancer that is limited to the organ in which it began, without evidence of spread.

Regional is cancer that has spread beyond the original (primary) site to nearby lymph nodes or organs and tissues.

Distant is cancer that has spread from the primary site to distant organs or distant lymph nodes.

Unknown is used to describe cases for which there is not enough information to indicate a stage.

In addition, it is common for cancer to return months or years after the primary tumor has been removed. Cancer that recurs after all visible tumor has been eradicated, is called recurrent disease. Disease that recurs in the area of the primary tumor is locally recurrent, and disease that recurs as metastases is referred to as a distant recurrence.

The tumor can be a solid tumor or a non-solid or soft tissue tumor. Examples of soft tissue tumors include leukemia (e.g., chronic myelogenous leukemia, acute myelogenous leukemia, adult acute lymphoblastic leukemia, acute myelogenous leukemia, mature B-cell acute lymphoblastic leukemia, chronic lymphocytic leukemia, polymphocytic leukemia, or hairy cell leukemia) or lymphoma (e.g., non-Hodgkin's lymphoma, cutaneous T-cell lymphoma, or Hodgkin's disease). A solid tumor includes any cancer of body tissues other than blood, bone marrow, or the lymphatic system. Solid tumors can be further divided into those of epithelial cell origin and those of non-epithelial cell origin. Examples of epithelial cell solid tumors include tumors of the gastrointestinal tract, colon, breast, prostate, lung, kidney, liver, pancreas, ovary, head and neck, oral cavity, stomach, duodenum, small intestine, large intestine, anus, gall bladder, labium, nasopharynx, skin, uterus, male genital organ, urinary organs, bladder, and skin. Solid tumors of non-epithelial origin include sarcomas, brain tumors, and bone tumors. Other examples of tumors are described in the Definitions section.

In some embodiments, the patient herein is subjected to a diagnostic test e.g., prior to and/or during and/or after therapy. Generally, if a diagnostic test is performed, a sample may be obtained from a patient in need of therapy. Where the subject has cancer, the sample may be a tumor sample, or other biological sample, such as a biological fluid, including, without limitation, blood, urine, saliva, ascites fluid, or derivatives such as blood serum and blood plasma, and the like.

The biological sample herein may be a fixed sample, *e.g.* a formalin fixed, paraffin-embedded (FFPE) sample, or a frozen sample.

Various methods for determining expression of mRNA or protein include, but are not limited to, gene expression profiling, polymerase chain reaction (PCR) including quantitative real time PCR (qRT-PCR), microarray analysis, serial analysis of gene expression (SAGE), MassARRAY, Gene Expression Analysis by Massively Parallel Signature Sequencing

(MPSS), proteomics, immunohistochemistry (IHC), etc. Preferably mRNA is quantified. Such mRNA analysis is preferably performed using the technique of polymerase chain reaction (PCR), or by microarray analysis. Where PCR is employed, a preferred form of PCR is quantitative real time PCR (qRT-PCR). In one embodiment, expression of one or more of the above noted genes is deemed positive expression if it is at the median or above, *e.g.* compared to other samples of the same tumor-type. The median expression level can be determined essentially contemporaneously with measuring gene expression, or may have been determined previously.

The steps of a representative protocol for profiling gene expression using fixed, paraffin-embedded tissues as the RNA source, including mRNA isolation, purification, primer extension and amplification are given in various published journal articles (for example: Godfrey *et al. J. Molec. Diagnostics* 2: 84-91 (2000); Specht *et al., Am. J. Pathol.* 158: 419-29 (2001)). Briefly, a representative process starts with cutting about 10 microgram thick sections of paraffin-embedded tumor tissue samples. The RNA is then extracted, and protein and DNA are removed. After analysis of the RNA concentration, RNA repair and/or amplification steps may be included, if necessary, and RNA is reverse transcribed using gene specific promoters followed by PCR. Finally, the data are analyzed to identify the best treatment option(s) available to the patient on the basis of the characteristic gene expression pattern identified in the tumor sample examined.

Detection of gene or protein expression may be determined directly or indirectly.

One may determine expression or amplification of hepsin in the cancer (directly or indirectly). Various diagnostic/prognostic assays are available for this. In one embodiment, hepsin overexpression may be analyzed by IHC. Parafin embedded tissue sections from a tumor biopsy may be subjected to the IHC assay and accorded a hepsin protein staining intensity criteria as follows:

Score 0 no staining is observed or membrane staining is observed in less than 10% of tumor cells.

Score 1+ a faint/barely perceptible membrane staining is detected in more than 10% of the tumor cells. The cells are only stained in part of their membrane.

Score 2+ a weak to moderate complete membrane staining is observed in more than 10% of the tumor cells.

Score 3+ a moderate to strong complete membrane staining is observed in more than 10% of the tumor cells.

In some embodiments, those tumors with 0 or 1+ scores for hepsin overexpression assessment may be characterized as not overexpressing hepsin, whereas those tumors with 2+ or 3+ scores may be characterized as overexpressing hepsin.

Alternatively, or additionally, FISH assays may be carried out on formalin-fixed, paraffin-embedded tumor tissue to determine the presence or and/or extent (if any) of hepsin amplification or translocation in the tumor.

Hepsin activation may be determined directly (e.g., by phospho-ELISA testing, or other means of detecting phosphorylated receptor) or indirectly (e.g., by detection of activated downstream signaling pathway components, detection of receptor dimers (e.g., homodimers, heterodimers), detection of gene expression profiles and the like.

Methods for detection of nucleic acid mutations are well known in the art. Often, though not necessarily, a target nucleic acid in a sample is amplified to provide the desired amount of material for determination of whether a mutation is present. Amplification techniques are well known in the art. For example, the amplified product may or may not encompass all of the nucleic acid sequence encoding the protein of interest, so long as the amplified product comprises the particular amino acid/nucleic acid sequence position where the mutation is suspected to be.

A sample comprising a target nucleic acid can be obtained by methods well known in the art, and that are appropriate for the particular type and location of the tumor. Tissue biopsy is often used to obtain a representative piece of tumor tissue. Alternatively, tumor cells can be obtained indirectly in the form of tissues/fluids that are known or thought to contain the tumor cells of interest. For instance, samples of lung cancer lesions may be obtained by resection, bronchoscopy, fine needle aspiration, bronchial brushings, or from sputum, pleural fluid or blood. Mutant genes or gene products can be detected from tumor or from other body samples such as urine, sputum or serum. The same techniques discussed above for detection of mutant target genes or gene products in tumor samples can be applied to other body samples. Cancer cells are sloughed off from tumors and appear in such body samples. By screening such body samples, a simple early diagnosis can be achieved for diseases such as cancer. In addition, the progress of therapy can be monitored more easily by testing such body samples for mutant target genes or gene products.

Means for enriching a tissue preparation for tumor cells are known in the art. For example, the tissue may be isolated from paraffin or cryostat sections. Cancer cells may also be separated from normal cells by flow cytometry or laser capture microdissection. These, as well as other techniques for separating tumor from normal cells, are well known in the art. If

the tumor tissue is highly contaminated with normal cells, detection of mutations may be more difficult, although techniques for minimizing contamination and/or false positive/negative results are known, some of which are described hereinbelow. For example, a sample may also be assessed for the presence of a biomarker (including a mutation) known to be associated with a tumor cell of interest but not a corresponding normal cell, or vice versa.

In some instances, the cancer does or does not overexpress hepsin. Hepsin overexpression may be determined in a diagnostic or prognostic assay by evaluating increased levels of hepsin present on a cell (e.g. via an immunohistochemistry assay; IHC). Alternatively, or additionally, one may measure levels of hepsin-encoding nucleic acid in the cell, e.g. via fluorescent in situ hybridization (FISH; see WO98/45479 published October, 1998), southern blotting, or polymerase chain reaction (PCR) techniques, such as real time quantitative PCR (RT-PCR). Aside from the above assays, various in vivo assays are available to the skilled practitioner. For example, one may expose cells within the body of the patient to an antibody which is optionally labeled with a detectable label, e.g. a radioactive isotope, and binding of the antibody to cells in the patient can be evaluated, e.g. by external scanning for radioactivity or by analyzing a biopsy taken from a patient previously exposed to the antibody.

Chemotherapeutic Agents

The combination therapy of the invention can further comprise one or more chemotherapeutic agent(s). The combined administration includes coadministration or concurrent administration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities.

The chemotherapeutic agent, if administered, is usually administered at dosages known therefor, or optionally lowered due to combined action of the drugs or negative side effects attributable to administration of the antimetabolite chemotherapeutic agent. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner.

Various chemotherapeutic agents that can be combined are disclosed herein.

Formulations, Dosages and Administrations

The therapeutic agents used in the invention will 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 subject 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, the drug-drug interaction of the agents to be combined, and other factors known to medical practitioners.

Therapeutic formulations are prepared using standard methods known in the art by mixing the active ingredient having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences (20^{th} edition), ed. A. Gennaro, 2000, Lippincott, Williams & Wilkins, Philadelphia, PA). Acceptable carriers, include saline, or buffers such as phosphate, citrate and other organic acids; antioxidants including ascorbic acid; 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, asparagines, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN™, PLURONICS™, or PEG.

Optionally, but preferably, the formulation contains a pharmaceutically acceptable salt, preferably sodium chloride, and preferably at about physiological concentrations. Optionally, the formulations of the invention can contain a pharmaceutically acceptable preservative. In some embodiments the preservative concentration ranges from 0.1 to 2.0%, typically v/v. Suitable preservatives include those known in the pharmaceutical arts. Benzyl alcohol, phenol, m-cresol, methylparaben, and propylparaben are preferred preservatives. Optionally, the formulations of the invention can include a pharmaceutically acceptable surfactant at a concentration of 0.005 to 0.02%.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsule prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, *supra*.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g., films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

The therapeutic agents of the invention are administered to a human patient, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. An *ex vivo* strategy can also be used for therapeutic applications. Ex vivo strategies involve transfecting or transducing cells obtained from the subject with a polynucleotide encoding a hepsin antagonist. The transfected or transduced cells are then returned to the subject. The cells can be any of a wide range of types including, without limitation, hemopoietic cells (e.g., bone marrow cells, macrophages, monocytes, dendritic cells, T cells, or B cells), fibroblasts, epithelial cells, endothelial cells, keratinocytes, or muscle cells.

For example, if the hepsin antagonist is an antibody, the antibody is administered by any suitable means, including parenteral, subcutaneous, intraperitoneal, intrapulmonary, and intranasal, and, if desired for local immunosuppressive treatment, intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. In addition, the antibody is suitably

administered by pulse infusion, particularly with declining doses of the antibody. Preferably the dosing is given by injections, most preferably intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic.

In another example, the hepsin antagonist compound is administered locally, e.g., by direct injections, when the disorder or location of the tumor permits, and the injections can be repeated periodically. The hepsin antagonist can also be delivered systemically to the subject or directly to the tumor cells, e.g., to a tumor or a tumor bed following surgical excision of the tumor, in order to prevent or reduce local recurrence or metastasis.

Administration of the therapeutic agents in combination typically is carried out over a defined time period (usually minutes, hours, days or weeks depending upon the combination selected). Combination therapy is intended to embrace administration of these therapeutic agents in a sequential manner, that is, wherein each therapeutic agent is administered at a different time, as well as administration of these therapeutic agents, or at least two of the therapeutic agents, in a substantially simultaneous manner.

The therapeutic agent can be administered by the same route or by different routes. For example, the anti-hepsin antibody in the combination may be administered by intravenous injection while a chemotherapeutic agent in the combination may be administered orally. Alternatively, for example, both of the therapeutic agents may be administered orally, or both therapeutic agents may be administered by intravenous injection, depending on the specific therapeutic agents. The sequence in which the therapeutic agents are administered also varies depending on the specific agents.

Depending on the type and severity of the disease, about 1 μg/kg to 100 mg/kg of each therapeutic agent is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to about 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 is sustained until the cancer is treated, as measured by the methods described above. However, other dosage regimens may be useful.

The present application contemplates administration of the hepsin antibody by gene therapy. See, for example, WO96/07321 published March 14, 1996 concerning the use of gene therapy to generate intracellular antibodies.

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, 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 when combined with another composition(s) 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, such as cancer. 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 agent. The article of manufacture in this embodiment of the invention may further comprise a package insert indicating that the first and second antibody compositions can be used to treat a particular condition, e.g., cancer. 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.

The following are examples of the methods and compositions of the invention. It is understood that various other embodiments may be practiced, given the general description provided above.

EXAMPLES

Materials and Methods

Reagents and proteins: The synthetic para-nitroanilide substrates S2765 (= DiaPharma FXa substrate), S2266, S2288, S2366, S2444 were from DiaPharma (Westchester, OH), Chromozym TH from Roche Diagnostics (Indianapolis, IN), Spectrozyme fIXa® (#299) and Spectrozyme® FVIIa from American Diagnostica (Greenwich, CT). 3,4-dichloro-isocoumarin, BSA and Tween-20 were from Sigma-Aldrich.

Plasmin and factor XIa were from Haematologic Technologies Inc. (Essex Junction, VT), plasma kallikrein from Calbiochem (La Jolla, CA), Factor VII and Factor XIIa from Enzyme Research (South Bend, IN), urokinase-type plasminogen activator (uPA) from

American Diagnostica, pro-uPA from Fitzgerald Industries Int. (Concord, MA). Rat laminin was from Millipore (Temecula, CA). Hepsin (human), matriptase, marapsin, hepatocyte growth factor activator (HGFA), pro-hepatocyte growth factor (pro-HGF), the Kunitz domain inhibitor-1 (KD1) derived from HGFA-inhibitor-1 (HAI-1) and HAI-2 were recombinantly expressed and purified as described previously (Kirchhofer et al., 2003; Kirchhofer et al., 2005; Li et al., 2009; Moran et al., 2006; Peek et al., 2002; Shia et al., 2005). As control antibodies we used anti-HGFA Fab or IgG (Fab40, Fab58, Fab75, IgG75) generated by antibody phage display (Ganesan et al., 2009; Wu et al., 2007).

M13-KO7 helper phage was from New England Biolabs. Maxisorp immunoplates plates were from Nalgen NUNC International (Naperville, IL). Dynabeads® MyOne Streptavidin was from Invitrogen (Carlsbad, CA). 3,3', 5,5'-Tetramethyl-benzidine/H_2O_2 (TMB) peroxidase substrate was from Kirkegaard and Perry Laboratories, Inc. NeutrAvidin and Streptavidin were from Pierce Biotechnology, Inc.

Cloning, Expression, and Purification of Prostasin: The extracellular domain of the human prostasin (Ala^{33}-Leu^{321}) harboring a C-terminal flag-tag was expressed using the baculovirus expression system with T. ni cells. After 72 hour incubation, the supernatant was harvested and cleared medium was loaded on an anti-Flag M2 antibody-agarose column (Sigma). The bound protein was eluted with 100 mM glycine, pH 3.0 and immediately neutralized with 1M Tris pH 8.0. The obtained zymogen form of prostasin was activated with recombinant matriptase at room temperature for 16 hours. Thereafter, the activated two-chain prostasin was purified by removing the $(His)_8$-tagged ("His8" disclosed as SEQ ID NO: 59) matriptase on a Ni-NTA column. Prostasin was further purified by size-exclusion chromatography using a S-200 column.

Cloning, Expression, and Purification of mouse Hepsin: The extracellular domain of the mouse hepsin (Ser^{45}-Pro^{416}) harboring a C-terminal His-tag was expressed using the baculovirus expression system with T. ni cells, under similar conditions as human hepsin (Moran et al., 2006). After 72 hour incubation, the supernatant was harvested and cleared medium was loaded on a Ni-NTA column (Qiagen), which was pre-equilibrated with a buffer containing 50 mM Tris-HCl, pH 8.0, 300 mM NaCl. The bound protein was eluted with a buffer containing 50 mM Tris-HCl, pH 8.0, 300 mM NaCl and 250mM imidazole.

Cloning, expression and purification of recombinant MSP and KD1: Recombinant MSP was expressed in Chinese Hamster Ovary cells as described (Wahl et al., 1997). KD1 was expressed and purified as described (Shia et al., 2005).

Library construction for Fab phage display: The library, designated as YSGX library, was constructed as previously described using a phagemid for Fab-phage display (pF1359) (Lib D in (Fellouse et al., 2007)). The diversity of the library is around 2 x 10^{10}.

Selection and characterization of phage displayed anti-hepsin Fabs : For phage display experiments, we used biotinylated hepsin (=biotin-hepsin) and biotinylated hepsin in complex with 3,4-dichloro-isocoumarin (DCI) (=biotin-hepsin:DCI). Hepsin was biotinylated by using Sulfo-NHS-LC-Biotin kit (Pierce, Catalog #21327) according to manufacturer's instructions. Some of the biotinylated hepsin was used to form a biotin-hepsin:DCI complex by incubation with 100 μM DCI and by maintaining this DCI concentration during subsequent panning experiments. Prior experiments with DCI showed that a 40 minute exposure of hepsin to 25-50 μM DCI completely inhibited hepsin activity in enzymatic assays with S2765 substrate. For the first round of panning, 10 μg of biotin-hepsin or biotin-hepsin:DCI was incubated with 1ml YSGX library at the concentration of 1 x 10^{13}pfu/ml at 4°C for 1.5 h in the absence or presence of 100 μM DCI, respectively. The phage that bound to the target was captured for 15 minutes with 100 μl Dynabeads® MyOne Streptavidin. The bound phage was eluted with 0.1 M HCl, immediately neutralized and then amplified following the standard protocol (Sidhu et al., 2000). From the second round forward, 2 μg of biotin-hepsin or biotin-hepsin:DCI was incubated with 400 μl of amplified phage at 4°C for 1.5 hours without or with 100 μM DCI, respectively. The phage that bound to biotin-hepsin or biotin-hepsin:DCI was captured for 15 minutes with Maxisorp Immunoplates (NUNC) coated with NeutrAvidin or streptavidin (alternatively between rounds) and blocked with blocking buffer (PBS, 0.5%(w/v) BSA). After five rounds of selection, phage were produced from individual clones grown in a 96-well format and the culture supernatants were diluted threefold in PBS, 0.5% (w / v) BSA, 0.1% (v / v) Tween 20 (PBT buffer). The diluted phage supernatants were incubated for 1 hour with biotin-hepsin or biotin-hepsin:DCI that was immobilized on Neutravidin-coated 384-well Maxisorp Immunoplates (NUNC). The plates were washed six times with PBS, 0.05% (v / v) Tween 20 (PT buffer) and incubated 30 minutes with horseradish peroxidase / anti-M13 antibody conjugate (1:5000 dilution in PBT buffer) (Pharmacia). The plates were washed six times with PT buffer and twice with PBS, developed for 15 min with 3,3',5,5'-tetramethyl-benzidine/H_2O_2 peroxidase substrate (Kirkegaard & Perry Laboratories), quenched with 1.0 M H_3PO_4 and absorption measured spectrophotometrically at 450 nm.

A single-point phage competition ELISA was used to identify the phage-displayed Fabs that binds to the same epitope of hepsin where KD1 binds. Individual clones grown in 96-well format and the culture supernatants were diluted fivefold in PBT buffer with or without 200 nM KD1 and incubated with biotin-Hepsin that was immobilized on NeutrAvidin-coated 384-well Maxisorp Immunoplates (NUNC) for 1 hour. The plate was washed and the bound phage was detected by anti-M13-HRP as described above. For each clone, the ratio of A_{450} in absence over in presence of 200 nM KD1 was calculated. Any clones with the ratio over 2 were considered as targeting to the similar epitope as KD1. Such clones were subjected to a detailed phage competition ELISA with KD1, in which a series concentration of KD1 (start from 10μM, 1:3 serial dilution) was used to compete with phage-displayed Fab bound to Hepsin.

Anti-hepsin Fab25 and IgG25 expression and purification: Generally, throughout this application, the IgG form of antibody 25 is designated with prefix Ab and the Fab form of antibody 25 is designated with prefix Fab. A stop codon was introduced between the heavy chain and gene 3 on the phagemid encoding the Fab25. The resulting phagemid was transformed into *E. Coli.* strain 34B8. The single colony was grown overnight at 37°C in 30 ml LB medium supplemented with 50 μg/ml of Carbenicilin. Five ml of the culture was inoculated into 500 ml of complete C.R.A.P. medium (to make 1 liter: 3.57g $(NH_4)_2SO_4$, 0.71g NaCitrate-$2H_2O$, 1.07g KCl, 5.36g Yeast Extract, 5.36g Hycase SF. Add up to 872ml deionized water. Adjust pH with KOH to 7.3. Autoclave. Cool to 55°C and add 110ml 1M MOPS pH 7.3, 11ml 50% Glucose and 7ml 1M $MgSO_4$) supplemented with Carbenicilin (50 μg/ml) and grown at 30°C for 24 hours. The Fab25 protein was purified using protein A agarose resin.

The variable domain of light chain and heavy chain of the selected Fabs were cloned into a pRK5-based plasmid with human light chain or heavy chain (human IgG1) constant domain for transient expression in Chinese hamster ovary (CHO) cells. The IgG25 protein was purified by use of protein A agarose chromatography.

Enzymatic assays with synthetic substrates: Fab25, IgG25, control Fab, or control IgG were incubated with hepsin (1 nM for human and 2 nM for mouse hepsin) in Hepes buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.01% Triton X-100) for 40 min at room temperature. For experiments with 3,4-dichloro-isocoumarin (DCI), hepsin (1 nM) was incubated with DCI in Hepes buffer containing 0.5 % DMSO for 40 min. S2765 was added (final concentration of 0.2 mM) and the linear rates of the increase in absorbance at

405 nm measured on a kinetic microplate reader (Versamax, Molecular Devices, Sunnyvale, CA). Data were expressed as fractional enzyme activity (v_i/v_o) and fitted to a four parameter regression curve fitting program (Kaleidagraph Version 3.6, Synergy Software) to determine the IC_{50} values. Values are the average ± SD of three independent experiments. K_i^{app} values for Fab25 and IgG25 were calculated by fitting the data to the equation for tight binding inhibition systems (Morrison, 1969; Olivero et al., 2005).

To examine Fab25 specificity, a panel of 9 trypsin-like serine proteases were incubated with 1 μM Fab25 for 40 min in Hepes buffer. The appropriate chromogenic substrates were added and the linear rates of the increase in absorbance at 405 nm measured on a kinetic microplate reader. The concentrations of each enzyme and the chromogenic substrate used were as follows: 1 nM Hepsin – 0.5 mM S2765, 0.5 nM matriptase – 0.5 mM S2765, 5 nM prostasin – 0.5 mM S2765, 2 nM plasmin – 0.5 mM S2366, 2 nM plasma kallikrein – 0.5 mM S2366, 0.5 nM Factor XIa – 0.5 mM S2366, 5 nM FXIIa – 0.5 mM S2288, 5 nM uPA – 0.5 mM S2444, 50 nM marapsin – 0.2 mM Spectrozyme® FVIIa, 10 nM HGFA – 0.2 mM Spectrozyme® FVIIa. Data were expressed as fractional enzyme activity (v_i/v_o) and were the average ± SD of 3 independent experiments.

A panel of commercially available pNA substrates, all having a P1 Arg residue, were used to examine hepsin inhibition by Fab25. The 8 substrates were S2765, S2266, S2288, S2366, S2444, Chromozym TH, Spectrozyme fIXa and Spectrozyme FVIIa. The assay was carried out as described above, except that the concentration of Fab25 and control Fab were fixed at 100 nM. The concentration of substrates was 0.5 mM. The data were expressed as per cent inhibition of uninhibited activity (no Fab) and are the average ± SD of four independent experiments.

Enzymatic assays with macromolecular substrates: To measure hepsin-mediated pro-uPA activation, Fab25 was serially diluted in HBSA buffer (20 mM Hepes, pH 7.5, 150 nM NaCl, 5 mM $CaCl_2$, 0.5mg/ml BSA) and incubated with hepsin for 35 min at room temperature. The enzymatic reaction was started by addition of pro-uPA. The concentrations of hepsin and pro-uPA in the reaction mixture were 0.5 nM and 100 nM, respectively. At 45 sec intervals, 50 μl aliquots were transferred to a 96-well plate containing 150 μl/well of the stop reagent HAI-2 (667 nM). After addition of S2444 (0.5 mM) the linear rates of the increase in absorbance at 405 nm was measured on a kinetic microplate reader. Data were expressed as fractional enzyme activity (v_i/v_o) and fitted to a four parameter regression curve fitting program (Kaleidagraph) to determine the IC_{50} values.

For Factor VII activation assays, hepsin was incubated with Fab25 or control Fab for 5 min in Hepes buffer before addition of factor VII. The concentrations in the reaction mixture were: 50 nM hepsin, 500 nM Fab and 8 μM factor VII. After 0.5 h and 2 h incubation at 37°C, aliquots were taken and analyzed by SDS-PAGE (4-20% gradient gel, InVitrogen) under reducing conditions. Gels were stained with SimplyBlue™ (InVitrogen).

In-vitro proteolytic processing of Fab25 by hepsin : Fab25 (3μM) was incubated with 10 nM hepsin for 24 h at room temperature, either in a low-pH buffer (100 mM Mes pH 6.0, 150 mM NaCl) or in a high pH buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl). The reaction was stopped by the addition of 20uL of 2X-sample buffer (with/without β-mercaptoethanol) and boiled at 95°C for 5 minutes. Proteolysis was monitored by gel mobility-shift on a 4-20% (w/v) polyacrylamide gradient gel stained with Coomassie brilliant blue.

Cell migration assay: Cell migration assays were performed as described previously (Tripathi et al., 2008), using a 24-well, 8.0 μm pore size FluoroBlok™ permeable supports (BD Biosciences, Bedford, MA). The underside of the filters were coated with either untreated or treated rat laminin (1 μg/ml). Laminin was co-incubated with hepsin or hepsin-Fab25 complex or phosphate buffered saline solution (PBS) overnight at 4°C. Inserts were then blocked with superblock buffer for 1 hour. DU145 human prostate cancer cells were trypsinized, resuspended in serum-free medium, washed twice with serum-free medium, and cells (20,000) were seeded in the upper chamber of inserts. After 5 hour incubation in 5% CO_2 and 37°C, cells remaining on the upper filter were scraped off gently using a cotton swab and the inserts were gently washed with PBS. Those cells that migrated to the lower chamber were fixed with 500 μl of Methanol for 30 minutes, air dried for 20 min and stained with 500 μl of 10μM YO-PRO-I (Invitrogen, Carlsbad, CA) for 10 minutes. The plate was washed with PBS and the fluorescence was measured in a plate reader (Spectramax M5, Molecular Devices, Sunnyvale, CA) using an excitation wavelength of 485 nm and emission wavelength of 555 nm. Subsequently, the plates were imaged with an inverted microscope (IX81, Olympus) connected to a CCD camera with a 10x objective.

Fab25 binding to hepsin: An Octet-RED system equipped with streptavidin SBC biosensor tips was purchased from ForteBio (Menlo Park, CA). Prior to the start of the experiment, the sensor tips were pre-wet for 15 minutes at 25°C in a buffer containing 50mM Hepes pH 7.5, 150mM NaCl and 0.05% tween-20. Biotinylated hepsin (7.5 μg/ml) was captured on to the streptavidin sensor for 5 minutes with agitation at 1000 rpm. The sensors

were briefly washed in buffer before dipping them on to the sample wells containing Fab25 (2-fold serial dilution starting from 200nM) and buffer control. Association was monitored for 10 minutes and dissociation was followed for 30 minutes. The data was fit to 1:1 binding model using Octet-RED analysis software.

Pro-MSP activation by cell surface expressed hepsin in LnCap cell: The LnCaP-34 cells were generated as described (Moran et al., 2006), to stably overexpress hepsin resulting in 5-fold increased hepsin cell surface expression and 3-fold increased hepsin enzymatic activity compared to the LnCaP-17 cells, which only express endogenous hepsin at relatively low levels, comparable to the parental LnCaP cells. Confluent LnCaP-17 and LnCap-34 cells cultured in 24-well plates were washed with serum-free RPMI-1640 medium and were incubated either alone or with different anti-hepsin inhibitors (1 μM of anti-hepsin antibody Fab25 / 1 μM of KD1 / 1 μM of Ac-KQLR-cmk ("KQLR" disclosed as SEQ ID NO: 12)) in serum-free RPMI-1640 medium for 15 minutes at 37°C prior to addition of ^{125}I-labeled pro-MSP (25 μg/ml). After incubation for 3 h at 37°C aliquots were removed and analyzed by SDS-PAGE (4-20% gradient gel) (Invitrogen, Carlsbad, CA) followed by exposure to X-ray films.

Pro-HGF activation by cell surface expressed hepsin in LnCap cell: Confluent LnCap-34 cells cultured in 24-well plates were washed with serum-free RPMI-1640 medium and were incubated either alone or with recombinant hepsin (10nM) or with different concentrations of Fab25(20 nM – 0.15 nM) in serum-free RPMI-1640 medium for 15 minutes at 37°C prior to addition of ^{125}I-labeled pro-HGF (25 μg/ml). After incubation for 3 h at 37°C aliquots were removed and analyzed by SDS-PAGE (4-20% gradient gel) (Invitrogen, Carlsbad, CA) followed by exposure to X-ray films.

Binding of Ab25 to hepsin by surface plasmon resonance: Surface plasmon resonance measurements were performed on a BIAcore™-3000 instrument (GE Health Care, NJ) to determine binding affinity of the Ab25. Rabbit anti-human IgG were chemically immobilized (amine coupling) on CM5 biosensor chips and Ab25 was captured to give approximately 350 response units (RU). For kinetics measurements, two-fold serial dilutions of active hepsin (1 nM to 500 nM) were injected in HBS-P buffer at 25°C with a flow rate of 30 μl/min. Association rates (k_a) and dissociation rates (k_d) were obtained by using a simple one-to-one Langmuir binding model (BIA-Evaluation) and the equilibrium dissociation constants (K_D) were calculated (k_d/k_a). Due to rapid kinetics, a steady state measurement was used to determine the affinity of pro-hepsin with Ab25. Two-fold serial dilution of pro-hepsin

(195 nM to 100 mM) was injected over captured antibody (Ab25) sensor chip to achieve maximal binding (R_{max}) and to reach the steady state equilibrium. The values of R_{eq} were calculated and plotted individually against C (concentration of pro-hepsin) using BIA-Evaluation to determine K_D.

Isothermal Titration Calorimetry: TC experiments were performed on a ITC_{200} instrument (GE healthcare). Fab25 and hepsin were purified separately on a size exclusion chromatographic column (Superdex S200, GE healthcare), using a buffer containing 50mM HEPES pH 7.5 and 150mM NaCl. The sample cell (204 μl) was loaded with hepsin (14 μM) and the Fab25 concentration in the syringe was 138 μM. A titration experiment typically consisted of 20 injections, each of 2 μL volume and 180 sec duration, with a 3 min interval between additions. The stirring rate was 1000 rpm. Raw data were integrated, corrected for nonspecific heats, normalized for concentration, and analyzed according to a 1:1 binding model assuming a single set of identical binding sites. (The isothermal titration curve was registered and analyzed using ORIGIN software provided with the ITC instrument. The data were integrated to provide a titration curve; and, by using a nonlinear least-squares fit, the binding constant K_A, the heat of binding (ΔH), and the stoichiometry of binding were extracted from the curve.

Results/Discussion

To identify anti-hepsin antibodies that block hepsin enzymatic activity, we employed solution sorting against biotinylated hepsin without inhibitor (biotin-hepsin) and against biotinylated hepsin in complex with DCI (biotin-hepsin:DCI). DCI is a mechanism-based serine protease inhibitor, which occupies the S1 pocket by forming covalent monoacyl or diacyl adducts with the catalytic Ser195 and His57 (chymotrypsinogen numbering) (Fig. 9A) (Powers et al., 1989). A molecular model of hepsin:DCI complex based on the crystal structure of factor-D:DCI complex (PDB code 1DIC) (Cole et al., 1998) indicated that the aromatic ring of the DCI inhibitor would occupy the S1 pocket. Our previous findings (Wu et al., 2007) indicated that function-blocking anti-HGFA antibodies derived from our Fab phage-display libraries do not occupy the S1 pocket. Therefore, hepsin-bound DCI may not interfere with antibody binding, but could exert beneficial influences on the active site conformation, favoring interactions with antibody. The active site of trypsin-like serine proteases is formed by several intrinsically mobile loops (the 'activation domain') (Huber and Bode, 1978). In particular, the 220-loop forms part of the S1 pocket and can adopt various conformational states in some serine proteases (Johnson et al., 2005; Shia et al., 2005;

Spraggon et al., 2009; Wilken et al., 2004). Only the apo-structures revealed such unusual loop conformations, whereas the co-crystal structures with an active site inhibitor showed properly formed active sites, most likely due to stabilizing forces applied by the inhibitor (Arni et al., 1994; Shia et al., 2005; Spraggon et al., 2009). The hepsin apo-structure is unknown and all published hepsin structures are co-crystal structures with an active site inhibitor (Herter et al., 2005; Somoza et al., 2003).Therefore, we reasoned that occupancy of the S1 pocket by DCI may apply stabilizing forces on potential loop flexibility, favoring antibody recognition. Enzymatic assays showed that DCI concentrations above 20 μM resulted in complete hepsin inhibition after a 40 min incubation period (Fig. 9). Therefore, the phage sorting experiments against biotinylated hepsin:DCI complex was carried out in the presence of 100 μM DCI.

A minimalist synthetic antibody library with restricted chemical diversity at complementary determining regions (CDR), designated as YSGX library (Fellhouse et al, 2007), was used to search for an inhibitory antibody against hepsin by solution sorting, in which biotin-hepsin or biotin-hepsin:DCI complex was incubated with the phage-displayed Fab library. Panning against biotin-hepsin:DCI resulted in one hepsin-binding clone, designated HpsFab25 (also termed "Fab25"), which became dominant after 5 rounds of selection. The HVR sequences of Fab25 are shown in Figure 1. The CDR-H3 of HpsFab25 is very long (21 residues) and HpsFab25 contained three Lys residues.

Elisa experiments were performed to test whether Fab25 binding was inhibited by KD1, a hepsin inhibitor that binds to hepsin active site. As shown in Figure 10, KD1 inhibited Fab25-phage binding to hepsin in a concentration-dependent fashion, suggesting that Fab25 bound to or near the active site region of hepsin and, thus, may interfere with enzyme activity.

The ability of Fab25 to inhibit human and mouse activity was tested using synthetic hepsin substrate, S2765. As shown in Figure 11, Fab 25 inhibited human and mouse hepsin activity in a concentration-dependent manner, reaching complete inhibition at the highest concentrations tested. The calculated K_i^{app} was 4.1 ± 1.0 nM (n=3) for human hepsin and 329 ± 51 nM (n=3) for murine hepsin. Additional experiments with IgG25 showed that it inhibited human hepsin with a K_i^{app} of 11.3 ± 1.7 nM (n=3), while a control IgG had no effect (data not shown). The results showed that Fab25 inhibited human hepsin about 80-fold more potently than murine hepsin. This suggested that the antibody binding site is not fully conserved in mouse hepsin. Because the protease domain of mouse hepsin has neither

insertions nor deletions compared to human hepsin, the reduced potency of Fab25 was probably due to changes in amino acids that are important for the interaction with Fab25.

Binding affinity of Fab25 to human hepsin was determined using Octet RED analysis. The Kd was 2.55 +/- 0.45 nM.

Specificity of Fab 25 was tested by asking whether it inhibited enzymatic activity of a panel of trypsin-like serine proteases. The panel of trypsin-like serine proteases included some of the closest protease domain homologues of hepsin, such as plasma kallikrein, prostasin, marapsin and plasmin. The results (Figure 12) demonstrated that Fab25 had an exclusive specificity towards hepsin, since it did not affect enzymatic activity of the examined serine proteases at a concentration that was 250-fold greater than the determined K_i^{app} value for hepsin.

The effect of Fab25 on hepsin-mediated processing of pro-uPA and Factor VIII was determined. As shown in Figure 13A, Fab25 inhibited hepsin activity towards pro-uPA with an IC_{50} value of 3.3 ± 0.4 nM (n=3), which was comparable to its potency in the pNA assay (Table 1). At Fab concentrations above 100 nM the inhibition was complete and comparable to the strong inhibition in the factor VII activation assay (Figure 13B). Factor VII is a relatively poor substrate for hepsin, requiring high hepsin concentrations in the assay (50 nM). Therefore, even though Fab25 was used at 500 nM this resulted in a relatively low Fab25:hepsin ratio (10:1) compared to the pro-uPA activation experiments (ratios up to 600:1), which may explain that inhibition during the extended 2 h experiment was not complete.

Hepsin preferentially cleaves substrates after a P1 Arg (Herter et al., 2005), yet also recognizes substrates with P1 Lys (Moran et al., 2006). Therefore, we considered the possibility that hepsin may cleave the Fab CDR-H3 loop, which contained three Lys residues and was unusually long. Precedence comes from recent studies on the anti-matriptase antibody E2, which underwent cleavage by matriptase after a P1-Arg residue in a long CDR-H3 loop (Farady et al., 2008). Therefore, Fab25 was exposed to hepsin for prolonged time periods under different pH conditions. Fab25 migrated as a 50 kDa band under non-reducing and as a 25 kDa band under reducing conditions and no low molecular weight degradation products could be indentified (Figure 14). Therefore, Fab25 was resistant to hepsin proteolysis when exposed to hepsin for prolonged time periods. This conclusion is consistent with our assumption that the biotin-hepsin:DCI complex favored selection of phage-displayed Fab that bind outside the S1 pocket.

Laminin was recently established to be a substrate for hepsin (Tripathi et al). The study also suggested that cleavage of laminin by hepsin may physiologically enhance migration of the prostate cancer cell line DU145. We examined whether Fab25 inhibited laminin-dependent migration of DU145 cell. The result s of this experiment (Figure 15) demonstrated that Fab25 effectively neutralized the proteolytic activity of hepsin and thus blocked the processing of its substrate laminin.

We also tested the ability of Fab25 to inhibit hepsin activity towards a panel of synthetic substrates. The results shown in Table 1 demonstrated that Fab25 inhibited hepsin-mediated cleavage of all pNA substrates by >90%. Because the pNA substrates occupy the S1-S3 subsites on hepsin, it can be concluded that the antibody strongly interfered with substrate interactions at these subsites. The finding that despite the chemical diversity of the pNA substrates' P2 and P3 positions, inhibition was >90% for all substrates argues for a strong antibody effect at the S2 and/or S3 sites rather than for subtle influences at these sites. Whether or not these effects are allosteric in nature or direct steric hindrance remains to be elucidated. Furthermore, because Fab25 was selected against DCI-inhibited hepsin, it is unlikely that Fab would inhibit hepsin by directly occupying the S1 pocket.

Table 1. Inhibition by Fab25 of hepsin activity towards a panel of synthetic substrates. Hepsin was incubated with 100 nM Fab25 or control Fab for 40 min before addition of 8 different pNA substrates. The initial linear velocities were measured on a kinetic microplate reader and enzyme activity was expressed as percent of hepsin activity without Fab present.

Table 1

Substrate	Fab25[a] (% of control)[b]	Control Fab[a] (% of control)[b]
S2765	2.2 ± 0.7	100.4 ± 2.3
S2266	3.0 ± 1.3	97.3 ± 3.9
S2288	2.5 ± 0.8	98.5 ± 1.9
S2366	3.4 ± 0.5	99.9 ± 2.7
S2444	1.7 ± 0.9	99.5 ± 1.8
Chromozyme TH	8.4 ± 1.2	99.0 ± 4.6
Spectrozyme FIXa	-1.6 ± 2.1	112.1 ± 8.5
Spectrozyme FVIIa	3.4 ± 0.8	101.8 ± 4.8

[a] 100 nM Fab in reaction mixture
[b] control was hepsin enzymatic without Fab

Fab25 was shown to consistently inhibit hepsin activity by >90% in all functional assays using a variety of synthetic and macromolecular substrates.

Proteolytic processing of pro-MSP by natively expressed hepsin on the cell surface was monitored on the LnCap-34 cell line (Moran et al., 2006). LnCap-34 cells expressing hepsin was capable of processing the ^{125}I-pro-MSP (Fig.16). The proteolytic activity of pro-MSP processing was mainly due to hepsin as all the three hepsin inhibitors (Ac-KQLR-chloromethylketone ("KQLR" disclosed as SEQ ID NO: 12), KD1 and Fab25) effectively blocked the proteolytic cleavage.

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Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, the descriptions and examples should not be construed as limiting the scope of the invention.

WHAT IS CLAIMED IS:

1. An isolated anti-hepsin antibody, wherein a monovalent form of the antibody binds human hepsin with an affinity of less than or equal to 10 nM or better, wherein a monovalent form of the antibody binds mouse hepsin with an affinity of less than or equal to 330 nM, and wherein the antibody binds hepsin present in a complex comprising hepsin and a serine protease inhibitor that binds the human hepsin S1 subsite.

2. The antibody of claim 1, wherein the serine protease inhibitor is 3,4-dichloro-isocoumarin (DCI).

3. The antibody of claim 1, wherein the antibody binds a Kunitz domain binding region of hepsin.

4. The antibody of claim 1, wherein the antibody blocks substrate binding to hepsin subsite 2 and/or hepsin subsite 3.

5. The antibody of claim 1, wherein the antibody inhibits hepsin-mediated activation of macrophage stimulating protein (MSP).

6. The antibody of claim 1, wherein the antibody inhibits laminin-dependent cell migration.

7. The antibody of claim 1, wherein the antibody comprises: at least one, two, three, four, five, and/or six hypervariable region (HVR) sequences selected from the group consisting of:

(a) HVR-L1 comprising sequence RASQSVSSAVA (SEQ ID NO:1);

(b) HVR-L2 comprising sequence SASSLYS (SEQ ID NO:2);

(c) HVR-L3 comprising sequence QQYYSSYYLLT (SEQ ID NO:3);

(d) HVR-H1 comprising sequence GFNFSYSYMH (SEQ ID NO:4);

(e) HVR-H2 comprising sequence ASIYSYYGSTYYADSVKG (SEQ ID NO:5); and

(f) HVR-H3 comprising sequence ARSDSWSYKSGYTQKIYSKGLDY (SEQ ID NO:6).

8. The antibody of claim 7, wherein the antibody comprises (a) HVR-H3 comprising sequence ARSDSWSYKSGYTQKIYSKGLDY (SEQ ID NO:6); (b) HVR-L3 comprising sequence QQYYSSYYLLT (SEQ ID NO:3); and HVR-H2 comprising sequence ASIYSYYGSTYYADSVKG (SEQ ID NO:5).

9. The antibody of claim 7, wherein the antibody comprises (a) HVR-H1 comprising sequence GFNFSYSYMH (SEQ ID NO:4); (b) HVR-H2 comprising sequence ASIYSYYGSTYYADSVKG (SEQ ID NO:5); and (c) HVR-H3 comprising sequence ARSDSWSYKSGYTQKIYSKGLDY (SEQ ID NO:6).

10. The antibody of claim 9, further comprising (a) HVR-L1 comprising sequence RASQSVSSAVA (SEQ ID NO:1); (b) HVR-L2 comprising sequence SASSLYS (SEQ ID NO:2); and (c) HVR-L3 comprising sequence QQYYSSYYLLT (SEQ ID NO:3).

11. The antibody of claim 7, wherein the antibody comprises (a) HVR-L1 comprising sequence RASQSVSSAVA (SEQ ID NO:1); (b) HVR-L2 comprising sequence SASSLYS (SEQ ID NO:2); and (c) HVR-L3 comprising sequence QQYYSSYYLLT (SEQ ID NO:3).

12. The antibody of claim 9 or 11, further comprising a heavy chain variable domain or light chain variable domain framework sequence shown in Figure 3, 4, 5, or 6.

13. The antibody of claim 1, comprising (a) a VH sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO:10; or having at least 95% sequence identity to the amino acid sequence of SEQ ID NO:9; or (c) a VH sequence as in (a) and a VL sequence as in (b).

14. The antibody of claim 13, comprising a VH sequence of SEQ ID NO:10.

15. The antibody of claim 13, comprising a VL sequence of SEQ ID NO:9.

16. The antibody of claim 13, comprising a VH sequence of SEQ ID NO:10 and a VL sequence of SEQ ID NO:9.

17. The antibody of any of the preceding claims, which is a full length IgG1 antibody.

18. The antibody of any of the preceding claims, wherein the antibody comprises human κ subgroup consensus framework sequence.

19. The antibody of any of the preceding claims, wherein the antibody comprises heavy chain human subgroup III consensus framework sequence.

20. Isolated nucleic acid encoding the antibody of any of the preceding claims.

21. A host cell comprising the nucleic acid of claim 20.

22. A method for making an antibody comprising culturing the host cell of claim 21 so that the antibody is produced.

23. The method of claim 22, further comprising recovering the antibody from the host cell culture.

24. An immunoconjugate comprising the antibody of claim 1 and a cytotoxic agent.

25. A pharmaceutical formulation comprising the antibody of claim 1 and a pharmaceutically acceptable carrier.

26. The pharmaceutical formulation of claim 25, further comprising an additional therapeutic agent.

27. The antibody of claim 1 for use as a medicament.

28. The antibody of claim 1 for use in treating cancer.

29. The antibody of claim 1 for use in treating an immune disorder.

30. The antibody of claim 1 for use in inhibiting cell migration.

31. The antibody of claim 1 for use in inhibiting hepsin enzymatic activity.

32. Use of the antibody of claim 1 in the manufacture of a medicament.

33. The use of claim 32, wherein the medicament is for treatment of cancer.

34. The use of claim 32, wherein the medicament is for treatment of an immune disorder.

35. The use of claim 32, wherein the medicament is for inhibiting cell migration or inhibiting hepsin enzymatic activity.

36. A method of making an anti-serine protease antibody, comprising selecting antibody that bind serine protease present in a complex comprising (a) serine protease; and (b) a serine protease inhibitor that binds a S1 subsite.

37. The method of claim 36, wherein the serine protease is hepsin.

38. The method of claim 36, wherein the serine protease inhibitor is 3,4-dichloro-isocoumarin.

39. The method of claim 36, wherein the serine protease inhibitor binds hepsin catalytic amino acid residues Ser195 and His57, whereby hepsin is inactivated.

40. The method of claim 36, wherein prior to selecting the antibody, the antibody is incubated with serine protease and serine protease inhibitor.

41. The method of claim 37, further comprising selecting antibodies that compete for hepsin binding with a Kunitz domain.

42. The method of claim 41, wherein the Kunitz domain is KD1.