CELL MODULATION

FIELD OF THE INVENTION

The present invention provides molecules for use in modulating cell growth and/or activity and for use in methods of treating or preventing cancer, its migration and/or spread.

BACKGROUND TO THE INVENTION

It is well known that some lectins, which are glycoproteins of non-immune origin, exhibit an ability to induce apoptosis in malignant cells and thus demonstrate anti-cancer properties. This phenomenon occurs, in part, through the interaction of these lectins with specific glycan receptors on immune cells (Yau *et al.*, 2015).

One such lectin is Viscumin from mistletoe, which is a toxin that binds to cellular receptors that are glycosylated with α2,6 sialyllactose (Müthing *et al.*, 2002). Viscumin is a 57kDa heterodimer, comprising of two subunits A and B. The A subunit exerts its toxic effect by disabling ribosomes, thereby interrupting protein production, whereas the B subunit exhibits glycan binding function. The lectin demonstrates picomolar cytotoxicity *in vitro* and *in vivo*, and has a recommended dose upper limit of 6 μg/kg in clinical trial subjects (half-life of 13 mins) (Zwierzina *et al.*, 2011).

Another plant lectin that has demonstrated an ability to prevent cell migration and growth (and hence an anti-cancer property) is the *Maackia amurensis* seed lectin, or MASL (Ochoa-Alvarez *et al.*, 2012; Astarita *et al.*, 2012). This lectin is also cytotoxic exhibiting nanomolar potency (~300nM), and exerts its effects through the binding of podoplanin, an α2,3 sialylated mucin-type transmembrane glycoprotein, that is overexpressed in a variety of human cancers (Kato *et al.*, 2005; Schacht *et al.*, 2005; Shibahara *et al.*, 2006).

The treatment of cell proliferation and differentiation disorders, including, for example cancer, demands the provision of additional molecules that are well tolerated in their hosts and have therapeutic potential.

SUMMARY OF THE INVENTION

The present disclosure is based on the finding that molecules which bind sialic acid also modulate aspects of cell growth and/or cell activity.

Throughout this specification, the terms "comprise", "comprising" and/or "comprises" is/are used to denote that aspects and embodiments of this invention "comprise" a particular feature or features. It should be understood that this/these terms may also encompass aspects and/or embodiments which "consist essentially of" or "consist of" the relevant feature or features.

In a first aspect, there is provided a sialic acid binding molecule for use in a method of modulating cell growth and/or cell activity.

In a second aspect, there is provided a method of modulating cell growth and/or activity, said method comprising contacting a cell with a sialic acid binding molecule. The method may be an *in vitro* method.

The term "modulating" may embrace any increase or decrease in one or more aspects of cell growth and/or activity. In other words, a sialic acid binding molecule described herein may either inhibit certain aspects of cell growth and/or activity or may induce or stimulate other aspects of cell growth and/or activity.

The terms "growth" and "activity" as applied to cells may embrace processes and/or phenomena associated with one or more of cell proliferation, cell viability, cell migration, cell metabolism, cell differentiation and/or cell morphology/phenotype. The terms "growth" and/or "activity" may further include the response of a cell to certain exogenous and/or endogenous factors or stimuli including, for example, responses to certain compounds of the immune system, cytokines, chemokines and one or more environmental factors (light, temperature, pressure, mechanical stress and the like). Thus, the sialic acid binding molecules disclosed herein may be used to modulate (inhibit, decrease or increase) levels of cell responsiveness.

Given that sialic acid binding molecules have been shown to modulate cell growth and activity (as described above), it will be appreciated that there are also a number of related medical and veterinary applications and uses for these sialic acid binding molecules. For example, a sialic acid binding molecule may be applied to the treatment and/or prevention of diseases in which aberrant cell growth, and/or aberrant cell activity is a factor.

Thus, there is provided a sialic acid binding molecule for use in treating and/or preventing a disease and/or condition caused, contributed to and/or characterised by aberrant cell growth and/or activity.

Further, there is provided use of a sialic acid binding molecule for the manufacture of a medicament for the treatment and/or prevention of a disease and/or condition caused, contributed to and/or characterised by aberrant cell growth and/or activity.

There is also provided a method of treating and/or preventing a disease and/or condition caused, contributed to and/or characterised by aberrant cell growth and/or activity, said method comprising the step of administering a therapeutically effective amount of a sialic acid binding molecule to a subject in need thereof.

Diseases which are caused, contributed to or characterised by aberrant cell growth and/or activity may include, for example cell proliferation and/or differentiation disorders including, those referred to or classified as benign or malignant conditions. For example, the term "cell proliferation and/or differentiation disorders" may include those diseases and/or conditions collectively referred to as "cancer". The term "cancer" may include, but is not limited to, those cancers referred to as forms of breast cancer, bone cancer, brain cancer (gliomas), pancreatic cancer, lung cancer, prostate cancer, skin cancer, ovarian cancer,

cervical cancer, head and neck cancers and bowel/colon cancer. The term "cancer" may also include those diseases and/or conditions collectively referred to as "leukaemias" (both chronic and acute) and any cancer affecting a mucosal/mucosal associated surface or tissue.

As such, a sialic acid binding molecule described herein may find application in the treatment and/or prevention of cancer.

Thus, there is provided a sialic acid binding molecule for use in treating and/or preventing cancer.

Further, there is provided use of a sialic acid binding molecule for the manufacture of a medicament for the treatment and/or prevention of cancer.

There is also provided a method of treating and/or preventing cancer, said method comprising the step of administering a therapeutically effective amount of a sialic acid binding molecule to a subject in need thereof.

A subject in need thereof or indeed a subject to be administered a sialic acid binding molecule disclosed herein or a medicament comprising the same, may be any subject suffering (or suspected as suffering) from (i) a cell proliferation and/or differentiation disorder, (ii) cancer, (iii) any other disease and/or condition described herein; or (iv) a disease or condition caused, contributed to or characterised by aberrant cell growth and/or activity. Additionally, or alternatively, any subject may be a subject predisposed or susceptible to (i) a cell proliferation and/or differentiation disorder, (ii) cancer, (iii) any other disease and/or condition described herein; or (iv) a disease or condition caused, contributed to or characterised by aberrant cell growth and/or activity.

It should be understood that the treatment of a cell proliferation and/or differentiation disorder, a cancer or a disease or condition caused or contributed to (or characterised by) aberrant cell growth and/or activity, may involve the use of one or more sialic acid binding molecule(s) of this disclosure to treat, ameliorate or reduce, one or more symptoms of those diseases. By way of example, the symptoms of a disease such as cancer may include, for example, the presence of tumours and/or cell masses. As such, the sialic acid binding molecules described herein may be used to modulate (for example stop, retard, inhibit or reduce) tumour formation and/or the metastasis thereof. The sialic acid binding molecules may also be used to reduce the overall size of a tumour. Certain tumours, including those that are large and/or aggressive, are often easier to surgically remove if they have first been reduced in size. Typically, chemo- and/or radiotherapy based treatments might be used to reduce the size of a tumour but treatments such as this may be replaced by and/or supplemented with sialic acid binding molecule based treatments. As stated, molecules which exhibit sialic acid molecule binding activity exhibit an ability to modulate cell growth and/or activity and therefore, without wishing to be bound by theory, the mechanism

underpinning the ability of a sialic acid binding molecule to affect the size of a tumour may be rooted in the cell proliferation, differentiation and/or metabolism modulating effects of the molecule.

In view of the above, the successful treatment of a tumour may therefore be characterised by a reduction in tumour size, a reduction in an observed or detectable/detected level of tumour metastasis, angiogenesis within tumorigenic tissue and/or tissue invasion.

Thus, sialic acid binding molecules may be for use in methods of modulating (for example inhibiting, restricting or reducing) tumour growth, development and/or metastasis in subjects in need thereof. The sialic acid binding molecules described herein may be formulated as compositions for use in modulating tumour growth, development and/or metastasis or used in the manufacture of medicaments for achieving the same. This disclosure also provides a sialic acid binding molecule for use in treating a tumour. Further, described is the use of a sialic acid binding molecule for the manufacture of a medicament for treating a tumour. Also, the disclosure provides a method of treating a tumour, said method comprising administering a sialic acid binding molecule to a subject (or tumorigenic tissue) in need thereof.

As defined earlier, the term "a subject in need thereof" may embrace any subject suspected as having a tumour or diagnosed with a tumour and/or subjects that are identified as being predisposed and/or susceptible to tumours.

The present disclosure therefore provides various applications for sialic acid binding molecules which have been identified as modulators of cell growth and/or activity. Any given sialic acid binding molecule may be identified as a modulator of cell growth and/or activity via the various experiments and assays described in the examples section of this patent application. For example, the cell wound scratch assay is one example of an assay that may be adapted to determine whether any given "test" molecule exhibits the necessary ability to modulate cell growth and/or activity.

Thus, the invention relates to those sialic acid binding molecules which via a cell activity modulation assay (for example a cell wound scratch assay) exhibit an ability to modulate cell growth and/or activity. To this end, the disclosure further provides a method of identifying sialic acid binding molecules for use in methods of modulating cell growth and/or activity or for the various medical and/or veterinary applications described herein, said method comprising subjecting a test compound to an assay capable of reporting an effect of the test compound on cell growth and/or activity, wherein the test compound is a sialic acid binding compound and any if the assay reports that the compound has an effect on any aspect of cell growth and/or activity, the compound may be useful in the treatment and/or prevention of diseases and/or conditions of the type described herein. The assay which is

capable of reporting an effect of the test compound on cell growth and/or activity may be a cell wound scratch assay as described herein.

The present disclosure relates to molecules and compounds which exhibit an ability to bind to sialic acid. These molecules may take any form and/or belong to any class of molecule or compound (for example they may be proteins, peptides, carbohydrates, antibodies and the like) and term "sialic acid" embraces all forms of *N*- or *O*-substituted neuraminic acid and includes all synthetic, naturally occurring and/or modified forms thereof. Sialic acids may be found as components of cell surface molecules, glycoproteins and glycolipids. Most often, sialic acids are present at the end (terminal regions) of sugar chains connected to cell membranes and/or proteins. For example, some cells of the human upper respiratory tract comprise α-2,6-linked sialic acid receptors and other cells of the upper and lower respiratory tracts comprise α-2,3-linked sialic acid receptors. The sialic acid family encompasses a number (approximately 50) of derivatives that may result from acetylation, glycolylation, lactonisation and methylation at C4, C5, C7, C8 and C9. All such derivatives are to be embraced by the term "sialic acid".

Furthermore, sialic acids are found linked α(2,3) or α(2,6) to Gal and GalNAc or α(2,8) or α(2,9) to another sialic acid. Accordingly, it is important to understand that while the term "sialic acid" is used throughout this specification, it encompasses all derivatives, analogues or variants (either naturally occurring or synthetically generated) thereof as well as monomers, dimers, trimers, oligomers, polymers or concatamers comprising the same.

Thus, a sialic acid binding molecule of this disclosure (and for use as described herein) comprises a moiety which exhibits an affinity for sialic acid – including all forms of sialic acid described above and any form of sialic acid present on the surface of a cell, for example a mammalian cell. These various forms of sialic acid may be collectively referred to as "sialic acid moieties"

The sialic acid binding molecules of this disclosure exhibit an affinity for sialic acid and as such they may bind/couple to and/or associate with one or more sialic acid moieties. Thus, the term "sialic acid binding molecule" may further encompass any fragment of a whole sialic acid binding molecule which retains an ability to bind to or otherwise couple or associate with a sialic acid moiety.

Sialic acid binding molecules for use may comprise a single sialic acid binding molecule (a monomeric or monovalent molecule, for example) or, alternatively, two or more sialic acid binding molecules - which two or more molecules may be the same or different – a polymeric or multivalent molecule, for example.

A sialic acid binding molecule for use may comprise, consist essentially of or consist of, one or more of the sialic acid binding molecules known as "carbohydrate binding

modules" (CBMs). CBMs suitable for use exhibit an affinity for sialic acid. Carbohydrate binding modules are classified into families and CBMs classed as members of the family 40 CBMs (CBM40) may be useful. The family 40 CBMs embrace molecules of approximately 200 residues and are often found at the N-terminus of GH33 sialidases. They may also be found inserted in the β-propeller of GH33 sialidases.

Exemplary carbohydrate binding modules for use may comprise the sialic acid binding domain of *Vibrio cholerae* NanH sialidase (*Vc*CBM: a CBM40) and/or the equivalent (or homologous) domain from *Streptococcus pneumoniae* NanA sialidase (*Sp*CBM: also a CBM40). Of course, similar or homologous sialic acid binding modules present in other organisms are to be encompassed within the scope of the term "CBM".

An exemplary *Vibrio cholerae* NanH sialidase amino acid sequence is deposited under accession umber A5F7A4 and is reproduced below as SEQ ID NO: 1 (781 amino acids).

MRFKNVKKTA LMLAMFGMAT SSNAALFDYN ATGDTEFDSP AKQGWMQDNT NNGSGVLTNA
DGMPAWLVQG IGGRAQWTYS LSTNQHAQAS SFGWRMTTEM KVLSGGMITN YYANGTQRVL
PIISLDSSGN LVVEFEGQTG RTVLATGTAA TEYHKFELVF LPGSNPSASF YFDGKLIRDN
IQPTASKQNM IVWGNGSSNT DGVAAYRDIK FEIQGDVIFR GPDRIPSIVA SSVTPGVVTA
FAEKRVGGGD PGALSNTNDI ITRTSRDGGI TWDTELNLTE QINVSDEFDF SDPRPIYDPS
SNTVLVSYAR WPTDAAQNGD RIKPWMPNGI FYSVYDVASG NWQAPIDVTD QVKERSFQIA
GWGGSELYRR NTSLNSQQDW QSNAKIRIVD GAANQIQVAD GSRKYVVTLS IDESGGLVAN
LNGVSAPIIL QSEHAKVHSF HDYELQYSAL NHTTTLFVDG QQITTWAGEV SQENNIQFGN
ADAQIDGRLH VQKIVLTQQG HNLVEFDAFY LAQQTPEVEK DLEKLGWTKI KTGNTMSLYG
NASVNPGPGH GITLTRQQNI SGSQNGRLIY PAIVLDRFFL NVMSIYSDDG GSNWQTGSTL
PIPFRWKSSS ILETLEPSEA DMVELQNGDL LLTARLDFNQ IVNGVNYSPR QQFLSKDGGI
TWSLLEANNA NVFSNISTGT VDASITRFEQ SDGSHFLLFT NPQGNPAGTN GRQNLGLWFS
FDEGVTWKGP IQLVNGASAY SDIYQLDSEN AIVIVETDNS NMRILRMPIT LLKQKLTLSQ
N

The CBM region of SEQ ID NO: 1 is from amino acid residue 25 to 216 – this sequence may be SEQ ID NO: 2.

An exemplary *Streptococcus pneumoniae* NanA sialidase amino acid sequence has been deposited under accession number P62575 and is reproduced below as SEQ ID NO: 3 (1035 amino acids).

MSYFRNRDID IERNSMNRSV QERKCRYSIR KLSVGAVSMI VGAVVFGTSP VLAQEGASEQ
PLANETQLSG ESSTLTDTEK SQPSSETELS GNKQEQERKD KQEEKIPRDY YARDLENVET
VIEKEDVETN ASNGQRVDLS SELDKLKKLE NATVHMEFKP DAKAPAFYNL FSVSSATKKD
EYFTMAVYNN TATLEGRGSD GKQFYNNYND APLKVKPGQW NSVTFTVEKP TAELPKGRVR
LYVNGVLSRT SLRSGNFIKD MPDVTHVQIG ATKRANNTVW GSNLQIRNLT VYNRALTPEE

VQKRSQLFKR SDLEKKLPEG AALTEKTDIF ESGRNGKPNK DGIKSYRIPA LLKTDKGTLI
AGADERRLHS SDWGDIGMVI RRSEDNGKTW GDRVTITNLR DNPKASDPSI GSPVNIDMVL
VQDPETKRIF SIYDMFPEGK GIFGMSSQKE EAYKKIDGKT YQILYREGEK GAYTIRENGT
VYTPDGKATD YRVVVDPVKP AYSDKGDLYK GNQLLGNIYF TTNKTSPFRI AKDSYLWMSY
SDDDGKTWSA PQDITPMVKA DWMKFLGVGP GTGIVLRNGP HKGRILIPVY TTNNVSHLNG
SQSSRIIYSD DHGKTWHAGE AVNDNRQVDG QKIHSSTMNN RRAQNTESTV VQLNNGDVKL
FMRGLTGDLQ VATSKDGGVT WEKDIKRYPQ VKDVYVQMSA IHTMHEGKEY IILSNAGGPK
RENGMVHLAR VEENGELTWL KHNPIQKGEF AYNSLQELGN GEYGILYEHT EKGQNAYTLS
FRKFNWDFLS KDLISPTEAK VKRTREMGKG VIGLEFDSEV LVNKAPTLQL ANGKTARFMT
QYDTKTLLFT VDSEDMGQKV TGLAEGAIES MHNLPVSVAG TKLSNGMNGS EAAVHEVPEY
TGPLGTSGEE PAPTVEKPEY TGPLGTSGEE PAPTVEKPEY TGPLGTAGEE AAPTVEKPEF
TGGVNGTEPA VHEIAEYKGS DSLVTLTTKE DYTYKAPLAQ QALPETGNKE SDLLASLGLT
AFFLGLFTLG KKREQ

The CBM region of SEQ ID NO: 3 is from amino acid residue 121 to 305 – this sequence may be SEQ ID NO: 4.

Thus, CBMs for use as sialic acid binding molecules in the various aspects and embodiments of this disclosure may comprise a protein or peptide having the sequence of SEQ ID NO: 1 or 2 or a sialic acid binding fragment thereof.

A useful sialic acid binding molecule may comprise a proteinaceous moiety encoded by the sialic acid binding domain of the *nan*H gene (encoding sialidase) of *V. cholerae* (as provided by SEQ ID NO: 1) or an equivalent or homologous gene present in another organism (for example the equivalent/homologous *nan*A sialidase gene of *S. pneumoniae*: see SEQ ID NO: 3).

A sialic acid binding molecule for use may comprise from about residue 1, 5, 10, 15, 25 or 30 (i.e. from 1-30 or from any amino acid residue there between) to about residue 150, 175, 200, 210, 216, 220-781 (to any residue from 150 to 781 including any residue therebetween) of the *V. cholerae* sialidase molecule of SEQ ID NOS: 1 and 2. For example, a sialic acid binding molecule for use may comprise a peptide having a sequence corresponding to residue 25 to about residue 216 of SEQ ID NO: 1 above.

A further suitable sialic acid binding molecule may comprise a protein or peptide having the sequence of SEQ ID NO: 3 or 4 or a sialic acid binding fragment thereof. For example, a useful sialic acid binding molecule may comprise a proteinaceous moiety encoded by the sialic acid binding domain of the *Streptococcus pneumoniae nan*A gene (encoding sialidase). For example, a sialic acid binding molecule for use may comprise from about residue 80, 90, 100, 110, 120, 121 to 130 (i.e. from any of about residues 80 to 130 including any residue therebetween) to about residue 250, 275, 300, 305, 310, 320-1035 (i.e. to any residue from about 250-1035 including to about any residue therebetween) of the

S. pneumoniae sialidase molecule of SEQ ID NOS: 3 and 4. For example, a sialic acid binding molecule for use may comprise a peptide having a sequence corresponding to residue 121 to about residue 305 of SEQ ID NO: 3 above.

A sialic acid binding molecule for use may comprise one or more CBMs. For example, suitable sialic acid binding molecules may comprise single CBMs – for example, a single *Vc*CBM or a single *Sp*CBM. Alternatively, a sialic acid binding molecule for use may comprise a plurality or multiple (i.e. two or more) CBMs. Sialic acid binding molecules which comprise a plurality of CBMs may be termed "multivalent sialic acid binding molecules" or "multivalent CBMs". A multivalent CBM may, for example, comprise two or more *Vc*CBMs or two or more *Sp*CBMs. A multivalent CBM may comprise a mixture of different CBMs, for example one or more *Vc*CBMs with one or more *Sp*CBMs.

For example, multivalent CBM molecules, (for example a Vc4CBM molecule) may be prepared as constructs comprising multiple CBMs linked by amino acid/peptide linkers. Each CBM (for example VcCBM) may be linked to another by, for example, peptides comprising 5, 10 or 15 amino acids. By way of example any one or more of the following peptides may be used to link two or more CBMs to produce a multivalent CBM:

(i)	5 amino acid linkers:	ALNGS
		LQALG
		GGNSG
(ii)	10 amino acid linkers:	ALNGSGGGSG
		LQALGGGGSL
(iii)	15 amino acid linkers:	ALNGSGGGSGGGGSG

Thus, the various aspects and embodiments of this invention (uses, sialic acid binding molecules for use, methods and medicaments) may exploit sialic acid binding molecules which comprise, consist of or consist essentially of sialic acid binding molecules selected from the group consisting of:

(i) one or more *Vc*CBM(s);

(ii) one or more *Sp*CBM(s); and

(iii) a multivalent CBM.

The sialic acid binding molecules for use may further comprise an oligomerisation domain. Suitable oligomerisation domains may exhibit an ability to self-associate to form multimer structures, for example trimers. An oligomerisation domain for use may comprise any molecule with the above mentioned oligomerisation properties or any functional fragment thereof. For example, one or more (for example two) sialic acid binding molecules (for example CBMs) may be bound, coupled or fused to an oligomerisation domain – the resulting sialic acid binding molecule::oligomerisation domain "fusion" may then be used (with one or more other such "fusions") as a molecule for modulating cell growth and/or

activity and/or for treating or preventing any of the diseases and/or conditions disclosed herein.

Suitable oligomerisation domains may be derived from, for example, *P*seudomonas *aeruginosa* pseudaminidase. An exemplary *Pseudomonas aeruginosa* pseudaminidase sequence amino acid sequence has been deposited under accession number PAO579 and is reproduced below as SEQ ID NO: 5 (438 amino acids).

MNTYFDIPHR LVGKALYESY YDHFGQMDIL SDGSLYLIYR RATEHVGGSD GRVVFSKLEG
GIWSAPTIVA QAGGQDFRDV AGGTMPSGRI VAASTVYETG EVKVYVSDDS GVTWVHKFTL
ARGGADYNFA HGKSFQVGAR YVIPLYAATG VNYELKWLES SDGGETWGEG STIYSGNTPY
NETSYLPVGD GVILAVARVG SGAGGALRQF ISLDDGGTWT DQGNVTAQNG DSTDILVAPS
LSYIYSEGGT PHVVLLYTNR TTHFCYYRTI LLAKAVAGSS GWTERVPVYS APAASGYTSQ
VVLGGRRILG NLFRETSSTT SGAYQFEVYL GGVPDFESDW FSVSSNSLYT LSHGLQRSPR
RVVVEFARSS SPSTWNIVMP SYFNDGGHKG SGAQVEVGSL NIRLGTGAAV WGTGYFGGID
NSATTRFATG YYRVRAWI

The oligomerisation domain of SEQ ID NO: 5 is from amino acid residue 333 to 438 – this sequence may be SEQ ID NO: 6.

Thus an oligomerisation domain for use may comprise from about residue 250, 275, 300, 310, 320, 333, 340 to 350 (i.e. from about residue 250 to about residue 350 including from about any residue therebetween) to about residue 400, 410, 420, 430 or 438 (i.e. to about any residue from about residue 400 residue 438 including to about any residue therebetween) of the *P. aeruginosa* pseudaminidase trimerisation domain (*Pa*TD) provided by SEQ ID NO: 5. For example, a useful sialic acid binding molecule may exploit an oligomerisation domain comprising residues 333 to 438 of SEQ ID NO: 6.

A sialic acid binding molecule for use may comprise one or more of the CBM based molecules presented in Figure 1. For example, a suitable sialic acid binding molecule may comprise (consist essentially of, or consist of) two or more *Vc*CBMs optionally fused, bound or conjugated to an oligomerisation domain (such as a *Pa*TD or oligomerisation fragment thereof). The sialic acid binding molecule may comprise, consist or consist essentially of two fused (or bound) *Vc*CBMs which are in turn fused to an oligomerisation domain (see, for example, molecule *Vc2*CBMTD shown in Figure 1).

Other sialic acid binding domains for use may comprise two or more *Sp*CBMs optionally fused, bound or conjugated to an oligomerisation domain (such as a *Pa*TD or an oligomerisation fragment thereof). Sialic acid binding molecules for use may comprise, consist or consist essentially of two fused (or bound) *Sp*CBMs which are in turn fused to an oligomerisation domain (see, for example, molecule *Sp2*CBMTD shown in Figure 1).

Thus, this disclosure provides:

(i) a CBM for use in a method of modulating cell growth and/or cell activity; said method comprising contacting a cell with a CBM;

(ii) a method of modulating cell growth and/or activity, said method comprising contacting a cell with a CBM. The method may be an *in vitro* method;

(iii) a CBM for use in treating and/or preventing a disease and/or condition caused, contributed to and/or characterised by aberrant cell growth and/or proliferation;

(iv) use of a CBM for the manufacture of a medicament for the treatment and/or prevention of diseases and/or conditions caused, contributed to and/or characterised by aberrant cell growth and/or proliferation;

(v) a method of treating and/or preventing a disease and/or condition caused, contributed to and/or characterised by aberrant cell growth and/or proliferation, said method comprising the step of administering a therapeutically effective amount of a sialic acid binding molecule to a subject in need thereof;

(vi) a CBM for use in treating and/or preventing cancer;

(vii) use of a CBM for the manufacture of a medicament for the treatment and/or prevention of cancer; and

(viii) a method of treating and/or preventing cancer, said method comprising the step of administering a therapeutically effective amount of a sialic acid binding molecule to a subject in need thereof.

In any one of embodiments (i) to (viii) above, the CBM may be a multivalent CBM comprising two or more family 40 CBMs. For example, in any one of embodiments (i) to (viii) above, the CBM may comprise (consist essentially of, or consist of) two or more *Vc*CBMs and/or *Sp*CBMs optionally fused, bound or conjugated to an oligomerisation domain (such as a *Pa*TD or oligomerisation fragment thereof). The sialic acid binding molecule may comprise, consist or consist essentially of two fused (or bound) *Vc*CBMs or two fused (or bound) *Sp*CBMs which are in turn fused to an oligomerisation domain (see, for example, molecule *Vc2*CBMTD and *Sp2*CBMTD shown in Figure 1).

In view of the above, this disclosure provides:

(i) the sialic acid binding domain of *Vibrio cholerae* NanH sialidase (*Vc*CBM) and/or the sialic acid binding domain of *Streptococcus pneumoniae* NanA sialidase (*Sp*CBM) for use in a method of modulating cell growth and/or cell activity; said method comprising contacting a cell with the sialic acid binding domain of *Vibrio cholerae* NanH sialidase (*Vc*CBM) and/or the sialic acid binding domain of *Streptococcus pneumoniae* NanA sialidase (*Sp*CBM);

(ii) A method of modulating cell growth and/or activity, said method comprising contacting a cell with the sialic acid binding domain of *Vibrio cholerae* NanH sialidase (*Vc*CBM) and/or the sialic acid binding domain of *Streptococcus pneumoniae* NanA sialidase (*Sp*CBM). The method may be an *in vitro* method;

(iii) The sialic acid binding domain of *Vibrio cholerae* NanH sialidase (*Vc*CBM) and/or the sialic acid binding domain of *Streptococcus pneumoniae* NanA sialidase (*Sp*CBM) for use in treating and/or preventing a disease and/or condition caused, contributed to and/or characterised by aberrant cell growth and/or proliferation;

(iv) use of the sialic acid binding domain of *Vibrio cholerae* NanH sialidase (*Vc*CBM) and/or the sialic acid binding domain of *Streptococcus pneumoniae* NanA sialidase (*Sp*CBM) for the manufacture of a medicament for the treatment and/or prevention of diseases and/or conditions caused, contributed to and/or characterised by aberrant cell growth and/or proliferation;

(v) a method of treating and/or preventing a disease and/or condition caused, contributed to and/or characterised by aberrant cell growth and/or proliferation, said method comprising the step of administering a therapeutically effective amount of the sialic acid binding domain of *Vibrio cholerae* NanH sialidase (*Vc*CBM) and/or the sialic acid binding domain of *Streptococcus pneumoniae* NanA sialidase (*Sp*CBM) to a subject in need thereof;

(vi) the sialic acid binding domain of *Vibrio cholerae* NanH sialidase (*Vc*CBM) and/or the sialic acid binding domain of *Streptococcus pneumoniae* NanA sialidase (*Sp*CBM) for use in treating and/or preventing cancer;

(vii) use of the sialic acid binding domain of *Vibrio cholerae* NanH sialidase (*Vc*CBM) and/or the sialic acid binding domain of *Streptococcus pneumoniae* NanA sialidase (*Sp*CBM) for the manufacture of a medicament for the treatment and/or prevention of cancer; and

(viii) a method of treating and/or preventing cancer, said method comprising the step of administering a therapeutically effective amount of the sialic acid binding domain of *Vibrio cholerae* NanH sialidase (*Vc*CBM) and/or the sialic acid binding domain of *Streptococcus pneumoniae* NanA sialidase (*Sp*CBM) to a subject in need thereof.

Additionally, the disclosure provides:

(i) A CBM (as described herein) and/or the sialic acid binding domain of *Vibrio cholerae* NanH sialidase (*Vc*CBM) and/or the sialic acid binding domain of *Streptococcus pneumoniae* NanA sialidase (*Sp*CBM) for use in (a method of) modulating tumour growth;

(ii) Use of a CBM (as described herein) and/or the sialic acid binding domain of *Vibrio cholerae* NanH sialidase (*Vc*CBM) and/or the sialic acid binding domain of *Streptococcus pneumoniae* NanA sialidase (*Sp*CBM) in the manufacture of a medicament for use in modulating tumour growth; and

(iii) A method of modulating tumour growth, said method comprising administering a therapeutically effective or tumour growth modulating amount of a CBM (as described herein) and/or the sialic acid binding domain of *Vibrio cholerae* NanH sialidase (*Vc*CBM) and/or the sialic acid binding domain of *Streptococcus pneumoniae* NanA sialidase (*Sp*CBM), to a subject in need thereof.

Molecules which bind sialic acid and in particular sialic acid which is part of a cell bound receptor, may find further application as moieties which may be conjugated, bound or joined to or associated with, other entities for the purpose of targeting or delivering that entity to some tissue or cell. Molecules of this type may be otherwise known as "therapeutic warheads" or "conjugates". Without wishing to be bound by theory, the presence of sialic acid in certain cell receptors and membrane bound molecules, may allow the sialic acid binding molecule to be exploited as a means to deliver conjugated heterologous molecules (that is a molecule distinct from and different to the sialic acid binding molecule) to said cells or tissues comprising said cells. Thus, conjugated sialic acid binding molecules may be useful in the treatment of cancer, where sialic acid binding molecules (with affinity for sialic acids expressed on cancerous cells and/or tumours) may be used to direct therapeutic and/or cytotoxic moieties thereto.

By way of example, a sialic acid binding molecule as described herein (including any of the CBM based molecules) may be conjugated to moieties which are, for example, therapeutic and/or cytotoxic. Thus, the disclosure relates to sialic acid binding molecule conjugates.

Sialic acid binding molecule conjugates may comprise a sialic acid binding molecule of this disclosure conjugated (joined, bound or otherwise associated with) to a heterologous moiety. The heterologous moiety may comprise a therapeutic and/or cytotoxic moiety which may be conjugated to some part of the sialic acid binding molecule. For example, the heterologous moiety may be conjugated to one or both ends of the sialic acid binding molecule. The heterologous moiety may be additionally or alternately conjugated (or even fused) to an internal portion of the sialic acid binding molecule. It will be appreciated that however the heterologous moiety is to be conjugated to the sialic acid binding molecule, the moiety (nor its conjugation) should not (substantially) interfere with or ablate or reduce the sialic acid binding property of the sialic acid binding molecule.

As stated, the heterologous moiety may be a drug useful in the treatment of a disease which affects a cell or tissue comprising sialic acid. For example, the drug may be a chemotherapeutic drug for use in the treatment of cancer and the like. The heterologous moiety may be a cytotoxic moiety capable of killing or inducing apoptosis in, a cell. The heterologous moiety may comprise a molecule which is able to recruit specific cells to or into a particular tissue. For example, the heterologous moiety may be, for example, a T cell receptor (TCR) which may be used as a means to recruit T cells to, for example a tumour or cancerous tissue.

The present disclosure may provide compositions for use in the various uses, medicaments and methods described herein. As such, any of the sialic acid binding molecule(s) described herein may be formulated for use. For convenience, and with

reference to the section below describing compositions, formulations and the like it should be noted that both sialic acid binding molecules as described herein and any conjugates comprising the same (for example sialic acid binding molecule:: drug conjugates/fusions) shall be included under the general term "sialic acid binding molecule".

A sialic acid binding molecule (or molecules) may be formulated for use and as a therapeutic or pharmaceutical composition. The various compositions may comprise one or more of the sialic acid binding molecules described herein and any given treatment may require the administration (together, concurrently or separately) of one or more of these compositions.

The various sialic acid binding molecules described herein may be formulated for enteral (including oral), parenteral and/or topical administration and one of skill will appreciate that the precise formulation may vary depending on the route of administration.

Pharmaceutical compositions according to the present invention may be prepared conventionally, comprising substances that are customarily used in pharmaceuticals and as described in, for example, Remington's The Sciences and Practice of Pharmacy, 22nd Edition (Pharmaceutical Press 2012) and/or Handbook of Pharmaceutical Excipients, 7th edition (compiled by Rowe et al, Pharmaceutical Press, 2012) – the entire content of all of these documents and references being incorporated by reference.

A therapeutic or pharmaceutical composition of this disclosure (that is a composition comprising a sialic acid binding molecule and for use in any of the medicaments or methods described herein – including the methods of or medicaments for, modulating cell growth and/or activity and/or treating cancer) may be formulated together with one or more pharmaceutically acceptable excipients, carriers, adjuvants and buffers. The compositions can be administered, e.g. orally (including mucosally), parentally, enterally, intramuscularly, subcutaneously, intravenously or via any other routes useful to achieve the desired effect (in this case effects which include, modulation of cell growth/activity, treatment or prevention of diseases/conditions associated with the same and/or cancer and/or modulation of tumour growth). As stated, depending on the chosen route of administration, the exact composition of the formulation may vary.

A therapeutic or pharmaceutical formulation comprising a sialic acid binding molecule and for administration to a subject may be coated, encapsulated or enveloped in a material which protects the sialic acid binding molecule from the action of enzymes, acids and other natural compounds/conditions (including, for example, compounds (including antibodies), cells and processes of the immune system) which may inactivate or denature the compound and/or its sialic acid binding properties.

Among the various standard and conventional excipients that may be available for use in compositions comprising sialic acid binding molecules, are those pharmaceutically

acceptable organic or inorganic carrier substances which are suitable for parenteral, enteral, oral (including mucosal) and other routes of administration that do not deleteriously react with the sialic acid binding molecule(s).

Where the sialic acid binding molecules are to be formulated for parental administration, the compositions may be sterile.

The composition may comprise an oil-based or aqueous solution, a suspension and/or an emulsion.

In other embodiments, the composition may take the form of an implant, such as for example a (dissolvable or biodegradable) film, pessary or implant (including suppositories).

The pharmaceutical preparations comprising sialic acid binding molecules may be mixed with stabilizers, wetting agents, emulsifiers, salts (for use in influencing osmotic pressure), buffers and/or other substances that do not react deleteriously with the active compounds.

One or more of the sialic acid binding molecules described herein may be formulated for and administered, orally. As stated, oral administration would include mucosal administration which would itself would include administration intranasally and/or by inhalation.

Compositions for use may include solid dosage forms which are suitable for oral administration. These may include, for example capsules, tablets, pills, powders, and granules. In any given solid dosage form, the sialic acid binding molecule (or any conjugate comprising the same) may be admixed with at least one inert pharmaceutically-acceptable excipient. Examples of suitable excipients will be known to one of skill in this field but may include, for example fillers or extenders, humectants, wetting agents, binders, disintegrating agents, solution retarders, absorption accelerators, adsorbents, lubricants or mixtures thereof. A tablet, pill or capsule may further comprise a buffering agent. Solid dosage forms such as tablets, dragees, capsules, pills and/or granules also can be prepared with coatings and shells, such as coatings which protect against the gastrointestinal environment and/or stomach acid.

A solid dosage form may contain opacifying agents, and can also be formulated so as to ensure the delayed release of the active agent (in this case the sialic acid binding molecule or a conjugate comprising the same) in or to a specific part of the intestinal tract.

Solid compositions for oral administration can be formulated in a unit dosage form, each dosage containing an appropriate dose of the sialic acid binding molecule (or conjugate comprising the same). The exact amount of sialic acid binding molecule (or conjugate comprising the same) contained within any given solid dosage form will vary depending on the intended use. a solid composition may contain a "unit dose" – a unit dose containing a quantity of sialic acid binding molecule (or conjugate containing the same)

calculated to produce the desired effect (for example modulation of cell growth and/or activity) over the course of a treatment period.

Liquid dosage forms for oral administration may (as stated) include emulsions, solutions, suspensions, syrups, and elixirs. In addition to the compound or composition, the liquid dosage forms may contain inert diluents commonly used in the art, such as water or other solvents, solubilizing agents and emulsifiers.

The sialic acid binding molecules may be used in any suitable amount. As stated, the sialic acid binding molecules may be formulated for oral, mucosal or parenteral administration and as such, the precise formulation may depend on the intended route of administration. The amount of sialic acid binding molecule present in any given dose may be in the region of 0.1 µg -1000 µg. For example, amounts of about 0.1 µg, 0.2 µg, 0.3 µg, 0.4 µg, 0.5 µg, 1 µg, 10 µg, 20 µg, 25 µg, 50 µg, 100 µg, 200 µg, 300 µg, 400 µg, 500 µg, 600 µg, 700 µg, 800 µg or 900 µg. The selected amount of sialic acid binding molecule may be formulated in a specifc volume of a pharmaceutically acceptable excipient, diluent and/or buffer. The volume of excipient, diluent or buffer may be about 10 µl to 5 ml. For example, the required amount of sialic acid binding molecule (CBM) may be combined (or formulated) with about 15 µl, 20 µl, 25 µl, 30 µl, 35 µl, 40 µl, 45 µl, 50 µl, 55 µl, 60 µl, 65 µl, 70 µl, 75 µl, 80 µl, 85 µl, 90 µl, 95 µl, 100 µl, 200 µl, 250 µl, 300 µl, 400 µl, 500 µl, 600 µl, 700 µl, 800 µl, 900 µl, 1 ml, 2 ml, 3 ml or 4ml. For example, an amount of 100 µg sialic acid binding molecule may be combined with about 250 µl of excipient to yield a final concentration of sialic acid concentration of 400 µg/ml. Doses at concentrations of about 0.1 µg/ml-1 mg/ml may be used including, for example, doses at 5 µg/ml, 10 µg/ml, 20 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml, 300 µg/ml, 500 µg/ml, 600 µg/ml, 700 µg/ml, 800 µg/ml or 900 µg/ml.

In use, a dose of a sialic acid binding molecule, administered as part of the treatment and/or prevention of a cell proliferation and/or differentiation disorder (for example cancer), may be administered multiple times over a number of days or weeks. For example, after an initial (or first) administration, a dose of a sialic acid binding molecule may be administered again at about (+/- 1 or 2 days) 3, 4, 5, 6, 7, 17, 21, 28 and/or 35 days later. On any given day, a specific dose of a sialic acid binding molecule may be administered 1, 2, 3 or more times. Each time, the sialic acid binding molecule may be administered (by whatever route is considered best to affect a suitable treatment or to induce prophylaxis against the development of a cell proliferation and/or differentiation disorder.

DETAILED DESCRIPTION

The present invention will now be described in detail by reference to the following Figures which show:

Figure 1. Building blocks of the multivalent CBM forms and their affinities for sialic acid. a, *Vc*CBM, residues 25-216 of the *V. cholerae* sialidase (PDB:1w0p) with α-2,3-sialyllactose drawn as spheres. **b,** *Sp*CBM, residues 121-305 of *S. pneumoniae* NanA sialidase with α-2,3-sialyllactose (PDB:4c1w). **c,** TD, the trimerisation domain, residues 333-438, of the *P. aeruginosa* pseudaminidase (PDB:2w38) in rainbow colors; the other two monomers in single colors. **d,** Multivalent forms: their molecular weights, valencies and binding affinities for α2,3-sialyllactose as determined by surface plasmon resonance (SPR) at 25°C (K_D values for *Vc*CBM, *Vc*2CBM and *Vc*3CBM had been reported previously (Connaris *et al*, 2009)). Tandem repeat CBMs, and oligomeric CBMs fused to TD are linked by a 5-amino linker (details in Connaris *et al*, 2014).

Figure 2: CBM dose analysis on A549 human lung carcinoma cells. Left panel: 10-fold serially diluted single dose (i.e. 400 µg to 0.04 µg per well) of *Vc*2CBMTD (top panel), and of *Sp*2CBMTD (bottom panel). The right panel is identical to the left, except that cells are given CBMs twice over three days. CBMs were diluted in serum-free DMEM and incubated with cells for 3h before removing and replaced with DMEM/5% FCS for cell maintenance. Mock wells represent cells that are given PBS only in DMEM/5% FCS.

Figure 3: Wound healing assay on A549 human lung carcinoma cells. Cells were scratched to create an artificial wound before treatment with PBS (A), 100 µg/ml *Sp*2CBMTD (B), or 100 µg/ml *Vc*2CBMTD (C), in serum-free DMEM. Cells were left for 24h, followed by actin staining to visualise cell cytoskeleton.

Figure 4: (A) The MTT assay absorbance indicating the metabolic activity of A549 human lung carcinoma cells after treatment with either 50 µg/ml of monomeric CBM-like domain (TcTs), or 5 µg/ml of hexameric CBMs (*Vc*2CBMTD or *Sp*2CBMTD) resuspended in serum-free DMEM or in serum-free DMEM with 2mM sialyllactose (+Sia) for 24h at 37degC, 5% CO_2. (B) Cell morphology of treated vs PBS only cells (control) after 24h.

Figure 5: **A.** Scratch assay data from a human lung cancer cell line (A549) using mCBM40s. Hexameric *Vc*2CBMTD and *Sp*2CBMTD were tested at 100 µg in a 250 µL volume representing a concentration of 400 µg/ml. Phase contrast images represent the wound healing assay after 1h and 72h, after cells were treated with PBS, *Vc*2CBMTD, or *Sp*2CBMTD, in serum--free DMEM. Yellow bar at bottom of each image indicate diameter of 400 µm. **B.** Graphs representing the percentage of wound healing (ii), and rate of wound closure (ii) for A549 cells in the absence and presence of mCBM40s after 72h. Dunnett's multiple comparison test was performed on the data. **** represents a statistical significance $p<0.0001$.

Figure 6: **A.** Scratch assay data from a human cervical cancer cell line (HeLa) using mCBM40s. Hexameric *Vc*2CBMTD and *Sp*2CBMTD were tested at 100 µg in a 250 µL

volume representing a concentration of 400 µg/ml. Fluorescent images represent the wound healing assay after 1h and 72h, after cells were treated with PBS, *Vc*2CBMTD, or *Sp*2CBMTD, in serum-free DMEM. Yellow bar at bottom of each image indicate diameter of 400 µm. **B.** Graphs representing the percentage of wound healing (ii), and rate of wound closure (ii) for HeLa cells in the absence and presence of mCBM40s after 72h. Dunnett's multiple comparison test was performed on the data. **** represents a statistical significance $p<0.0001$.

Figure 7: Scratch assay data for lung (A549), cervical (HeLa), breast (MDA.MB.231 and MCF-7) and colon (SW480 and SW620) cancer cell types in the presence and absence of Sp2CBMTD 100 µg (400 µg/mL). (a) Graph representing percentage of A549 cell wound closure in the presence and absence of *Sp*2CBMTD 100 µg after 72hrs. (b) Graph representing percentage of HeLa cell wound closure in the presence and absence of *Sp*2CBMTD 100 µg after 72hrs. (c). Graph representing percentage of MDA.MB.231 cell wound closure in the presence and absence of *Sp*2CBMTD 100 µg after 72hrs. (d). Graph representing percentage of MCF--7 cell wound closure in the presence and absence of *Sp*2CBMTD 100 µg after 72hrs. (e). Graph representing percentage of SW620 cell wound closure in the presence and absence of *Sp*2CBMTD 100 µg after 72hrs. (f). Graph representing percentage of SW480 cell wound closure in the presence and absence of *Sp*2CBMTD 100 µg after 72hrs. All data normalised to control. Unpaired t test performed on data, statistical significance (p) was reported between control and *Sp*2CBMTD ($p = 0.0001$) highlighted by ****.

Figure 8: Cell proliferation of HeLa cells in the presence and absence of hexameric CBM40s. Graph depicts cell number against time (h). *Vc*2CBMTD and *Sp*2CBMTD were tested at 100µg in a 250 µL volume representing a concentration of 400 µg/ml. PBS served as control.

Figure 9: Scratch assay data from a lung cancer cell line (A549) and a cervical cancer cell line (HeLa) using a tetrameric CBM40. *Vc*4CBM tested at 100 µg in a 250 µL volume representing a concentration of 400 µg/mL. A. Graphs representing percentage (i), and rate (ii), of A549 cell wound closure in the presence and absence of *Vc*4CBM after 72hrs. B. Graph representing percentage (i) and rate (ii) of HeLa cell wound closure in the presence and absence of *Vc*4CBM also after 72hrs. Unpaired t test was performed on all data with statistical significance (p) reported between control and *Vc*4CBM ($p = 0.0001$) as highlighted by control and *Vc*4CBM highlighted by ****.

Figure 10: Effect of component parts of engineered multimeric CBM40s on cell proliferation of cancer cell lines using a wound healing assay after 72h. Monomeric (*Vc*CBM, *Sp*CBM) and multimeric (*Vc*2CBMTD and *Sp*2CBMTD) CBM40s were tested at 100 µg in a 250 µL volume representing a concentration of 400 µg/mL. **A** Images representing wound healing

assay in A459 (a) and HeLa (b) cells after 1h and 72h, after treatment with PBS, *Sp*CBM, or *Vc*CBM in serum-free DMEM. Yellow bar at bottom of each image indicate diameter of 400 µm. **B** Graphs representing percentage (i) (ii), and rate (iii) (iv) of wound closure in the presence and absence of CBM after 72h in A549 and HeLa cell lines, respectively. Dunnett's multiple comparison test was performed on the data and a statistical significance (p) of 0.0001 is highlighted by **** and a p of 0.05 is highlighted by *.

Figure 11: Effect of component parts of engineered multimeric CBM40s on cell proliferation of A459 (A) and HeLa (B) cell lines using a wound healing assay after 72h. The trimerization domain (TD) was tested at 100 µg in a 250 µL volume representing a concentration of 400 µg/mL. (a) Graph representing percentage of A549 cell wound closure in the presence and absence of TD after 72hrs. Unpaired t test performed on data, no statistical significance (p) was reported between control and TD (p = 0.1201). (b) Graph representing percentage of HeLa cell wound closure in the presence and absence of TD after 72hrs. Unpaired t test performed on data, no statistical significance (p) was reported between control and TD (p = 0.1201). (c). Graph representing the rate of A549 cell wound closure in the presence and absence of CBM after 72hrs. Unpaired t test performed on data, a p of 0.05 was reported between control and TD (p = 0.0212) highlighted by *. (d). Graph representing the rate of HeLa cell wound closure in the presence and absence of TD after 72hrs. Unpaired t test performed on data, a p of 0.05 was reported between control and TD (p = 0.0338) highlighted by *.

Figure 12: Scratch assay data plus wash off for A549 cell line in the presence and absence of CBM after 72hrs followed by a further 72hrs after wash off. A one-way ANOVA and a Dunnett's multiple comparison test were performed on the data and a statistical significance (p) of 0.0001 is highlighted by ****. A *p* of 0.0002 is highlighted by ***. (a). The p of 0.0001, 0.0002 and 0.05 between the 72hr control is represented by ****, *** and * respectively. (a). Graph representing percentage of A549 cell wound closure in the presence and absence of CBM after 72hrs and 144hrs after a CBM wash-off at the 72hr time point (72hrs after wash). (b). Graph representing the rate of A549 cell wound closure in the presence and absence of CBM after 72hrs and 144hrs after a CBM wash-off at the 72hr time point (72hrs after wash).

Figure 13: Chemotaxis assay data from a lung cancer cell line (A549). A549 cells seeded at 8.0x 10^5 cells/well. Fluorescence measured after 24hrs background corrected. CBM tested at 100 µg in a 100 µL volume representing a concentration of 1mg/ml. Dunnett's multiple comparison test was performed on the data and a statistical significance (p) of 0.0001 is highlighted by ****.

Figure 14: Agglutination assay adapted from Hwang *et al* (1974). Graphs showing the absorbance at 546nm of A549 cells in the absence (control) and presence of mCBM40 (*Vc*4CBM, *Vc*2CBMTD and *Sp*2CBMTD all at 1 mg/mL) for 5 mins, 30 mins and 1hr **(A)**, and

at 1hr and 24hrs **(B)**. A PBS only control was also included. Graphs showing the absorbance **(C)**, and the rate of absorbance **(D)** of A549 cells at 546nm for every ten seconds over 20mins in the absence (control) and presence of mCBM40s after incubation on ice for 24hrs.

Figure 15: Caspase dependent apoptosis. Graph showing the normalised (background corrected) luminescence signal produced by luciferase following caspase cleavage of Caspase-Glo® 3/7 reagent in A549 cells in the presence and absence of CBMs for 4hrs, 24hrs and 72hrs. A one-way ANOVA and a Dunnett's multiple comparison test were performed on the data and a statistical significance (p) of ≤0.05 is highlighted by *.

Figure 16: Cell viability. Graph showing the normalised (background corrected) fluorescence signal produced by cleavage of GF-AFC substrate by A549 live cell protease in the presence and absence of CBMs for 4hrs, 24hrs and 72hrs. A one-way ANOVA and a Dunnett's multiple comparison test were performed on the data and a statistical significance (p) of ≤0.05 is highlighted by *.

Figure 17: Cell cytotoxicity data for lung cancer cell line A549 in the presence and absence of CBM. Graph showing the normalised (background corrected) fluorescence signal produced by cleavage of bis--AAF-R110 substrate by A549 dead cell protease in the presence and absence of CBMs for 4hrs, 24hrs and 72hrs. A one-way ANOVA and a Dunnett's multiple comparison test were performed on the data and a statistical significance (p) of ≤0.05 is highlighted by *.

Example 1

In an *in vitro* mCBM40 dosing experiment using confluent A549 human lung carcinoma cells and high concentrations of *Sp*2CBMTD (400-800 µg), little or no cell growth or activity was observed after 3 days. This was noted as the colour of the culture medium (DMEM/5% FCS), which would change colour as it is utilised, remained the same (see Figure 2).

This observation led to the hypothesis that our engineered sialic acid-recognising CBMs might affect cell (including cancer cell) proliferation and migration by targeting sialylated host cell receptors.

To further support this hypothesis a wound scratch assay to determine cell migration and proliferation and a cell viability (MTT) assay were performed on A549 human lung carcinoma cells. These cells were treated with CBMs for 24h. The results were compared to control cells that were given PBS only.

As seen in Figure 3, little or no wound healing was observed in CBM-treated wells compared to PBS after 24 h. PBS-treated cells demonstrated a scratch diameter of 250 µm with lone cells migrating into the artificial wound after 24h. In contrast, scratch diameters for both CBM-treated cells were roughly 710 µm, with changes in cell morphology likely associated with cell death or cell cycle arrest for the *Vc*2CBMTD-treated cells. There is also

poor cell migration into the wound by *Sp*2CBMTD-treated A549 cells. Interestingly, the formation of filopodia was observed with *Sp*2CBMTD, a phenomenom usually coupled with cell motility (Mattila and Lappalainen, 2008).

Metabolic activity decreased in cells that were given *Vc*2CBMTD in serum-free medium (absence of FCS), compared to cells that were given PBS only. Cell morphology of *Vc*2CBMTD-treated cells was also observed to have changed, with cells appearing more rounded (a sign of cell arrest) when compared to the control. Interestingly, cell metabolic activity and phenotype appeared to be reversed with the addition of 2mM sialyllactose. Similar concentrations of sialic acid can also be found in FCS, and this may be the reason why *Vc*2CBMTD-treated cells remained viable after 3 days compared to *Sp*2-treated cells, which appear to be unaffected by the presence of FCS, at least at the high dose of CBM used in the assay (Fig 2). Alternatively, it may be that free sialyllactose in the medium compete for the glycan binding sites of *Vc*2CBMTD, thus allowing A549 cells to recover and proliferate.

As for *Sp*2CBMTD-treated cells, there was also a slight (but not significant) decrease in metabolic activity. However, the lack of significance is likely due to the low concentration administered to the cells.

There was a slight recovery of cell growth in the presence of sialyllactose compared to *Vc*2-treated cells, but due to the nature of *Sp*2CBMTD, which is also known to stimulate pro-inflammatory mediators, it is likely that the phenomenon observed in Figure 2, could be due to its immunomodulatory role. It has been demonstrated that *Sp*2CBMTD increased a number of inflammatory mediators such as IFN-γ, VEGF, IP-10 and IL-8 and other cytokines that are known to be important in neutrophil and macrophage targeting (Govorkova *et al* (2015). This feature may be ideal for targeting the immune system to cancer cells. As for TcTs-treated cells, the CBM-like domain had little effect on cell metabolism and morphology.

Example 2

Wound healing and proliferation assay of cancer cell lines

Method: All CBM40 proteins were prepared as described in Connaris et al (2014)[1]. The following cancer cell lines, A549 (human lung), HeLa (cervical), MDA.MB.231 and MCF--7 (breast), and SW620 and SW480 (colon) were purchased from ATCC. All cells with the exception of HeLa cells were maintained in 10% FBS, DMEM (high glucose with 1% Penicillin/Streptomycin) and incubated at 37°C and 5% CO2. HeLa cells expressing GFP restricted to the nucleus (using IncuCyte NucLight Green Lentivirus Reagent Cat No. 4626 Essen Biosciences) were maintained in complete medium containing 0.5 µg/ml Puromycin. For wound healing assays, 24 well plates (Nunc) were seeded with cells (2.5 x 10^5 cells/well) and maintained in 500 µL of their respective maintenance medium and incubated at 37°C and 5% CO_2 for 24 hrs. Wells were washed with 500 µL of serum-free DMEM, prior to the

wound/scratch creation. A wound/scratch was created as a straight line down the middle of the well (running from the top to bottom of the well) in each well using a sterile 200 µL pipette tip (Axygen). Wells were then washed with 500 µL of serum-free DMEM prior to the addition of 250 µL of appropriately diluted CBMs, also prepared in serum-free DMEM. Serum-free medium was also included to wells as a control. Plates were incubated at 37°C and 5% CO_2 for 10-30 mins before a further addition of 250 µL of 4% FBS, DMEM to each well (2% FBS final concentration) so that the final CBM concentration ranged from 20-200 µg/ml. The IncuCyte ZOOM (Essen BioScience) apparatus was used to collect time-lapse images of each well. This was set to collect three images of the wound/scratch in each well every 1hr for 72 h. For data collection, the IncuCyte ZOOM 2016 software was used to measure and collate wound width measurements for each image (3 x wound measurements were performed manually). For the cell proliferation assay, GFP-labelled HeLa cells were grown in 24well plates over 72 h in the presence or absence of mCBM40s. Cell proliferation is monitored by analysing the occupied area (percentage of confluence) of cell images over time using the IncuCyte ZOOM apparatus. Cell proliferation is directly proportional to increase in confluence.

Results: Figures 5 and 6 illustrate the results of a wound--healing assay measuring the effect of different hexameric mCBM40s against different cancer cell types A549 and HeLa. The results indicate that hexameric CBM40s *Vc*2CBMTD and *Sp*2CBMTD significantly inhibited wound closure in both cancer types after 72h compared to cells that were left untreated. Multimeric CBMs also inhibited wound closure in other cancer types that displayed differential expression of sialylated receptors. The cell growth of both breast and colon cancers were found to be inhibited in a dose--dependent manner (Figure 7 illustrates the results from the highest dose of mCBM40s used against two metastatic cell types of breast and colon cancer). A difference in potency was observed between the highly metastatic and lowly metastatic breast cancer cell lines MDA.MB.231 and MCF-7. Multimeric CBMs were more potent against the MDA.MB.231 cell line. The potency did not differ much between the colon cancer cell lines SW620 and SW480 despite different glycosylation profiles. The percentage of wound covered for the two colon cancer cell lines and the low--grade breast cancer cell line after 72hrs was low (between 30% to 40%).

The inhibition of wound healing was due to the lack of cell proliferation over time as shown in Figure 8, using GFP-labelled HeLa cells that demonstrated reduced cell growth over time. The anti-proliferative effect of mCBM40s was also observed with a tetrameric version mCBM40, Vc4CBM in both A549 and HeLa cells (Figure 9).

To determine whether the anti-proliferative effect is a result of the multimeric nature of engineered CBMs, A549 and HeLa cell lines were treated with the monomeric versions of *Vc*- and *Sp*-based CBM40s (*Vc*CBM, *Sp*CBM, 100 µg each) and left to incubate for up to 72

h. The data was then compared with that of their multimeric counterparts. Figure 10 illustrates the results of a wound healing assay of A549 and HeLa cell lines after treatment with CBMs. At the dose used, multimeric CBMs had a significant effect on the rate of wound closure.

Furthermore, the non-CBM40 component of mCBM40s, that is, the trimerisation domain (TD), was also tested in a scratch assay to determine if this domain may contribute to the anti-proliferative effect seen in the scratch assays. Figure 11 demonstrates that the TD domain, when given at the same dose as the CBMs, does not appear to affect the cell growth or proliferation of A549 or HeLa cells, as the percentage and rate of wound closure was identical to untreated cells.

To establish if anti-proliferative activity of CBM proteins can be reversed after a wound scratch assay, a wash-off experiment was attempted. After 72h incubation, treated wells were washed twice with 0.5 mL of 2% FBS, DMEM (high glucose, 1% Penicillin/Streptomycin). The same medium (0.5 mL) was then added to each well and incubated for a further 72h. The EVOS FL (ThermoFisher Scientific) apparatus was used to manually collect three images of the wound/scratch in each well at 0 hrs and 72hrs. Image J software was used to manually measure and collate wound width measurements for each image (Image J measurement tool was calibrated against a scale bar for the EVOS FL). After removal of mCBM40s, cells were clearly seen to migrate and/or proliferate into the wound (Figure 12). The rate and percentage of wound closure was observed to revert to "normal"/control back after the wash off. The rate of wound closure for VcCBM2TD after wash off was observed to be slightly faster than that of the control condition after 72hrs. These observations suggest that mCBM40s can modulate the cell response to cancer by interacting with sialylated cell surface receptors.

Migration assay.

Method: A Boyden chamber assay was selected to determine if cellular migration is interrupted by multimeric CBM40s. The Boyden chamber assay (8µm, fluorometric format) was prepared as described in manufacturer's instructions (Cell Biolabs CytoSelect 96-well cell migration assay). Final CBM concentration ranged from 20-200 µg/ml.

Results: Figure 13 illustrate the results from the migration assay, which indicate that the CBMs tested inhibited migration of A549 cells. To eliminate the possibility that the lack of migration may be attributed to agglutination of A549 cells by mCBM40s when added directly to cell suspension, an agglutination assay was also performed using a spectrophotometric assay (Hwang *et al*, 1974)[2]. The rate of absorbance/sedimentation of A549 cells over time did not differ greatly between the conditions (Figure 14, A). This was further validated with a longer duration agglutination assay and a rate of absorbance/kinetics assay (Figure 14, B, C and D). This suggests that no agglutination of A549 cells (at 0.8 $x10^6$ cells/mL) in the

presence of 1mg/mL mCBM40 occurs. The inhibition of A549 cell chemotaxis can, therefore, be attributed to the direct inhibition of cellular migration and not cellular agglutination.

Cell apoptosis, viability and cytotoxicity assay.

Method: To determine if multimeric CBMs cause cell apoptosis, the Promega ApoTox-Glo™ Triplex Assay was used. This assay was performed using A549 cells. 10,000 cells per well were seeded 24hrs prior to experiment. CBMs and controls were added to wells to a final volume of 100µL with final CBM concentrations of 0.1 – 1 mg/ml. Cells were cultured in conditions for test exposures of 4hrs, 24hrs, 48hrs and 72hrs (Figure 8).

Results: After 48hrs, *Vc*2CBMTD (at 400µg) showed elevated luminescence signal corresponding to activation of caspase 3 (a hallmark of apoptosis). All the other conditions at 4hrs, 24hrs and 48hrs showed similar luminescence levels to that of the untreated cells (Figure 11). The fluorescence signal for the cleaved AFC is lower in CBM conditions after 48hrs (Figure 12). This might be due to either reduced permeability of the substrate, cells entering into a dormant state and/or cell cycle arrest. During most cytotoxic events, viability and cytotoxic measures will be inversely proportional; a reduction in viability without an increase in cytotoxicity is seen for compounds/drugs that alter normal cell division (i.e. cell cycle arrest) without producing membrane integrity changes. A number of the CBMs showed a decreased level of fluorescence for R110 indicating low levels of cytotoxicity (Figure 13). Results from this cell viability, cytotoxicity and apoptosis experiment suggest that in the presence of mCBM40s, A549 cells are in cell cycle arrest or a state of dormancy.

Discussion

The results identify that CBMs (*Vc*4CBM, *Vc*2CBMTD and *Sp*2CBMTD), but not the non-CBM component (TD), inhibit cellular behaviours required for wound healing (proliferation and migration) and are effective against multiple cancer types as indicated by experiments using breast, colon, cervical and lung cells. Agglutination of the A549 cell line was not observed. The inhibition of both migrating and proliferating cells in the wound-healing assay can be reversed via a wash-off. The results obtained from the Promega ApoTox-Glo™ Triplex Assay suggest that CBMs cause cell cycle arrest/or dormancy in the A549 cell line.

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References for Example 2

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Claims

1. A sialic acid binding molecule for use in a method of modulating cell growth and/or cell activity.

2. The sialic acid binding molecule of claim 1, for use of claim 1, wherein one or more of cell viability, cell migration, cell metabolism, cell differentiation and/or cell morphology/phenotype are modulated.

3. A sialic acid binding molecule for use in treating and/or preventing a disease and/or condition caused, contributed to and/or characterised by aberrant cell growth, differentiation, proliferation and/or activity.

4. The sialic acid binding molecule of claim 3, for use of claim 3, wherein the diseases which are caused, contributed to or characterised by aberrant cell growth, differentiation, proliferation and/or activity are selected from the group consisting of cell proliferation and/or differentiation disorders; and cancer.

5. A sialic acid binding molecule for use in the treatment and/or prevention of cancer.

6. The sialic acid binding molecule of claims 3 or 4, for use of claims 3 or 4, wherein the cancer is lung cancer, cervical cancer, colon cancer and/or breast cancer.

7. The sialic acid binding molecule of any preceding claim, for use of any preceding claim, wherein the sialic acid binding molecule comprises one or more family 40 carbohydrate binding module(s).

8. The sialic acid binding molecule of any preceding claim, for use of any preceding claim, wherein the sialic acid binding molecule comprises the sialic acid binding domain of *Vibrio cholerae* NanH sialidase and/or the sialic acid binding domain of *Streptococcus pneumoniae* NanA sialidase.

9. The sialic acid binding molecule of claim 8, for use of claim 8, wherein the *Vibrio cholerae* NanH sialidase comprises the amino acid sequence of SEQ ID NO: 1 or 2.

10. The sialic acid binding molecule of claim 8, for use of claim 8, wherein the *Streptococcus pneumoniae* NanA sialidase comprises the amino acid sequence of SEQ ID NO: 3 or 4.

11. The sialic acid binding molecule of any preceding claim, for use of any preceding claims, wherein the sialic acid binding molecule is a multimeric CBM.

12. The sialic acid binding molecule of claim 11, for use of claim 11, wherein the multimeric CBM comprises two or more *Vibrio cholerae* NanH sialidases and/or two or more *Streptococcus pneumoniae* NanA sialidases.

13. The sialic acid binding molecule of claim 11 or 12, for use of claim 11 or 12, wherein the multimeric CBM comprises 2, 3, 4, 5, 6, 7, 8, 9 or 10 *Vibrio cholerae* NanH sialidases and/or *Streptococcus pneumoniae* NanA sialidases.

14. The sialic acid binding molecule of any one of claims 11, 12 or 13, for use of any one of claims 11, 12 or 13, wherein the multimeric CBM is *Vc*4CBM, *Vc*2CBMTD and/or *Sp*2CBMTD.

15. A sialic acid binding molecule for use in the treatment and/or prevention of cancer, wherein the sialic acid binding molecule comprises:

(i) one or more sialic acid binding domain(s) of *Vibrio cholerae* NanH sialidase and/or one or more sialic acid binding domain(s) of *Streptococcus pneumoniae* NanA sialidase;

(ii) a multimeric CBM;

(iii) a multimeric CBM comprising two or more *Vibrio cholerae* NanH sialidases and/or two or more *Streptococcus pneumoniae* NanA sialidases; and/or

(iv) *Vc*4CBM, *Vc*2CBMTD and/or *Sp*2CBMTD.

16. An *in vitro* method of modulating cell growth and/or cell activity, said method comprising contacting a cell or population of cells with a sialic acid binding molecule so as to modulate cell growth and/or cell activity.