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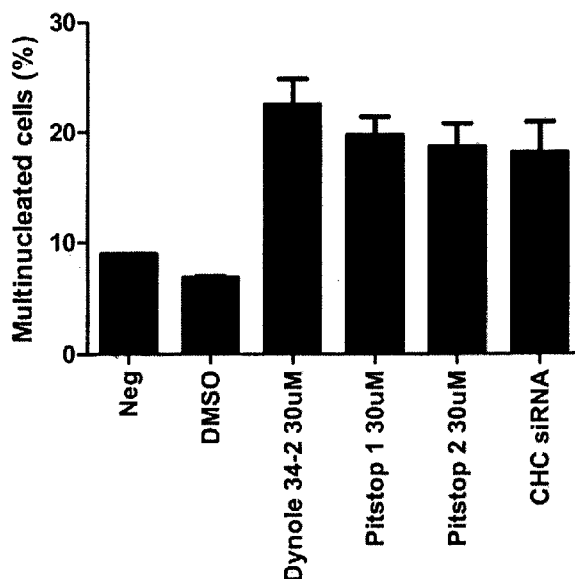
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(54) Title: INHIBITION OF CLATHRIN

FIGURE 12

(57) **Abstract:** Inhibitors are provided for inhibiting the activity of clathrin. In particular, binding site(s) on the clathrin terminal domain (TD) for binding inhibitors have been identified. The binding sites are defined by amino acid(s) in the group Ile 52, Ile 62, Ile 80, Phe 91, and Ile 93 of the clathrin TD (SEQ ID No. 1). In at least some forms, the binding site may be further defined by amino acid(s) in the group Ile 66, Arg 64, Leu 82 and Lys 96 of SEQ ID No. 1, or by Val 50 of SEQ ID No. 1. There are also provided methods for prophylaxis or treatment of disease and conditions responsive to the inhibition of clathrin.



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INHIBITION OF CLATHRIN

FIELD OF THE INVENTION

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The present invention relates to the inhibition of clathrin and in particular though not exclusively, to the inhibition of clathrin-mediated endocytosis (CME) and cell division (mitosis). The inhibition of clathrin has application to the prophylaxis or treatment of various diseases and physiological conditions.

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BACKGROUND OF THE INVENTION

Clathrin-mediated endocytosis (CME) is a major process by which cargo is internalized into intracellular vesicles, thereby regulating the cell surface levels and endocytic uptake of important plasma membrane proteins. These include nutrient and growth factor receptors, ion channels, adhesion proteins, and synaptic vesicle (SV) membrane proteins in the nervous system. The clathrin based endocytic machinery is also hijacked by pathogens such as bacteria and viruses to get access to the cell interior. In addition, clathrin plays a function in traffic between the trans-Golgi network and the endo-lysosomal system, a pathway related to lysosomal storage disorders among other diseases. Assembly of clathrin-coated pits (CCPs) at plasma membrane sites is likely initiated by the recruitment of early-acting endocytic proteins. These factors serve as scaffolds for the co-assembly of heterotetrameric AP-2 complexes, which coordinate recognition of transmembrane cargo with the recruitment of endocytic accessory proteins. Early endocytic structures are presumably stabilized by an assembling clathrin coat built from soluble clathrin triskelia comprising three heavy and three light chains. The central building block of triskelia is the 190 kDa clathrin heavy chain, which forms an extended three-legged structure. The N-terminal β -propeller domain (referred to as the terminal domain or TD) at the distal end of the leg adopts a WD40-like fold (ter Haar et al., 2000), while the C terminus is near the vertex of the triskelion (Brodsky et al., 2001).

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The clathrin cage has been postulated to serve at least two major functions in the endocytic process: (i) stabilizing deformed membrane domains, thereby facilitating the

transition to invaginated pits, and (ii) providing an interaction hub for the recruitment of accessory factors that regulate progression of endocytosis. Indeed structural and proteomic studies have revealed a surprisingly simple architecture of accessory protein-clathrin interactions. Most of these factors, including amphiphysins, Eps15/ Eps15R, OCRL, and arrestins, harbor so-called clathrin box motifs, simple degenerate peptides that bind to a structurally well-defined site on the TD of clathrin heavy chain (ter Haar et al., 2000), consistent with the clathrin TD functioning as a central protein-protein interaction hub within the endocytic network.

Clathrin has a central role in many cellular processes. Besides CME, clathrin has involvement in mitosis. Mitosis is the stage of cell division where the chromosomes segregate and two new daughter cells form. Segregation is achieved via kinetochores which mediate attachment of chromosomes to spindle microtubules (MTs) known as the mitotic spindle. In its triskelia form clathrin binds the mitotic spindle, providing a structural lattice (Royle et al, 2005). The localisation of clathrin to the mitotic spindle is dependent on Aurora A, TACC3, and ch-TOG. Inhibitors of Aurora A target the mitotic spindle and are currently in pre-clinical or clinical studies as cancer treatments.

The inhibition of clathrin thus has application to the prophylaxis or treatment of a wide range of diseases and conditions. Inhibitors of clathrin would not only be important tools for unravelling the role of clathrin during mitosis but in at least some forms, have application as anti-mitotic compounds useful for the treatment of cancer and cell proliferative conditions. There is an ongoing need to identify molecular reagents that can selectively inhibit clathrin function and acutely interfere with CME and/or mitosis. Whilst small molecule inhibitors of dynamin (a protein with a central role in endocytosis) have been reported, it is believed that no specific inhibitors of clathrin have previously been described in the literature.

SUMMARY OF THE INVENTION

Broadly stated, the invention stems from the surprising finding that the clathrin terminal domain (TD) has an unexpected central role in clathrin-coated pit (CCP) dynamics. In particular, the invention relates, to the identification and characterization of a specific binding site on the clathrin TD enabling the inhibition of clathrin-mediated endocytosis (CME) in cells and more generally, inhibition of clathrin function. The

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identification of the binding site facilitates the rational design of inhibitors for targeted binding to the clathrin TD for the inhibition of clathrin.

In an aspect of the invention there is provided a clathrin inhibitor wherein the inhibitor binding to a binding site of the terminal domain (TD) of clathrin forms a complex with the clathrin TD, the binding site of the clathrin TD being defined by at least one or more of amino acids Ile 52, Ile 62, Ile 80, Phe 91 and/or Ile 93 of SEQ ID No. 1.

In another aspect of the invention there is provided a complex of a clathrin inhibitor and the terminal domain (TD) of clathrin or a fragment of the clathrin TD, the inhibitor binding to a binding site of the clathrin TD, and the binding site being defined by at least one or more of amino acids Ile 52, Ile 62, Ile 80, Phe 91 and/or Ile 93 of SEQ ID No. 1.

Typically, the binding of the clathrin inhibitor to the clathrin TD is provided by binding interactions of the inhibitor with at least some of amino acids Ile 52, Ile 62, Ile 80, Phe 91 and/or Ile 93 of SEQ. No. 1.

Typically, the clathrin inhibitor interacts with all of amino acids Ile 52, Ile 62, Ile 80, Phe 91, and Ile 93 of SEQ ID No.1.

In at least some embodiments, the binding site of the clathrin TD for the clathrin inhibitor is defined by one or more of further amino acids Ile 66, Arg 64, Leu 82 and/or Lys 96 of SEQ ID No. 1, and the binding of the inhibitor is provided by binding interactions of the inhibitor with the one or more further amino acids.

Typically, in this embodiment, the clathrin inhibitor interacts with all of amino Ile 66, Arg 64, Leu 82 and Lys 96 of SEQ ID. No. 1.

In another embodiment, the binding site can be further defined by amino acid Val 50 of SEQ ID No. 1 and the binding of the inhibitor is provided by binding a interaction of the inhibitor with that amino acid.

The binding interactions between the clathrin inhibitor with the clathrin TD can comprise direct contact of the inhibitor with at least some of the amino acids defining the binding site of the clathrin TD for the inhibitor.

Typically, the above binding interactions between the inhibitor and the clathrin TD involve direct contact between the inhibitor and respective of the amino acids defining the binding site of the clathrin TD.

Typically, the binding of the inhibitor to the clathrin TD is stabilised by one or more hydrogen bonds between the inhibitor and the clathrin TD. The hydrogen bonds can be direct hydrogen bonds between the inhibitor and the clathrin TD and/or hydrogen bonds mediated by water.

5 In another aspect of the invention there is provided a method for inhibiting clathrin, comprising contacting clathrin with an effective amount of at least one clathrin inhibitor for forming a complex with the terminal domain (TD) of clathrin in accordance with one or more embodiments of the invention, or a prodrug or physiologically acceptable salt of the inhibitor.

10 A complex in accordance with the invention can be formed *in vitro* or *in vivo*, and in some forms, may be isolated or at least partially purified.

As such, animal cells or tissues can be treated with the inhibitor *in vitro* or *in vivo* for the inhibition of clathrin, and the invention expressly extends to the prophylaxis or treatment of diseases or conditions responsive to the inhibition of clathrin, or for
15 which the inhibition of clathrin may otherwise be beneficial.

Accordingly, in another aspect there is provided a method for inhibiting clathrin in a mammal, comprising administering to the mammal an effective amount of at least one clathrin inhibitor for forming a complex with the terminal domain (TD) of clathrin in accordance with one or more embodiments of the invention.

20 Typically, the clathrin inhibitor(s) are administered for the inhibition of CME and/or inhibition of cell division.

In another aspect there is provided a method of screening a putative clathrin inhibitor, comprising:

25 fitting a model of a putative inhibitor of clathrin to a representation of a binding site for the inhibitor on the terminal domain (TD) of clathrin;

modelling interaction of the inhibitor with the binding site to evaluate whether the inhibitor forms a complex with the clathrin TD in accordance with one or more embodiments of the invention; and

30 evaluating whether the inhibitor may inhibit clathrin on the basis of the modelling, the formation of the complex being indicative of the capacity of the inhibitor to inhibit clathrin.

In still another aspect there is provided a method for providing a clathrin inhibitor, comprising:

designing the inhibitor to bind to a binding site for the inhibitor on the terminal domain (TD) of clathrin;

5 modelling interaction of the inhibitor with the binding site; and

evaluating whether the inhibitor forms a complex with the binding site of the clathrin TD in accordance with one or more embodiments of the invention on the basis of the modelling, the formation of the complex being indicative of capacity of the inhibitor to inhibit clathrin.

10 In this embodiment, the designing of the inhibitor can involve fitting the inhibitor into the binding site of the clathrin TD.

A range of inhibitors that bind to the clathrin TD binding site and inhibit clathrin function (e.g., in CME or mitosis) have been identified by the inventors.

Advantageously, the identification and characterisation of the clathrin TD binding site
15 as described herein facilitates the rational design of inhibitors for use in embodiments of the invention.

In yet another aspect of the invention there is provided a clathrin inhibitor compound of Formula I to Formula V as described herein, or a prodrug or pharmaceutically acceptable salt of the compound.

20 In another aspect of the invention there is provided a pharmaceutical composition comprising at least one compound of Formula I to Formula V, or a prodrug or pharmaceutically acceptable salt of the compound, together with a pharmaceutically acceptable carrier.

In another aspect of the invention there is provided a method for inhibiting
25 clathrin, comprising contacting clathrin with an effective amount of a compound of Formula I to Formula V, or a prodrug or physiologically acceptable salt of the compound.

In another aspect of the invention there is provided a method for inhibiting clathrin in an animal, comprising administering to the mammal an effective amount of a
30 compound of Formula I or Formula V to the mammal, or a prodrug or pharmaceutically acceptable salt thereof.

In another aspect there is provided a clathrin inhibitor embodied by the invention for use in inhibiting clathrin in a mammal, or a prodrug or physiologically acceptable salt of the inhibitor.

5 In another aspect of the invention there is provided the use of a clathrin inhibitor embodied by the invention in the manufacture of a medicament for inhibiting clathrin in a mammal in need thereof, or a prodrug or physiologically acceptable salt of the inhibitor.

10 In another aspect of the invention there is provided the use of a compound of Formula I to Formula V in the manufacture of a medicament for prophylaxis or treatment of a disease or condition as described herein.

By the term "terminal domain of clathrin" and variations thereof such as "clathrin TD" and "clathrin terminal domain" as used herein is meant the N-terminal β -propeller region of clathrin heavy chain 1, such as defined by amino acids 2-479 of SEQ ID No. 1 (Genbank Accession No. NM-004859.3, National Center for
15 Biotechnology Information, National Institutes of Health, Rockville Pike, Bethesda, Maryland, USA), and fragments thereof providing the binding site for a clathrin inhibitor in accordance with the invention.

By the term "clathrin inhibitor" as used herein and variations thereof such as "inhibitor of clathrin", is meant a compound that interacts with the clathrin TD in
20 accordance with the invention to at least partly inhibit the functional activity of clathrin, examples of which are the inhibition of clathrin in clathrin-mediated endocytosis (CME) as may be expressed by a reduction in CME and/or the level of CME in cells, and the inhibition of cell growth and proliferation by inhibiting mitosis. The inhibition of clathrin in accordance with the invention can be total or partial inhibition of the
25 functional activity of clathrin.

By the term "agent" as used herein is meant a clathrin inhibitor as described herein, or a composition or preparation comprising a clathrin inhibitor.

By the term "binding" as used herein in the context of binding of the clathrin inhibitor with the binding site of the clathrin terminal domain, is meant association of
30 the clathrin inhibitor with the binding site through binding interactions of the inhibitor with at least some the amino acids defining the clathrin TD binding site. A binding interaction of the inhibitor with the clathrin TD can, for example, comprise direct contact of the inhibitor with amino acid(s) of the clathrin TD that define the binding

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site, the formation of one or more direct hydrogen bonds between the inhibitor and the clathrin TD, and/or the formation of one or more hydrogen bonds between the inhibitor and the clathrin TD mediated by water.

5 The identification of the clathrin TD binding site and provision of inhibitors of clathrin in accordance with one or more embodiments of the invention, not only facilitates the dissection of the mechanisms of endocytic pathways and CME and CCP dynamics in research, but has application to modulation of cell signalling, inhibition of entry of viruses and other pathogenic agents into cells, inhibition of synaptic transmission (such as in epilepsy) and more generally, the targeted inhibition of clathrin
10 for the prophylaxis or treatment of a wide variety of diseases or physiological conditions. In addition to this role in CME, as described above, these inhibition of clathrin has application to inhibiting mitosis, and hence in the prophylaxis or treatment of cancer and more generally, cell proliferative conditions. Moreover, inhibitors, compounds and methods embodied by the invention have application to use in research
15 into clathrin function.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers, integers or steps.

20 Any discussion of documents, acts, materials, devices, articles or the like that has been included in this specification is solely for the purpose of providing a context for the invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the invention as it existed in Australia or elsewhere before the priority date of this
25 application.

The features and advantages of the invention will become further apparent from the following detailed description of exemplary embodiments of the invention together with the accompanying drawings.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS

Figure 1: Amino acid sequence for Homo sapiens clathrin heavy chain 1 (SEQ ID No. 1; Genbank Accession No. NM-004859.3 (GI: 11527063), National
5 Centre for Biotechnology Information, National Institutes of Health, Rockville Pike, Bethesda, Maryland, USA). The clathrin TD of Homo sapiens clathrin heavy chain 1 is defined by amino acid residues 2-479 of SEQ ID. No 1. Amino acid 1 of SEQ ID No. 1 is an initiator methionine residue.

Figure 2: A ligplot showing interaction of the clathrin inhibitor pitstop 1 with
10 the clathrin TD.

Figure 3: A ligplot showing interaction of the clathrin inhibitor pitstop 2 with the clathrin TD.

Figure 4: Pitstops compete with clathrin box ligands for a common site on the clathrin TD. (A, C) Ribbon representations of the clathrin TD in top view. The blades
15 of the TD- β -propeller are numbered from 1 to 7. Both inhibitors bind specifically to the clathrin-box binding site (between blades 1 and 2 of the clathrin TD). (B) Close-up view of the binding site for pitstop 1. The inhibitor and amino acids of the binding groove are shown in ball-and-stick mode. Hydrogen bonds formed at both ends of pitstop 1 are indicated by broken lines. The non-polar portion of pitstop 1 is
20 sandwiched between Phe91 and the Arg64 side chains. On one side of the non-polar portion of pitstop 1 parallel π - π stacking interactions take place with the aromatic ring of Phe91. On the other end polar π -stacking interactions take place between the guanidinium group of Arg64 (δ^+ dipole) and the π -electrons of the aromatic rings (δ^- dipole) of the inhibitor. (D) Close-up view of the binding site for pitstop 2. The
25 inhibitor and amino acids are shown in ball-and-stick mode. Bidentate hydrogen bonds at 3.0 Å distance are formed between the guanidinium group of Arg64 and O and N atoms of the central portion of pitstop 2. The aromatic ring of Phe91 is located in the same position as in the inhibitor-free form of the clathrin TD and stacks against the edge of the bromobenzene of pitstop 2. (E, F) For comparison, pitstops (surface
30 representations) are superimposed with the clathrin box-containing AP-3 β 3-hinge peptide (sequence AVSLLDLDA) (SEQ ID No. 2). Pitstop 1 and pitstop 2 are shown in solid form. Binding of the inhibitors to the clathrin TD blocks the association of the β 3-hinge peptide and similar clathrin-box ligands.

Figure 5: Pitstop 2 reversibly inhibits clathrin-mediated endocytosis.

(A) Pitstop 2 reversibly inhibits Tf uptake. HeLa cells were incubated with Alexa⁵⁶⁸-Tf in the presence of DMSO or 30 μ M pitstop 2. Tf uptake is seen to resume after washout of the drug for 1 h. Scale bar, 10 μ m. (B) Reversibility and dose-dependence of pitstop 2-mediated inhibition of Tf uptake. Tf internalization after washout is not significantly different from uptake in the DMSO control (SEM; $n = 3$ independent experiments; * $p < 0.05$, *** $p < 0.0001$). (C) Pitstop 2 inhibits EGF uptake. HeLa cells were incubated with Alexa⁴⁸⁸-EGF in the presence of DMSO or 30 μ M pitstop 2. Data represent SEM ($n = 3$ independent experiments; *** $p < 0.0001$). (D) Pearson's correlation between Alexa⁴⁸⁸-EGF and AP-2. Data represent SEM ($n = 3$ independent experiments; * $p < 0.05$). (E) Pitstop 2 blocks HIV infection. Shown is the HIV Tat-driven luciferase activity in cells treated with increasing concentrations of pitstop 2 during infection. HIV Tat-driven luciferase activity in mock-treated cells was set as 100 % infection.

Figure 6: Ultrastructural analysis of intermediates observed in pitstop 2-treated cells. (A) Representative examples of clathrin-coated structures observed at the plasma membrane of Cos7 cells treated with 0.1 % DMSO or 30 μ M pitstop 2. Scale bar, 200 nm. Morphological groups were: shallow CCPs (stage 1), non-constricted u-shaped CCPs (stage 2), constricted Ω -shaped CCPs (stage 3), or structures containing complete clathrin coats (stage 4). (B) Bar diagram displaying the numbers of clathrin-coated endocytic intermediates observed along the perimeter of Cos7 cells treated with 0.1 % DMSO or 30 μ M pitstop 2.

Figure 7: Pitstop 1 inhibits activity-induced SV recycling in lamprey reticulospinal synapses. (A, B) Electron micrographs of reticulospinal synapses microinjected with pitstop 1 and stimulated at 5 Hz for 20 min. Note the depletion of SVs (sv) at sites of release and large membrane expansions and pockets (asterisks), which in some sections (e.g., in B) surrounded the active zone (marked with thick arrows). Boxed area containing clathrin-coated pits (CCPs) is shown as inset at higher magnification in A. (C) EM-image of a control non-injected synapse from the same spinal cord preparation. (D, E) Constricted CCPs from a control non-injected axon and a synapse microinjected with pitstop 1. Note the similar shape of CCPs and the presence of clathrin coats in both cases. (F) Electron micrograph of a synapse microinjected with pitstop 1 but kept at rest. (G, H, I) Bar diagrams showing the

difference in numbers of SVs (Nsv), curvature index (CI), and the number of clathrin-coated intermediates (Nccp) between control synapses stimulated at 5 Hz (white bars) and pitstop 1-microinjected stimulated synapses (black bars), respectively. ax-
axoplasmic matrix; d-dendrite. (J) Bar diagram showing the relative abundance of

different stages of coated endocytic intermediates (see above description for Fig. 6A) in control synapses stimulated at 5 Hz (white bars) and stimulated synapses microinjected with pitstop 1 (black bars). Scale bars for (A-C, and F), 0.5 μ m and (D, E), 100 nm.

Figure 8: Treatment with the pitstops 1 and 2 causes mitotic failure.

The percentage of cells trapped in mitosis increased with increasing concentrations of the pitstop compounds. At 30 μ M pitstop 1 more than triples the mitotic index from 1% in untreated controls and 2% in DMSO treated to 7%, and at 10 μ M, pitstop 2 increases the mitotic index to 5%, while clathrin depletion with siRNA increased the mitotic index to 11%.

Figure 9: Pitstops 1 and 2 affect the mitotic spindle. (A) The triskelial clathrin molecule provides a structural lattice for the mitotic spindle. In the absence of clathrin the spindle collapses becoming narrow. The graph shows the percentage of cells with narrow spindles when treated with pitstops. The inhibition of clathrin by the pitstop compounds gave the same narrow spindle phenotype as clathrin depleted cells. (B) Graph quantifying spindle width as a ratio of the total cell width shows that upon pitstop treatment the spindles significantly narrow. (C) Images from fluorescence microscopy showing the narrow spindle phenotype caused by treatment with pitstops. Both pitstop 1 and 2 caused the spindle microtubules to collapse as evidenced by tubulin staining. The same type of collapsed spindles is seen in cells depleted of clathrin by siRNA. In untreated control cells, or cells treated with vehicle DMSO, the dynamin (and thereby endocytosis) inhibitor dynole 34-2, or depletion of epsin 1 by siRNA did not cause the spindle to collapse demonstrating the observed effect of pitstops is not solely due to endocytosis inhibition.

Figure 10: The pitstop compounds affect K fibre organisation during metaphase. Graph showing percentage of cells with disrupted HURP localisation.

HURP is a marker for K fibres, which are a unique subset of the spindle microtubules that bind to the kinetochores of chromosomes during metaphase. Depletion or inhibition of clathrin by siRNA or the pitstop compounds caused disruption to HURP staining.

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Figure 11: The pitstops activate the metaphase checkpoint. (A) Representative images of (A) and (A') showing the co localisation of CENPB (red), MAD2 (green), and DNA (DAPI, blue), white arrows in zoom shows active checkpoints. HeLa cells where synchronised at the G2/M boarder by the cdk1 inhibitor RO-3306, then released in the presence of the indicated drug or control for 90 min, then fixed and stained for CENPB (red), MAD2 (green) and DNA (DAPI, blue). Captured images were decovoled and the channels combined. (B) The number of co-localisations between CENPB and MAD2 were scored for $n \geq 30$ cells per sample, and are shown on the dot graph; the median is represented by the line. Inhibition by the pitstop compounds increased the number of active checkpoints per cell. (C) HeLa cells were depleted of either clathrin heavy chain (CHC) or epsin by siRNA and scored as in (B). Depletion of CHC increased the number of active checkpoints. $N \geq 30$ cells per sample, and are shown on the dot graph; the median is represented by the line.

Figure 12: Pitstops induce multinucleation. The level of multinucleation (indicative of failed cytokinesis) more than doubled in cell populations treated with pitstop 1 or pitstop 2, or in cells depleted of clathrin by treatment by specific siRNA.

Figure 13: Crystal data for the terminal domain of clathrin complexed with pitstop 1.

Figure 14: Crystal data for the terminal domain of clathrin complexed with pitstop 2.

Figure 15: Crystal data for amino acids in pocket of the terminal domain of clathrin defining the binding site of clathrin for pitstop 1.

Figure 16: Crystal data for amino acids in pocket of the terminal domain of clathrin defining the binding site of clathrin for pitstop 2.

DETAILED DESCRIPTION OF EXEMPLARY EMBODIMENTS OF THE INVENTION

In this specification, the term "alkyl" used either alone or in a compound word such as alkylaryl refers to a straight chain, branched or mono- or polycyclic alkyl. Examples of straight chain and branched alkyl include methyl, ethyl, propyl, *iso*-propyl, butyl, and the like. Examples of cyclic alkyls include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl and cycloheptyl.

The term "alkenyl" refers to a straight chain, branched or cyclic alkenyl with one or more double bonds. Examples of alkenyl ethenyl, propenyl, and butenyl.

Typically, the alkyl or alkenyl will be a linear alkyl or linear alkenyl substituent. Most typically, the alkyl or alkenyl will have 10 carbon atoms or less in its longest chain and most typically, will be a lower alkyl or lower alkenyl. A lower alkyl or lower alkenyl as described herein is to be taken to have 6 carbon atoms or less in the longest chain of the alkyl or alkenyl group. Hence, a lower alkyl or lower alkenyl as described herein may have 5, 4, 3, 2 or 1 carbon atom(s) in its longest chain.

The term "alkoxy" refers to an alkyl group with an oxygen radical substituent. Examples include methoxy, ethoxy and n-propoxy. The term "lower alkoxy" refers to an alkoxy with 6 atoms or less in the longest alkyl chain of the alkoxy group (e.g., 5, 4, 3, 2 or 1 carbon atoms). An alkoxy substituent as described herein will typically be a methoxy group.

The term "aryl" used either alone or in compound words such as "alkylaryl", refers to a single, polynuclear, conjugated or fused aromatic hydrocarbon or aromatic heterocyclic ring system. Examples of aryl groups include phenyl and naphthyl. When the aryl comprises a heterocyclic aromatic ring system, the aromatic heterocyclic ring system can contain one or more heteroatoms independently selected from N, O and S and will normally contain 5 or 6 ring members. Typically, an aryl group of a compound embodied by the invention or utilised in a method embodied by the invention will be selected from the group consisting of phenyl, naphthyl and benzyl.

The term "halo" as used herein refers to a halogen atom. The halogen atom is typically selected from the group consisting of F, Cl, Br and I.

The term "heteroaryl" refers to a heterocyclic aromatic ring system.

Heterocyclic, heteroaryl, aryl and other ring systems (eg., single and fused ring systems) as described herein can have from 5 to 7 ring atoms unless specified otherwise, and may contain one or more double bonds. Moreover, the ring or rings may independently be saturated and/or optionally, have one or more substituents.

By the term terminally substituted as used herein in the context of an alkyl or alkenyl is meant that the end carbon atom of the longest chain of the alkyl or alkenyl that is remote from the group substituted by the alkyl or alkenyl is substituted.

Methodology for screening a putative inhibitor of clathrin and the designing of an inhibitor of clathrin in accordance with embodiments of the invention are well

known to a person skilled in the art. Computer programs and systems for this are commercially available, and any conventionally known such methodology and/or modelling software can be utilised (e.g., AutoDock 4.2, AutoDock Vina (The Scripps Research Institute, La Jolla, CA, USA). Whilst the screening or modelling of clathrin inhibitors and their interaction with the clathrin TD binding site may at least in part be accomplished manually with graphical representations and 3-dimensional modelling, computer based systems are the preferred option. Such computer assisted modelling can involve the provision of an initial structural representation of an inhibitor and electronically modelling it into a representation of the clathrin TD binding site, such as an electron density map of the binding site to determine whether the fit of the putative inhibitor forms the desired complex with the binding site or whether optimisation of the structure of the putative inhibitor is desirable. The designing of an inhibitor of clathrin may also involve modelling the inhibitor to at least partially fit or conform to the structure of a pharmacore or other clathrin inhibitor as described herein.

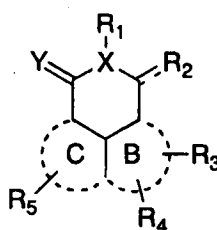
The design and optimisation of the structural features of the putative inhibitor can involve modification of the backbone scaffold of the inhibitor and/or modification (e.g., replacement), exclusion or addition of scaffold substituents to alter the interaction of the inhibitor with the binding site. This can take into account steric, lipophilic, and/or charge considerations (e.g., attraction and/or repulsion) to provide for formation of a complex with the clathrin TD in accordance with embodiments of the invention and/or to alter (i.e., increase or decrease) the affinity with which the inhibitor binds to the clathrin TD binding site. The design and modelling of the inhibitor can also take into account the formation of hydrogen bonds between the inhibitor and the clathrin TD wherein the hydrogen bond(s) can be formed directly between the inhibitor and the clathrin TD or be mediated by water, or be a mixture of those two possibilities. The design of compounds and modelling of ligand binding is for instance described in United States Patent Application Publication No. 2004/0219653, US 2009/0275047, US 2005/0170431 and US 2010/0247569, the entire contents of all of which are incorporated herein in their entirety by cross-reference.

Further, the crystal structure of a 55 kD fragment of the the terminal domain (TD) of the heavy chain of rat clathrin consisting of amino acid residues 1-494 and the methodology for obtaining same has previously been reported (ter Haar et al, 1998), as has the crystal structure for a fragment comprising amino acid residues 1-363 of the

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terminal domain TD of clathrin heavy chain respectively complexed with a peptide derived from β -arrestin 2 or the β -subunit of the AP-3 complex (the crystal structural data for which is available under ID codes 1C9L and 1C9I from the RBSC Protein Data Bank, Rutgers, the State University of New Jersey, Taylor Road, Piscataway, NJ, USA) (ter Haar, 1998; ter Haar, 2000), reference to which can also be had for the design and provision of clathrin inhibitors in accordance with the invention, and the contents of all of which are also expressly incorporated herein in their entirety by cross-reference.

A compound for use in a method of the invention may, for example, be a compound of Formula I, or a prodrug or pharmaceutically acceptable salt thereof, wherein:



Formula I

and wherein:

R_1 is alkyl, alkenyl, alkylaryl, aryl, or a ring having from 5 to 7 ring atoms including from 0 to 3 heteroatoms typically selected from O, N or S, wherein the alkyl, alkenyl, alkylaryl, aryl, and ring group are optionally substituted; and

R_2 is O, S or NH; or

R_1 , X, and R_2 form a ring A, the ring having from 5 to 7 ring atoms including from 0 to 3 heteroatoms typically selected from O, N or S and being optionally substituted; and

X is N or a carbon atom;

Y is O, S, or NH;

ring B and ring C each independently have 5 to 7 ring atoms including from 0 to 3 heteroatoms typically selected from O, N or S; and

R_3 , R_4 , and R_5 are each independently H or an optional substituent.

In instances R_1 is a ring substituent having from 5 to 7 ring atoms, the ring may include zero or one or more double bonds. Generally, the ring will have 6 ring atoms. Typically, the ring will not include any hetero ring atoms.

Typically, R_1 is a lower alkyl, lower alkylaryl, ~~heteroaryl~~, or aryl group, wherein the lower alkyl, lower alkylaryl or aryl group is optionally substituted. The aryl group may be a heteroaryl group.

In some embodiments, R_1 may be a C_1 - C_5 alkyl group and typically, a C_1 - C_3 alkyl, C_2 - C_3 alkyl, or C_1 - C_2 alkyl. The C_1 - C_5 alkyl group when substituted, will typically be terminally substituted.

In one or more embodiments, R_1 may be a lower alkyl terminally substituted with a ring having from 5 to 7 ring atoms including from 0 to 3 heteroatoms typically selected from O, N and S. This ring may include zero or one or more double bonds.

10 Typically, this ring has 6 ring atoms.

In at least some embodiments in which R_1 is a lower alkylaryl, the lower alkylaryl can be a C_1 - C_2 alkylaryl and typically, is a C_1 - C_2 alkylphenyl. The C_1 - C_2 alkylphenyl can be unsubstituted. When substituted, the phenyl ring of the C_1 - C_2 alkylphenyl is typically selected from the group consisting of 2-substituted phenyl, 3-substituted phenyl, and 4-substituted phenyl groups. Most typically, the C_1 - C_2 alkylphenyl is a benzyl group.

15

When R_1 is an aryl group, the aryl is typically phenyl. When substituted, the phenyl can be selected from the group consisting of 2-substituted phenyl, 2,4-substituted phenyl, and 2,5-substituted phenyl groups.

20 Substituents of R_1 may, for instance, be independently selected from the group consisting of hydroxyl, sulfhydryl, amino, nitro, halo, cyano, lower alkoxy, lower alkyl, $N(CH_3)_2$, CO_2H , SO_3H , PO_4H . For example, R_1 may be a C_1 - C_3 alkoxy, C_1 - C_3 alkanol, or a C_1 - C_3 alkylthiol and more usually, a C_1 - C_2 alkoxy, C_2 - C_3 alkanol, or C_2 - C_3 alkylthio. Typically, R_2 in a compound of Formula I is O. In at least some embodiments,

25 ring A of a compound of Formula I may be substituted with one or more substituents selected from alkyl, alkenyl, alkylaryl and aryl, wherein the alkyl, alkenyl, alkylaryl and aryl substituents are optionally substituted.

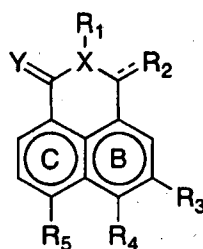
Each of ring A and ring B of a compound of Formula I may each independently be an aryl group, or a heteroaryl group having from 1 to 3 hetero ring atoms typically selected from O, N and S.

30

Typically, rings B and C of Formula I each have 6 ring atoms. Most typically, rings B and C form a naphthyl group wherein the naphthyl group is optionally substituted.

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In at least some embodiments, a compound of Formula I has the structure of Formula IIa as follows:

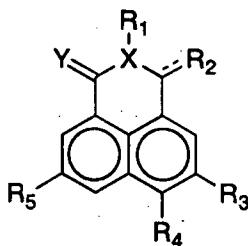


Formula IIa

5

wherein X, Y, R₁, R₂, R₃, R₄ and R₅ are as for Formula I.

In at least some embodiments, a compound of Formula I has the structure of Formula IIb as follows:



Formula IIb

10

wherein X, Y, R₁, R₂, R₃, R₄ and R₅ are as for Formula I. Typically, X in a compound of Formula I, IIa or IIb is N.

15 Typically, Y in a compound of Formula I, IIa or IIb is O or S and preferably, is O.

Typically, R₃ and R₄ of a compound of Formula I, IIa or IIb are independently selected from H, halo, cyano, hydroxyl, sulfhydryl, nitro, amino, alkoxy, alkyl, alkylaryl, aryl, NHR', NR'₂, CO₂H, SO₃H and PO₄H.

20 Most typically, R₃ and R₄ of a compound of Formula I, IIa or IIb are independently selected from H, halo, cyano, hydroxyl, sulfhydryl, nitro, amino, alkyl, alkylaryl, aryl, NHR', NR'₂, CO₂H, SO₃H and PO₄H.

In some embodiments, R₅ of a compound of Formula IIb is selected from H, halo, cyano, hydroxyl, sulfhydryl, nitro, amino, alkoxy, alkyl, alkylaryl, aryl, NHR',
25 NR'₂, CO₂H, SO₃H and PO₄H.

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Most typically, R_5 of a compound of Formula IIb is selected from H, halo, cyano, hydroxyl, sulfhydryl, nitro, amino, alkyl, alkylaryl, aryl, NHR' , NR'_2 , CO_2H , SO_3H and PO_4H . Typically, each R' of a compound of Formula I, IIa or IIb is independently H, alkyl, alkenyl, aryl, or alkylaryl, wherein the alkyl, alkenyl, aryl or alkylaryl is optionally substituted.

Typically, at least one of R_3 and R_4 of a compound of Formula I, IIa or IIb is other than H.

In some embodiments, R_5 of a compound of Formula IIa is H, aryl, naphthyl, benzyl or tetralin, wherein the aryl, naphthyl, benzyl and tetralin are optionally substituted.

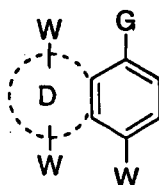
When R_5 is aryl, the aryl group is typically phenyl or naphthyl.

Typically, ring A of a compound of Formula I, IIa and IIb has 5 or 6 ring atoms and most typically, 5 ring atoms. Most typically, ring A is an imadazolyl group.

Typically, in compounds of Formula I, IIa or IIb in which R_1 , X and R_2 form ring A, R_2 is NR_6 and R_6 forms ring A with R_1 and X.

Typically, ring A has an aryl group substituent, the aryl group being fused with ring A. The aryl group fused with ring A will normally have 6 ring atoms and most typically is a phenyl group. The phenyl group is optionally substituted. Typically, ring A when substituted with phenyl forms a benzimidazol group.

In at least some embodiments, R_5 of a compound of Formula I or IIa is a substituent of Formula III, as follows:



Formula III

wherein:

G is a bond with ring C;

ring D has from 5 to 7 ring atoms optionally including from 0 to 3 heteroatoms selected from N, O and S; and

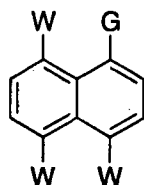
each W is independently H, halo, nitro, amino, hydroxyl, sulfhydryl, CO_2H , SO_3H , or PO_4H .

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Typically, ring D is a carbocyclic or aryl group. Most typically, ring D has 6 ring atoms.

Typically, at least one W substituent of Formula III is other than H.

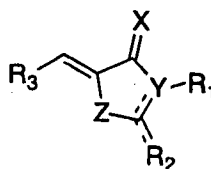
Typically, R₅ of compound of Formula I or IIa is a substituent group of Formula
5 IV as follows:



Formula IV

10 wherein each W is independently H, CO₂H, SO₃H, or PO₄H.

In another form, a compound for use in a method embodied by the invention may be a compound of Formula V, or a prodrug or pharmaceutically acceptable salt thereof, wherein:



15

Formula V

and wherein:

X is O, S, or NH;

Y is O, N or a carbon atom;

Z is O, S or NH;

20 R₁ is H, CO₂H, SO₃H, PO₄H, alkyl, alkenyl, alkylcarboxy, or alkylaryl;

R₂ is O, S, NH, NHR', NHS(=O)₂R', S(=O)₂R', sulfonyl, NHC(=O)NH, NHC(=S)NH, or NHC(=O);

R' is H, alkyl, alkenyl, alkylaryl, aryl, the alkyl, alkenyl, alkylaryl and aryl group being optionally substituted; and

25 R₃ is aryl or a polycyclic group having at least 2 fused rings each independently having from 5 to 7 ring atoms, the aryl and the polycyclic group being optionally substituted.

Typically, R_1 in a compound of Formula V is H or a lower alkyl or lower alkenyl, wherein the lower alkyl or lower alkenyl group is optionally substituted and generally, terminally substituted.

When R_1 of Formula V is lower alkyl, the lower alkyl is typically a C_1 - C_3 alkyl and more preferably, a C_1 - C_2 alkyl. In at least some embodiments, the lower alkyl is terminally substituted, preferably with a carboxy or thiocarboxy group (e.g., a lower alkylcarboxy or lower alkylthiocarboxy).

Substituents of R_1 in a compound of Formula V, may for instance, be selected from the group consisting of hydroxyl, sulfhydryl, amino, nitro, halo, cyano, CO_2H , SO_3H and PO_4H .

Typically, R_2 in a compound of Formula V is S, NH, $HN-S(=O)_2-R'$, $S(=O)_2R'$, or $NHC(=S)NH$, and R' is alkyl, alkenyl or aryl, wherein the alkyl, alkenyl or aryl is optionally substituted. Typically, the lower alkyl and alkenyl are lower alkyl and lower alkenyl, respectively.

Typically R' of $HN-S(=O)_2-R'$ is aryl and most typically, phenyl, benzyl naphthyl, or tetralin.

In at least some embodiments, R_3 of Formula V is phenyl or an aryl group with 2 fused rings each independently having from 5 to 7 ring atoms including from 0 to 3 heteroatoms selected from O, N and S.

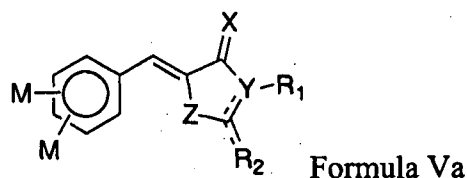
When R_3 of Formula V is a polycyclic group, R_3 will typically have 2 fused rings at least one of which is an aryl group having 6 ring atoms. Typically, each ring of the aryl group has 6 ring atoms.

R_3 of Formula V may, for example, be optionally substituted and selected from the group consisting of phenyl, naphthyl, benzopyranyl, quinoliny, quinoxaliny, quinoxoliny, cinnoliny, benzofuranyl, isobenzofuranyl, indolyl, isoindolyl, benzothiophenyl, benzo[c]thiophenyl, benzimidazolyl, purinyl, indazolyl, benzoxazolyl, benzoisoxazolyl, and benzothiazolyl.

Typically, the R_3 group is selected from phenyl, naphthyl, and benzopyranyl (e.g., benzopyran-4-one). When R_3 is phenyl, in a compound of Formula V, the phenyl may be mono, di, tri or tetra-substituted (e.g., trihydroxy phenyl or tetrahydroxy phenyl). Suitable such substituents may, for example, be selected from the group of substituents as defined for M in Formula Va below.

-20-

In at least some embodiments, a compound of Formula V has the structure of Formula Va as follows:

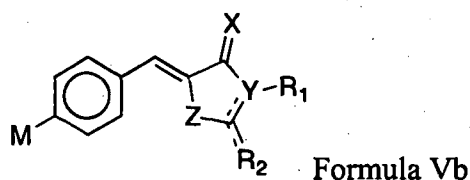


5 wherein each M is independently selected from H, halo, hydroxy, sulfhydryl, nitro, amino, cyano, CO₂H, SO₃H, PO₄H, OR', NHR', NR'₂, SO₂R', SO₂NH₂, wherein R' is as defined as Formula II. Typically, at least one M substituent is other than hydrogen.

Typically, when M is OR', the R' is a lower alkyl or lower alkenyl, the lower
10 alkyl or alkenyl being optionally substituted. When substituted, the alkyl or alkenyl group is optionally terminally substituted. The terminal substituent of R' may, for example, be selected from the group consisting of amino, nitro, halo, cyano, hydroxy, sulfhydryl, and typically, is amino.

Typically, the phenyl group shown in Formula Va is selected from the group
15 consisting of 4-substituted phenyl, and 3,5-substituted phenyl groups.

In at least some embodiments, a compound of Formula Va may have the structure of Formula Vb as follows:



20

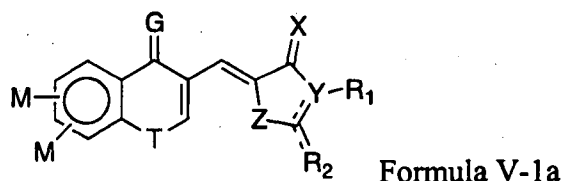
Typically, M in a compound of Formula Vb is halo, nitro or -OR'NH₂, wherein R' is a lower alkyl or lower alkenyl.

When an M substituent is -OR'NH₂ in a compound of Formula Va or Vb, R' is typically a C₁-C₄ alkyl and preferably, a C₃ alkyl.

25

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In other embodiments, a compound of Formula V may have the structure of Formula V-1a as follows:



5

wherein:

G is O or S;

T is O, S, N or a carbon atom; and

each M is independently as defined for Formula Va.

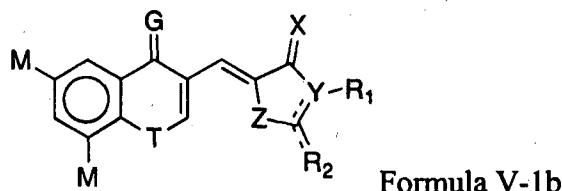
10

Typically, at least one M substituent of a compound of Formula V-1a is other than hydrogen.

At least one M in a compound of Formula V-1a may be halo, nitro or $-OR'NH_2$, wherein R' is a lower alkyl or lower alkenyl, and $-OR'NH_2$ is typically a C_1 - C_4 alkyl and preferably, a C_3 alkyl.

15

Typically, a compound of Formula V-1a has the structure of Formula V-1b as follows:



20

Typically, T in a compound of Formula V-1a or V-1b is O, S or N.

The dashed line(s) in Formula I to Formula V-Ib above indicate a bond depending on the applicable atom(s) and/or substituent(s) involved.

25

It will be understood that all combinations and permutations of the compounds provided for by Formula I to Formula V-Ib as defined above are expressly encompassed, including compounds not specifically exemplified herein. Moreover, compounds of the invention in which substituents of R_1 of a compound of Formula I or II are replaced with a bioisostere may be provided and utilised in a method described

herein. This is also the case with substituents R₃, R₄ and W of compounds of Formula I and II, and M of compounds of Formula V, and all such compounds and their use is expressly encompassed. Bioisosteres are, for example, described in Lima and Barreiro, 2005, the contents of which is encompassed herein in its entirety by cross-reference.

Clathrin inhibitory activity of compounds may be determined utilising methodology and assays described herein. In addition, compounds useful as clathrin inhibitors in embodiments of the invention can be identified utilising compound library screening strategies, such strategies being well known to a person skilled in the art. The compounds identified as L1 and L2 in Table 1 below were identified utilising this technique and were validated by assaying for selective inhibition of clathrin TD-amphiphysin association as described herein

Examination of lead compounds L1 and L2 (see Table 1 below) led to the development of minimalist pharmacores. From these pharmacores synthetic methodologies were developed that gave rise to focused compound libraries that are exemplified in Table 2 and Table 3.

Table 1: Initial compound inhibitors identified by compound library screening

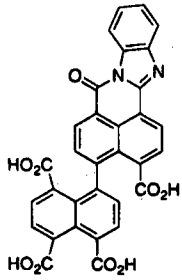
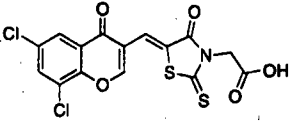
ID	Structure	IC ₅₀ (μM)
L1		6
L2		4

Table 2: Naphthalamide library compounds

ID	Structure	IC ₅₀ (μM)
KAM-6-C12 (Pitstop 1)		18
KAM-6-C17		~ 80
KAM-6-C21		~ 50
KAM-6-D31		~ 100
KAM-6-D32		12
KAM-6-D36		14
KAM-6-E32		1

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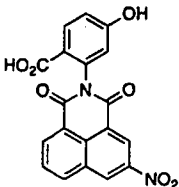
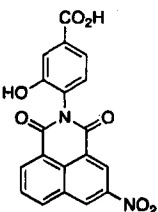
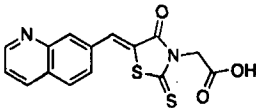
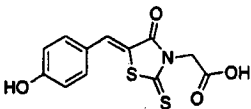
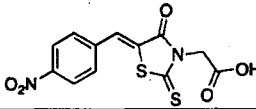
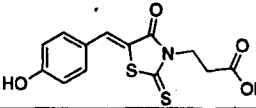
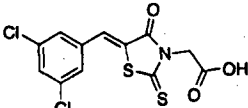
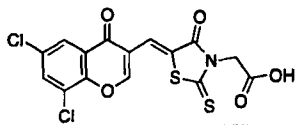
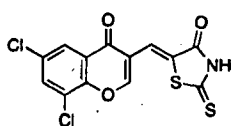
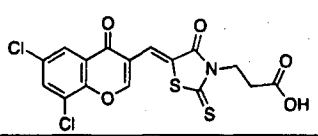
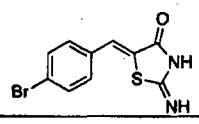
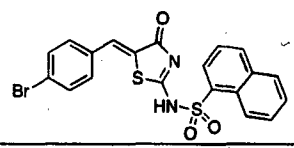
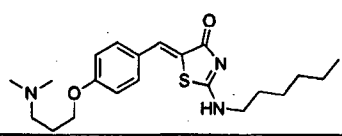
KAM-6-J23		~ 80
KAM-6-J27		~ 100

Table 3: Rhodanine compound library

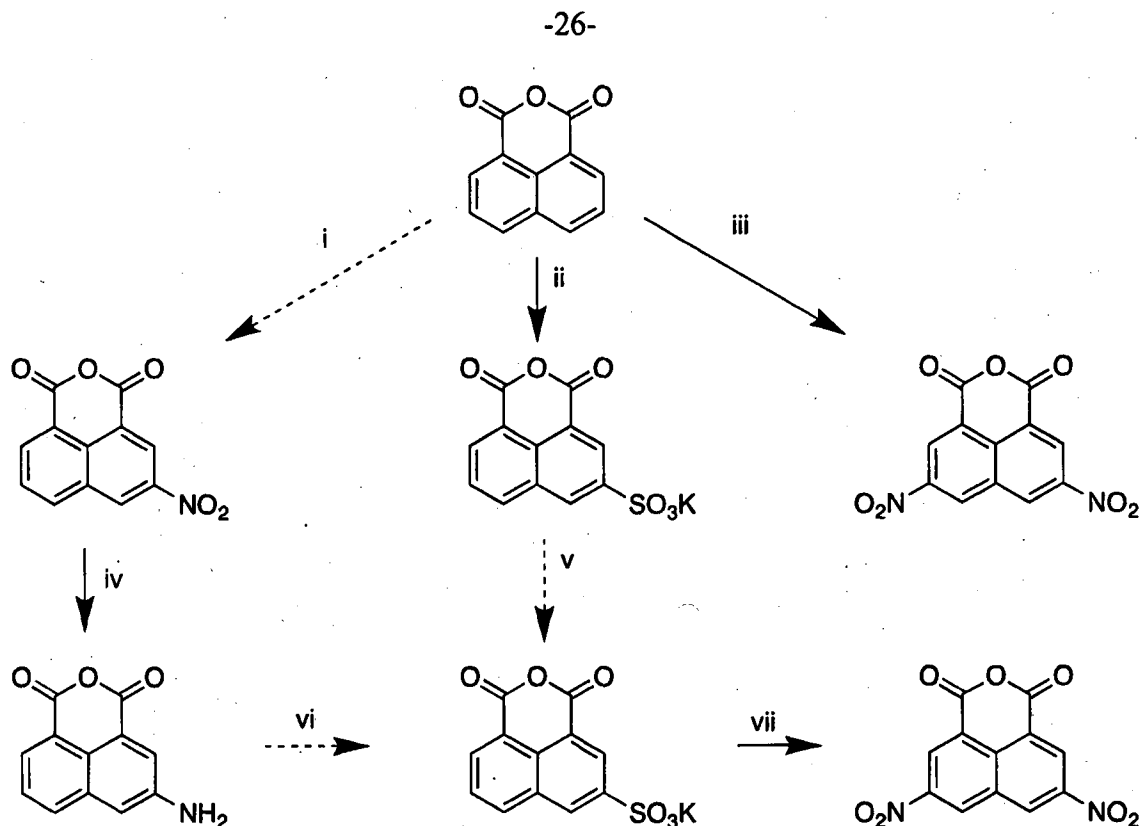
5

ID	Structure	IC ₅₀ (μM)
EH10		~ 60
EH13		~ 100
EH14		~ 100
EH22		~ 70
RODA 6		~ 70

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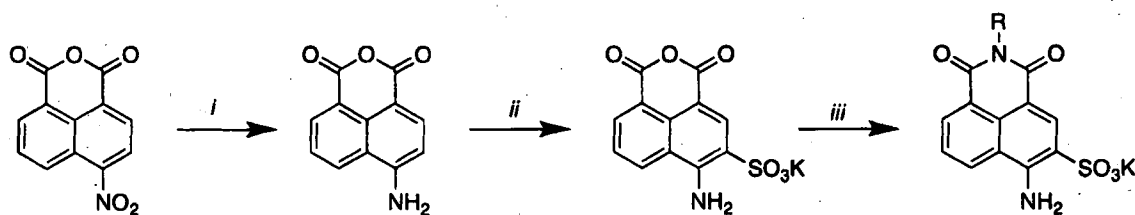
RODA 8		~ 10
ROD 8		~ 100
RODP 8		~ 10
MR1130		~ 30
MR1133/MR264 (Pitstop 2)		57
MR1153B2		~ 50

Examples of synthetic routes for the synthesis of compounds of Tables 2-3 are shown below.



Scheme 1. Reaction scheme for the synthesis of a range of substituted 1,8-naphthalic anhydrides from the commercially available 1,8-naphthalic anhydride.

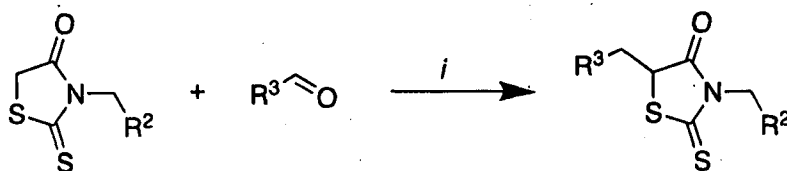
- 5 Reagents and conditions: (a) HNO_3 , H_2SO_4 , 17 hr; (b) (i) Fuming sulfuric acid (30 % SO_3), 90 °C, 30 min; (ii) Saturated KCl, 4 °C, 18 hr; (c) Conc. H_2SO_4 (95-98 %), conc HNO_3 (70 %), 60 °C, 90 min; (d) SnCl_2 , HCl, ethanol, 80 °C, 2 hr; (e) KOH, H_2O , 220 °C, 1 hr; (f) (i) NaNO_2 , HCl; (ii) H_2SO_4 , H_2O , heat; (g) $(\text{CH}_3)_2\text{SO}$, K_2CO_3 , acetone, 80 °C, 18 hr. Dashed arrows indicate that whilst synthetic preparation is possible,
- 10 commercial availability removed the need to synthesise these compounds.



Scheme 2. Reagents and Conditions: (i) SnCl_2 , Hcl, EtOH, 80°C, 2 hr; (ii) a.

- 15 Fuming sulfuric acid (oleum), 40-50°C, 3 hr; b. Kcl (sat), 4°C, 12 hr; (iii) RNH_2 , EtOH, 18 hr.

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Scheme 3. Reagents and Conditions: (i) catalytic piperidine, EtOH, μ Wave

Clathrin inhibitors according to the invention may be prepared by any conventional techniques known to a person skilled in the art, including by organic synthesis strategies, solid phase-assisted techniques or by commercially available automated synthesizers. Alternatively, conventional recombinant techniques alone or in combination with conventional synthetic approaches can be utilised.

Moreover, the clathrin inhibitors as described herein may be used for *in vitro* or *in vivo* diagnostic, prophylactic or therapeutic purposes.

Clathrin-mediated endocytosis (CME) is important for many cell trafficking and cell signaling pathways, and the inhibition of clathrin function has application in the prophylaxis or treatment of diverse diseases and conditions. It is known, for example, that endocytosis is a major contributor or direct cause of diverse human diseases and a list of vesicle trafficking-specific diseases has been published, see for example Aridor and Hannan (2000)-, and Aridor and Hannan (2002), the contents of which are incorporated herein by reference in their entirety. In particular, clathrin is involved in trafficking from the cell surface, and also plays a role in trafficking or fission events from the Golgi apparatus and endosomes. In the brain, diseases and conditions in which endocytosis plays a role include Alzheimer's disease. In Alzheimer's disease (AD) β -amyloid precursor protein (APP) is internalized from axonal cell surfaces in clathrin-coated vesicles, sorted away from recycling synaptic vesicles, and transported to endosomes and the cell soma. Mutations within the genes encoding AP180 and CALM, two clathrin binding proteins involved in CME that are displaced by the inhibitors described herein are directly implicated in Alzheimer's disease (Harold et al., 2009; Yao et al., 1999). The endosome is the first compartment along the CME pathway after internalization of APP or ApoE, and endosomal alterations are evident in pyramidal neurons in Alzheimer brain. Endocytic pathway activation is prominent in APP processing and β -amyloid formation and is an early feature of neurons in vulnerable regions of the brain in sporadic Alzheimer's disease.

Another example of disease involving CME relates to presynaptic synuclein protein which is a prime candidate for contributing to Lewy body diseases, including Parkinson's disease, Lewy body dementia and a Lewy body variant of AD. Exogenous synuclein causes neuronal cell death due to its endocytosis and formation of intracytoplasmic inclusions. Cell death and α -synuclein aggregates are direct consequences of its endocytosis in human neuroblastoma cells. Dent's disease (polycystic kidney disease) is yet another example of a disease involving endocytosis of CIC-5 chloride channel and endocytosis blockers are known to prevent its internalisation. Indeed, the prophylaxis or treatment of any physiological disorder amendable to the inhibition of CME as described herein is expressly encompassed by the invention. It is known, for instance, that human polyomavirus JCV is the etiologic agent of progressive multifocal leukoencephalopathy, a fatal central nervous system (CNS) demyelinating disease and its entry to neurons is blocked by endocytosis inhibitors such as chlorpromazine.

In addition, endocytosis has been implicated in epilepsy and CME plays a key role in synaptic vesicle (SV) recycling. Human epilepsies are heterogeneous conditions, broadly subdivided as genetic or acquired. Whatever the origin, seizures are associated with a massive burst of synaptic transmission. The genes identified to cause epilepsies mostly encode ion channels gated by voltage or neurotransmitters that are highly enriched in nerve terminals and controls their excitability. All these genes directly or indirectly regulate synaptic transmission. One common feature of all anti-epileptic drugs (AEDs) is that they reduce synaptic transmission. Synaptic transmission is the release of neurotransmitter by exocytosis from their storage compartments, synaptic vesicles, in presynaptic nerve terminals. It is controlled by the electrical properties of neurons and chemical transmission across the synapse. The nerve terminal is an electrically excitable structure which maintains a net negative membrane potential so that depolarization can activate voltage-sensitive Ca^{2+} channels to trigger exocytosis. Epilepsy results in the sustained and uncontrolled exocytosis from these terminals.

Currently available epilepsy therapy is unsatisfactory in many patients. Approximately 50% of patients fail to have their seizures controlled by the first medication prescribed and ~30% will continue to have seizures despite trials of multiple drugs. Most AEDs have been discovered using similar traditional methodologies -

screening of compounds in animal models of acute provoked seizures. Possibly as a result, most AEDs have similar principal mechanisms of action, such as inhibiting voltage gated sodium channels and/or enhancing GABAergic activity. A rare example of a new AED acting via a novel mechanism is levetiracetam (KeppraTM) which inhibits the putative SV refilling protein SV2A and inhibits exocytosis.

The massive burst of exocytosis that occurs during an epileptic seizure cannot be sustained for more than 1 minute without compensation by clathrin mediated synaptic vesicle endocytosis (SVE). SVE occurs after exocytosis and retrieves the empty SVs for reuse. Thus, SVE is the mechanism used to replenish SV supply. There are only ~200 SVs in a nerve terminal, enough storage for less than half a minute of maximal synaptic transmission. As such, sustained synaptic transmission (as occurs in a seizure) is a cycle of exocytosis and endocytosis of SVs – the SV cycle. Considerable evidence links most elements of the SV cycle directly or indirectly to epilepsy. Some SV cycle genes cause epilepsy in humans (e.g., synapsin), or epileptic-like seizures when genetically knocked-out in animals (e.g., SV2, amphiphysin or synaptojanin). A ubiquitous mechanism to stop a seizure is to block synaptic mechanisms that are associated with pathologically sustained neuronal burst firing. A reduction in synaptic transmission is the common feature of all anti-epileptic drugs (AEDs). For most AEDs the mechanistic basis of this reduction is uncertain. However, inhibition of clathrin mediated SVE by blocking clathrin may lead to an activity-dependent run-down in synaptic transmission reducing the availability of SVs to sustain or propagate a seizure. Indeed, a recent report has shown that SVE is the rate limiting step in synaptic transmission after a prolonged stimulation time. Targeting clathrin in accordance with an embodiment of the invention may therefore provide a number of advantages over standard therapy. In particular, conventional AEDs reduce synaptic transmission at all times, but a clathrin inhibitor may only exert effect at high frequency or after sustained stimulation that is, under conditions associated with a seizure. Inhibiting clathrin mediated SVE may also limit the effect to overactive neurons and thus have reduced side-effects by allowing physiological neurotransmission to occur unimpeded.

CME mediated pathways are also utilized by many pathogenic agents such as viruses, toxins and symbiotic microorganisms to gain entry into cells. For instance, botulism neurotoxins and tetanus neurotoxin are bacterial proteins that inhibit transmitter release at distinct synapses and cause two severe neuromuscular diseases,

tetanus and botulism. Their action is dependent on their internalisation via endocytosis into nerve terminals.

Cell division (mitosis) results in equal segregation of duplicated chromosomes into two independent daughter cells. Progression through mitosis is highly regulated and is thought to involve membrane trafficking proteins, such as those required for endocytosis (Schweitzer et al, 2005). Premature chromosome segregation (metaphase-anaphase transition) results in aneuploidy, which is a hallmark of many human cancers. This adverse situation is avoided by activation of the spindle assembly checkpoint (SAC), a cellular mechanism that monitors proper assembly of the mitotic spindle. The SAC delays anaphase onset until all chromosomes are stably attached to kinetochores (Musacchio and Salmon, 2007). The SAC is thus an active signal produced by improperly attached kinetochores, which is conserved in all eukaryotes. The SAC stops the cell cycle by negatively regulating CDC20, thereby preventing the activation of the polyubiquitylation activities of anaphase promoting complex. Kinetochores are a large protein assembly that mediates the attachment of chromosomes to spindle microtubules (MTs). These microtubules are known as kinetochore fibres (K-fibres). Clathrin in its triskelia form binds to the mitotic spindle to provide a structural lattice. Here it complexes with TACC3 and ch-TOG to stabilise the K-fibres to allow effective chromosome binding (Royle et al, 2005). Mitotic spindle proteins are recruited during metaphase in a temporal manner. Aurora A-mediated phosphorylation of TACC3 at Ser-558 is required to target TACC3 to the spindle. This in turn recruits ch-TOG and clathrin. Thus, the mitotic spindle localisation of clathrin is dependent on Aurora A and TACC3, and possibly ch-TOG.

Several prior art small molecule inhibitors targeting mitotic proteins are currently in pre-clinical or clinical studies as cancer treatments. Inhibitors of Aurora A are an example of such compounds as they activate the SAC leading to cell death. Clathrin spindle localisation is abolished in cells treated with Aurora A inhibitors (Booth et al, 2011). As such, the the anti-mitotic effects of these inhibitors may in part be due to indirectly blocking the mitotic function of clathrin. Hence, small molecule inhibitors of clathrin are not only useful molecular tools to unravel the role of clathrin during mitosis but in at least some embodiments, have application as anti-mitotic compounds for the treatment of cancer and cell proliferative conditions.

Many growth factor receptors (e.g., EGF-R) also require CME for internalisation and maintenance of cellular activities from signalling to cell growth. Blocking CME prevents cell proliferation in many of these examples and provides further evidence of the anti-cancer and ant-proliferative activity of clathrin inhibitors.

5 Inhibition of CME may also have application to the prophylaxis or treatment of pain. For instance, neuropathic pain typically develops when peripheral nerves are damaged through surgery (including spine surgery), bone compression in cancer, diabetes, viral infection (including shingles or HIV infection), AIDS, alcoholism, amputation, chemotherapy, facial nerve problems, or multiple sclerosis, and is a major
10 factor causing impaired quality of life for millions of people worldwide. Anti-convulsant drugs such as phenytoin and gabapentin are highly efficacious in treating neuropathic pain. These drugs act through modulation of synaptic vesicle transmission indicating the potential of the inhibition of clathrin mediated SVE in the treatment of this debilitating condition. That is, inhibition of synaptic vesicle endocytosis may halt
15 or limit pain signalling and thereby reduce or ameliorate the sensation of pain experienced.

As such, examples of specific diseases and conditions for which methods of the invention find application for the prophylaxis or treatment of include, but are not limited to, cell proliferative diseases and conditions, multifocal leukoencephalopathy,
20 polycystic kidney disease, β -amyloid associated diseases, Alzheimer's disease, neurodegenerative disorders, neuropsychiatric disorders, psychotic disorders, psychosis, bipolar disorders, schizophrenia, aberrant up-regulated neuronal excitation, seizures, neuropathic pain, migraine, mucopolidosis, cell proliferative diseases and conditions, neurological, diseases responsive to inhibition of clathrin, and diseases and conditions
25 mediated or otherwise associated with synaptic signal transmission, CME or SVE (e.g., such as epilepsy), or cell vesicle trafficking.

Cell proliferative diseases and conditions that may be treated in accordance with embodiments of the invention include cancer, skin conditions such as psoriasis and scleroderma, benign growths, and cardiovascular diseases and conditions including
30 atherosclerosis. The cancer may, for instance, be selected from the group consisting of carcinomas, sarcomas, lymphomas, leukaemias, and cancer of the liver, tongue, mouth, oropharynx, nasopharynx, gastrointestinal tract, stomach, small intestine, duodenum, colon, rectum, gallbladder, pancreas, larynx, trachea, bronchus, lung, breast, uterus,

cervix, ovary, vagina, vulva, prostate, testes, penis, bladder, kidney, thyroid and skin (e.g., melanoma and basal cell carcinoma) amongst others.

As indicated above, the inhibition of CME in accordance with one or more embodiments of the invention may also be useful in inhibiting entry of a range of pathogenic agents into cells and so be useful in the prophylaxis or treatment of diseases or conditions associated with this, non-limiting examples of which include nerve viral infection, botulinum neurotoxin A (BoNT/A) and C2 toxin from *Clostridium botulinum* (botulism), Toxin A (TcdA) and B (TcdB) from *Clostridium difficile* (antibiotic-associated diarrhoea), α -toxin (TcnA) from *Clostridium novyi* (necrosis, oedema), Lethal toxin (TcsL) from *Clostridium sordellii*, (toxic shock syndrome, sepsis), Tetanus neurotoxin (TeNT) from *Clostridium Tetani* (tetanus), diphtheria toxin from *Corynebacterium diphtheriae* (diphtheria), leukotoxin (LKT) from *Mannheimia haemolytica* (bovine respiratory disease), Dermonecrotic toxin (DNT) from *Bordetella* (swine atrophic rhinitis), *Chlamydia psittaci* and *Chlamydia trachomatis* (chlamydia), Anthrax toxin from *Bacillus anthracis* (Anthrax), *Listeria monocytogenes* (Listeriosis), *Yersinia pseudotuberculosis* (Pseudotuberculosis (yersinia)), *Staphylococcus aureus* (Golden staph infections), *Candida albicans* (candidiasis), *Porphyromonas gingivalis* (periodontitis), Uropathogenic *Escherichia coli* (urinary tract infection), Adeno-associated virus, Adenovirus (Respiratory infection), African swine fever virus (African swine fever), Bluetongue virus-1 (Bluetongue disease/ catarrhal fever), Chikungunya virus (CHIKV) (Chikungunya disease), Coxsackievirus B3 (CVB3) and A9 (CVA9) (Viral myocarditis/ CNS infection/ respiratory disease), Dengue virus 1 & 2 (DENV) (Dengue fever), Zaire Ebolavirus (Ebola hemorrhagic fever), Echovirus (EV1) from *Picornaviridae* (Echovirus disease), Equine infectious anemia virus (EIAV) (Equine infectious anemia), Feline infectious peritonitis virus (FIPV) (Feline infectious peritonitis), Hantaan virus (Korean hemorrhagic fever), Hepatitis B virus (HBV) (Hepatitis B), Hepatitis C virus (HCV) (Hepatitis C), Herpes Simplex virus (Herpes simplex), Kaposi's sarcoma-associated herpes-virus (KSHV) (Kaposi's sarcoma), Human immunodeficiency virus (HIV) (Acquired-immunodeficiency syndrome (AIDS)), Influenza virus (influenza), Jaagsiekte sheep retrovirus (JSRV) (Jaagsiekte/ ovine pulmonary adenocarcinoma), Junin arenavirus (JUNV) (Argentine hemorrhagic fever), Murine norovirus-1 (MNV-1) (murine norovirus disease), Bovine papillomavirus BPV1 (Carcinomas/ haemangio-endotheliomas of the bladder), Human

papillomavirus 31 (HPV31) and 16 (HPV16) (genital warts/ cervical cancer), Canine parvovirus (Parvo), Poliovirus (poliomyelitis), Human rhinovirus 2 (HRV) (common cold), Rotavirus (gastroenteritis), Semliki forest virus (SFV) (Semliki forest virus disease), Simian virus 40 (SV-40), Vesicular stomatitis virus (VSV) (vesicular stomatitis). The above list is to be taken as only indicative only of the pathogenic agents for which compounds may be administered in accordance with embodiments of the invention. More generally, further examples include for instance, so-called “zippering bacteria” which bind to cellular receptors and invade cells in a clathrin-dependent manner such as enteropathogenic *E. coli* bacteria, and reovirus.

Suitable pharmaceutically acceptable salts include acid and amino acid addition salts, base addition salts, esters and amides that are within a reasonable benefit/risk ratio, pharmacologically effective and appropriate for contact with mammalian tissues without undue toxicity, irritation or allergic response. Representative acid addition salts include hydrochloride, sulfate, bisulfate, maleate, fumarate, succinate, tartrate, tosylate, citrate, lactate, phosphate, oxalate and borate salts. Representative base addition salts include those derived from ammonium, potassium, sodium and quaternary ammonium hydroxides. Suitable salts also include alkali metal and alkali earth cation salts such as sodium, calcium, magnesium, and potassium salts, as well as ammonium and amine cation salts. The provision of such salts is well known to the skilled addressee. Suitable pharmaceutical salts are for example exemplified in S. M Berge et al, J. Pharmaceutical Sciences (1997), 66:1-19, the contents of which is incorporated herein in its entirety by cross-reference.

Prodrugs of compounds in embodiments of the invention include those in which groups selected from carbonates, carbamates, amides and alkyl esters have been covalently linked to free amino, amido, hydroxy or carboxylic groups of the compounds. Suitable prodrugs also include phosphate derivatives such as acids, salts of acids, or esters, joined through a phosphorus-oxygen bond to a free hydroxyl or other appropriate group. A prodrug can for example be inactive when administered but undergo *in vivo* modification into the active compound that binds to clathrin such that the function of clathrin is inhibited as a result of cleavage or hydrolysis of bonds or other form of bond modification of the compound post administration. The prodrug form of the active compound can have greater cell membrane permeability than the active compound thereby enhancing potency of the active compound. A prodrug can

also be designed to minimise premature *in vivo* hydrolysis of the prodrug external of the cell such that the cell membrane permeability characteristics of the prodrug are maintained or enhanced for optimum availability to cells and for systemic use of the compound.

5 Esterified prodrugs may for instance be provided by stirring a compound embodied by the invention with an appropriate anhydride or acid chloride (in molar excess) in a pyridine/N,N-dimethylformamide (DMF) solution in the presence of a suitable catalyst such as dimethylaminopyridine (DMAP). In some cases, the solution may need to be refluxed to drive the reaction to completion. On completion of the
10 reaction, the esterified product is purified by either recrystallization or by chromatography. Representative esters include C₁-C₇ alkyl, phenyl and phenyl(C₁₋₆) alkyl esters. Preferred esters include methyl esters. A clathrin inhibitor can be administered to a mammal in alone or be co-administered with one or more other therapeutic compounds or drugs conventionally used for treating the applicable disease
15 or disorders. By "co-administered" is meant simultaneous administration in the same formulation or in two different formulations by the same or different routes, or sequential administration by the same or different routes, wherein the administered drugs have overlapping therapeutic windows. By "sequential" administration is meant one is administered after the other.

20 The terminal domain (TD) of clathrin is highly conserved between mammalian species. For example, the amino acid sequence is conserved 100% (i.e. all amino acids are identical) between the terminal domain of bovine, human, rat, mouse, pig, and dog clathrin. Whilst methods and clathrin inhibitors in accordance with the invention have particular application to inhibiting the activity of human clathrin, the invention is not
25 limited thereto and extends to inhibiting clathrin activity of other mammals including but not limited to, bovine, ovine, porcine, rodent (e.g., mouse, rat, guinea pig), canine, feline, and primate clathrin.

 A clathrin inhibitor as described herein will generally be formulated into a pharmaceutical composition comprising the compound and a pharmaceutically
30 acceptable carrier. Pharmaceutical compositions include sterile aqueous solutions suitable for injection and sterile powders for the extemporaneous preparation of injectable solutions. Such injectable compositions will be fluid to the extent that syringability exists. Injectable solutions will typically be prepared by incorporating the

active(s) in the selected carrier prior to sterilising the solution by filtration. In the case of sterile powders, preferred methods of preparation are vacuum drying and freeze-drying techniques which yield a powder of the active and any additional desired ingredient from previously sterile filtered solutions thereof. As will be understood, a pharmaceutical composition in accordance with the invention can be provided in a form wherein the components of the composition are admixed with one another. Alternatively, in another embodiment, the clathrin inhibitor(s) can be provided partially or totally separately for combination with other components to form the composition, such as in the form of a kit.

For oral administration, the clathrin inhibitor can be formulated into any orally acceptable carrier deemed suitable. In particular, the inhibitor can be formulated with an inert diluent, an assimilable edible carrier or it may be enclosed in a hard or soft shell gelatin capsule. Moreover, a clathrin inhibitor can be provided in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions or syrups.

Clathrin inhibitors as described herein can also be formulated into topically acceptable preparations including creams, lotions or ointments for internal or external application. Topically acceptable compositions can be applied directly to the site of treatment including by way of dressings and the like impregnated with the preparation.

A pharmaceutical composition embodied by the invention can also incorporate one or more preservatives such as parabens, chlorobutanol, phenol, and sorbic acid. In addition, prolonged absorption of the composition may be brought about by the inclusion of agents for delaying absorption such as aluminium monostearate. Tablets, troches, pills, capsules and like can also contain one or more of the following: a binder such as gum tragacanth, acacia, corn starch or gelatine, a disintegrating agent such as corn starch, potato starch or alginic acid, a lubricant such as magnesium stearate a sweetening agent such as sucrose, lactose or saccharin; and a flavouring agent.

Pharmaceutically acceptable carriers include any suitable conventionally known physiologically acceptable solvents, dispersion media, isotonic preparations and solutions including for instance, physiological saline. Use of such ingredients and media for pharmaceutically active substances is well known. It is particularly preferred to formulate compositions in dosage unit form for ease of administration and uniformity of dosage. Clathrin inhibitors as described herein can also be provided in dosage unit form. A dosage unit form as used herein is to be taken to mean physically

discrete units, each containing a predetermined quantity of the selected compound(s) calculated to produce a therapeutic or prophylactic effect. When the dosage unit form is a capsule it can contain the active in a liquid carrier. Various other ingredients may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills or capsules may be coated with shellac, sugars or both.

Pharmaceutical compositions embodied by the invention will generally contain clathrin inhibitors in a range of from about at least 0.1% by weight up to about 80% w/w of the composition. The amount of the clathrin inhibitor(s) in the composition will be such that a suitable effective dosage will be delivered to the individual taking into account the proposed mode of administration. Preferred oral compositions will contain between about 0.1 μ g and 4000 mg of a clathrin inhibitor.

The dosage of clathrin inhibitor(s) administered will depend on a number of factors including whether the inhibitors are to be administered for prophylactic or therapeutic use, the disease or condition for which the active is intended to be administered, the severity of the condition, the age of the individual, and related factors including weight and general health of the individual as may be determined in accordance with accepted medical principles. For instance, a low dosage may initially be given which is subsequently increased at each administration following evaluation of the individual's response. Similarly, frequency of administration can be determined in the same way that is, by continuously monitoring the individual's response between each dosage and if necessary, increasing the frequency of administration or alternatively, reducing the frequency of administration.

Typically, a clathrin inhibitor as described herein will be administered at a dosage up to about 50 mg/kg body weight and preferably, in a range of from about 5 μ g/kg to about 100 μ g/kg body weight.

Routes of administration include but are not limited to respiratorily, intravenously, intraperitoneally, subcutaneously, intramuscularly, by infusion, orally, rectally, topically, by implant, or by other conventionally employed methods of administration. With respect to intravenous routes, particularly suitable routes are via injection into blood vessels which supply the target tissue to be treated. Suitable pharmaceutically acceptable carriers and formulations useful in compositions of the present invention may for instance be found in handbooks and texts well known to the skilled addressee, such as "Remington: The Science and Practice of Pharmacy (Mack

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Publishing Co., 1995)", the contents of which is incorporated herein in its entirety by reference.

The animal treated by a method embodied by the invention will typically be a mammal such as a member of the bovine, porcine, ovine or equine families, a laboratory test animal such as a mouse, rat, rabbit, guinea pig, cat or dog, or a primate or human being as outlined above. However, it will be understood non-mammalian species may also be treated with clathrin inhibitors as described herein for research or other purposes in accordance with the invention.

"Pitstop" is a word coined for use as a trademark by the Applicants in respect of chemical compounds and its use whether alone or in coined words such as "Pitstop 1" and "Pitstop 2" is expressly reserved for use by the Applicants for chemical compounds.

The invention will be further described herein after with reference to a number of non-limiting Examples.

EXAMPLE 1: Pitstop inhibitors of clathrin function

1. Methodology and reagents

Protocols and reagents utilised in the present study are described below unless indicated to the contrary.

1.1 Antibodies

The following antibodies were used in this study: clathrin (X22, Affinity Bioreagents), AP-2 (α -adaptin, Abcam), intersectin 1 (S750; kindly provided by Dr. Thomas Südhof, Howard Hughes Medical Institute, Stanford University, Palo Alto, USA), FCHo 1/2 (kindly provided by Dr. Harvey McMahon, MRC Laboratory of Molecular Biology, Cambridge, U.K.), dynamin 1/2/3 (Hudy-1, Upstate), EEA1 (BD transduction), Gadkin (Schmidt et al., 2009), transferrin receptor (Zymed), TGN46 (Serotec), GM130 (BD transduction), mannose-6-phosphate receptor (CI-M6PR, Affinity Bioreagents), AP-1 (α 1-adaptin, Sigma), CD63 (RFAC4, Millipore), and GST (peroxidase conjugate, Sigma).

1.2 DNA constructs

Fusion proteins encoding GST-clathrin heavy chain TD (amino acids 1-364), GST-AP-2 alpha-appendage (amino acids 702-938), and GST-AP-1 gamma-appendage (amino acids 690-822) were in pGEX4T-1 expression vectors and His₆-amphiphysin 1 (250-578), His₆-gadkin (amino acids 52-302), and His₆-stonin 2 (amino acids 1-242) were in pET28a.

1.3 Protein purification

Expression plasmids were transformed into *Escherichia coli* BL21-Codon PlusTM (DE3)-RP competent cells (Stratagene). His₆- or GST-tagged fusion proteins were expressed and purified using HIS-SelectTM nickel affinity gel (Sigma) or GST Bind® resin (Novagen) following the manufacturer's instructions.

1.4 ELISA-based binding assay

Purified His₆-tagged protein was diluted into screening buffer (20 mM HEPES pH 7.4, 50 mM NaCl, 1 mM DTT, 1 mM PMSF), added to a 384 well ELISA plate (high-binding PS Microplate, Greiner Bio-One) and bound for 1 h at RT. Non-specific binding was prevented by addition of 50 µl blocking buffer (20 mM HEPES pH7.4, 50 mM NaCl, 1 mM DTT, 1 mM PMSF, 2 % BSA, 2.5 % milk) followed by incubation overnight at 4°C. Following extensive washes (with 20 mM HEPES pH 7.4, 50 mM NaCl, 0.05 % Tween 20), chemical compounds diluted in DMSO (10 µl) were added and incubated together with GST-tagged protein for 1 h at RT in screening buffer. After 3 washes, HRP-coupled anti-GST antibodies were added in screening buffer and the plate was incubated for 15 min at RT. Following additional washes, 50 µl TMB substrate (Pierce Biotechnology) was added and the plate was incubated for 20 min before the reaction was terminated by adding 50 µl 1N sulphuric acid. The amount of bound protein was determined by photometric measurement in a plate reader. Relative binding was calculated as % of DMSO control.

1.5 Cell cycle analysis by flow cytometry

Cells (5x10⁵) were grown in 10 cm dishes. Following inhibitor treatment, cells (floating and adherent) were collected and single-cell suspensions were fixed in 80% ice-cold ethanol at -20°C for at least 16 h. Cells were stained with propidium iodide

and the cell cycle was analyzed as described previously (Joshi et al., 2010). Cell cycle profiles were acquired with a FACS Canto Flow Cytometer (Becton Dickinson) using FACS Diva software (v5.0.1) at 488 nm. Cell cycle profiles were analyzed using FlowJo software (v7.1).

5

1.6 Trypan blue exclusion assay

Cells were seeded in 10 cm dishes (1×10^5 cells/dish). On day 0 (24 h after seeding), cells in triplicate were treated in the presence or absence of pitstops at concentrations of 1, 3, 10 and 30 μ M. After 20 h, the cell number and viability were measured using a Vi-CELL XR cell viability analyzer as previously described (Joshi et al., 2010).

10

1.7 Lactate dehydrogenase (LDH) toxicity assay

Toxicity was assayed by determination of lactate dehydrogenase (LDH) activity. HeLa cells were seeded in 96 well plates. Asynchronously growing cells were treated in the presence or absence of pitstop 1 or pitstop 2 at the indicated concentration for 8h. The supernatant (50 μ l) was added to 100 μ l of LDH assay reagent (Sigma-Aldrich) and the reaction was allowed to develop for 20 min. Absorbance was measured at 490 nm and 690 nm (plate background absorbance). Values were normalized to drug/media background value and toxicity was calculated as a % of a 100% lysed cell control.

15

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1.8 MTT Assay

Growth inhibitory assays were carried out using an MTT assay. Cells in logarithmic growth were transferred to 96-well plates (100 μ l medium/well) at a density of 2500 cells/well for HeLa, HT29, H460, A431, and DU145 cells, 3000 cells/well for SW480, 3500 cells/well for MCF7, BE2-C and SJ-G2, and 2000 cells/well for A2780. On day 0, (24 h after plating) cells in duplicate were treated with or without pitstops. After 72 h drug exposure, cytotoxicity and growth inhibitory effects were evaluated using the MTT (3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl-tetrazolium bromide) assay. GI₅₀ values were calculated from the MTT dose response curve from three independent experiments, each performed in duplicate. GI₅₀ is the drug concentration at which cell growth is inhibited by 50% based on the difference between the optical density values on day 0 and those at the end of drug exposure.

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30

1.9 TUNEL assay

A TUNEL Apoptosis Detection Kit for Adherent Cells (FITC-labeled POD) from GenScript was used according to the provided manual.

5 1.10 Electron microscopy analysis of Cos7 cells and primary hippocampal neurons

Cos7 cells (80% confluent) were grown in plastic dishes. Growth media were replaced by optiMEM with 0,1% DMSO or 30 μ M pitstop 2 for 10 min. Cells were fixed with 2% glutaraldehyde (GA) in PBS. After scraping and pelleting, subsequent
10 electron microscopy preparation and morphometric analysis were performed as previously described (Ferguson et al., 2009). Briefly, after epoxy resin embedding and sectioning micrographs were taken along the cell perimeter at x20000. Images were combined in order to reconstruct cell perimeter and numbers of clathrin-coated intermediates were estimated.

15 Rat primary neuronal cultures (P1; DIV 14-21) were assayed either in incubation medium (resting conditions) or high potassium solution. Incubation medium contained (concentration in mM, pH 7.4): 170 NaCl/ 3.5 KCl/ 0.4 KH_2PO_4 , 20 TES(N-tris[hydroxyl-methyl]-methyl-2-aminoethane-sulfonic acid), 5 NaHCO_3 / 5 glucose/ 1,2 Na_2SO_4 / 1.2 MgCl_2 and 1.3 CaCl_2 ; at pH 7,4; high potassium solution was identical to
20 the above but containing 120 mM NaCl plus 50 mM KCl. All media contained glutamate receptor blockers CNQX (20 μ M) and AP5 (50 μ M). Neurons were preincubated with 0.1% DMSO or 30 μ M pitstop 2 for 5 min at RT. Cultures were then either left for an additional 10 min in the same solution before fixation or were stimulated with high potassium solution for 90 s and rinsed with incubation medium for
25 7 min to assess vesicle recovery in presence of 30 μ M pitstop 2 or 0.1% of DMSO as a control. After fixation with 2% GA, neurons were post-fixed in osmium tetroxide 1%, incubated with 1% aqueous uranyl acetate, dehydrated and flat-embedded into epoxy resin. Glass was removed with the help of liquid nitrogen. Ultrathin sections were viewed with a Zeiss TEM 912 and images were taken at x30000
30 magnifications. 30 synaptic boutons per condition were analyzed.

1.11 Microinjection of compounds into lamprey reticulospinal axons and electron microscopic analysis of the preparation

Spinal cord preparations from adult male and female lampreys (*Lampetra fluviatilis*) were used in this study. Lampreys were kept in an aerated fresh water aquarium at 4°C. Animals were treated according to the Swedish Animal Welfare Act (SFS 1988:534), as approved by the Local Animal Research Committee of Stockholm. All efforts were made to minimize animal suffering and to reduce the number of animals used. Following anesthetization by immersion in 0.1 g/l tricaine methanesulphonate (MS-222, Sigma) and decapitation, trunk segments of the spinal cord were dissected out and transferred to a cooled (7-9°C) bath containing oxygenated lamprey Ringer's solution. All dissection steps, injections, and physiological recordings were performed at 7-9°C in Ringer's solution containing: 2 mM HEPES, 109 mM NaCl, 2.1 mM KCl, 2.6 mM CaCl₂, 1.2 mM MgCl₂, 4 mM glucose, 0.5 mM L-glutamine. Reagents were introduced into borosilicate glass injection microelectrodes and microinjected into the reticulospinal axons with pressure pulses (5-15 psi) of 200 ms duration. Microinjections were monitored with a CCD camera (Roper Scientific, Tucson, AZ) or (Princeton Instruments, Trenton, NY) connected to a standard fluorescence upright microscope (Olympus BXH-50) with 10x and 40x water-immersion objectives. Membrane potentials of the impaled axons were monitored throughout injections.

Spinal cords were fixed during stimulation in 3% glutaraldehyde, 4% tannic acid in 0.1 M cacodylate buffer, pH 7.4, for 1 h, followed by incubation in the same fixative without tannic acid overnight. After postfixation in 1% osmium tetroxide for 1 h and dehydration in graded alcohol series, the specimens were embedded in Durcupan ACM (Fluka, Saint Quentin Fallavier, France).

Serial ultrathin sections were cut with a diamond knife (Diatome, Switzerland) on a Leica Ultracut UTC ultramicrotome (Leica, Sweden), mounted on formvar-coated copper slot grids, counterstained with uranyl acetate and lead citrate, and examined at 80 kV in a Tecnai 12 transmission electron microscope (FEI, Sweden). Effects of microinjections were analyzed in synapses cut at various distances from microinjection sites (i.e., different concentrations). For quantitative analysis, the number of synaptic vesicles (SVs) and coated pits and the membrane length of pockets or invaginations (curvature index) in the endocytic zone in injection experiments were determined from

middle sections of at least five serially cut synapses. The values for the numbers of SVs and coated pits were normalized to the length of the active zone as the number of SVs in the cluster is proportional to the length of the active zone in the reticulospinal synapse. The length of endocytic zone membrane invaginations and pockets was measured using the NIH ImageJ software.

1.14. Protein crystallography

GST-clathrin heavy chain TD was purified by GSH affinity chromatography (Qiagen). The GST-tag was cleaved off by thrombin and separated from TD via GST affinity chromatography. The cleaved TD was further purified by size exclusion chromatography using a Superdex S200 column (Amersham Biosciences) in 10 mM Tris, pH 7.5, 50 mM NaCl, and 4 mM dithiothreitol, concentrated up to 22 mg/ml (~0.5 mM), and supplied with a 4-fold molar surplus of pitstops 1 or 2.

Crystals were grown at 18°C using the sitting drop vapor diffusion method. The reservoir solution contained 20% PEG 3350, 150 mM potassium acetate, and 0.1 M Tris, pH 8.0, and drops were prepared by mixing 2 µl of reservoir and 2 µl of protein solution. Crystals formed within a few minutes and reached their final size in 1–2 days at 18 °C. The crystals were soaked briefly in a cryoprotection solution that was prepared by increasing the PEG 3350 concentration up to 40 % in 150 mM potassium acetate, and 0.1 M Tris, pH 8.0. Crystals were then mounted in nylon loops, and flash-cooled in liquid N₂. X-ray data were collected at Beamline BL1 at BESSY-II, Berlin, and processed using XDS (Kabsch, 2010) and Xscale. The phase problem was solved by molecular replacement using the CCP4 program MOLREP (Collaborative Computational Project, 1994) using the 2.7 Å structure of clathrin TD (Protein Data Bank code 1C9I) as a model (ter Haar., 1998; ter Haar et al., 2000). All water molecules and ligand atoms were omitted from the starting model. Subsequent cycles of refinement to 1.7 Å resolution were performed using Refmac5 (Murshudov et al., 1997). Pitstops 1 and 2 were built using the Dundee PRODRG2 server and fitted in the electron density (Schuttelkopf and van Aalten, 2004). Fig. 3A-F were produced with PyMOL software (see: <http://www.pymol.org/>).

1.15 Fluorescence microscopy of immunostained samples

Cos7 cells seeded on matrigel-coated glass coverslips were preincubated with 30 μ M pitstop 2 or DMSO (0.1%) in DMEM/ 0.1 % FCS/ 10 mM HEPES for 15 min, fixed in 4% PFA or methanol, and stained with antibodies as previously described (Schmidt et al., 2009). Cells were imaged using spinning disc confocal or total internal reflection microscopy (TIRFM). Pearson's correlations were obtained using Volocity software (Improvision).

1.16 Confocal and TIRF-based live imaging

For live cell microscopy, Cos7 cells stably expressing eGFP-clathrin LC (Gaidarov et al., 1999), or transiently expressing FCHo2-eGFP, AP-2 σ -eGFP (Gaidarov et al., 1999), and dynamin2-eGFP were used. CCP dynamics were imaged by TIRFM (Visitron) under the control of Slidebook 5 (3i Inc). Time series of 2 min (at 0.5 Hz) were acquired 5 min after addition of 30 μ M pitstop 2 or DMSO (0.1%) in HBSS media supplemented with 0.1 % FCS and 10 mM HEPES (pH 7.4) at 37°C. For lifetime analysis events were tracked manually.

Fluorescence recovery after photobleaching (FRAP) experiments were performed using a spinning disc confocal microscope (Perkin Elmer) controlled by Volocity (Improvision). Cells were imaged for 12 s, then bleached in a region of 15 x 15 μ m², and imaged for an additional 120 s at 0.5 Hz. Fluorescence recovery was analyzed by defining CCPs in the FRAP region before bleaching, followed by measuring the fluorescence intensity in the FRAP region over time. Intensity values were corrected for photobleaching in a non-bleached control area.

1.17. Ligand internalization and surface staining

HeLa or Cos7 cells seeded on MatrigelTM-coated glass coverslips were starved (2 h) before incubation with 20 μ g Alexa^{488/568}-transferrin or 100 ng Alexa^{595/488}-EGF for 15 min at 37°C. Washed cells were fixed in 4 % PFA, 4 % sucrose in PBS, pH 7.4 and processed for epifluorescence microscopy. Fluorescence levels were quantified using a Zeiss Axiovert200M fluorescence microscope under the control of Slide Book5 software (3i Inc., Göttingen, Germany). To analyze ligand sequestration into CCPs cells were incubated with labeled EGF in the presence or absence of 30 μ M pitstop 2 for 30 min at 8°C. Washed cells were fixed and stained for AP-2 α . Cells were imaged

using TIRFM and analyzed by Slidebook5 software. Colocalization was assessed by determining Pearson's correlation coefficients.

1.18 Shiga toxin internalization and retrograde traffic

5 HeLa cells were seeded on glass cover slips and starved overnight. The cells were washed once with ice-cold PBS and then incubated at 4°C for 30 min with 1 µg/ml Cy3-StxB (kindly provided by Dr. Ludger Johannes, Institute Curie, Paris, France) in DMEM/ 0.2% BSA/ 20 mM HEPES pH 7.4. After two washes cells were fixed immediately or incubated for 15 to 60 min in growth medium at 37°C before
10 fixation. Fixed cells were processed for immunofluorescence microscopy using antibodies against GM130. For quantifications, the total fluorescence of Cy3-StxB per cell was analyzed and the fraction localized to the Golgi was determined by quantifying the overlap with the GM130-positive area.

15 1.19 HIV infection

TZM-bl cells (Wei et al., 2002) were plated in 96 well plates and incubated for 2 h in serum-free medium prior to the experiment. Cells were preincubated with pitstop 2 or solvent for 30 min, and infections were carried out by adding different amounts of HIV-1 strain NL4-3 obtained from infected MT-4 cells. Virus-containing medium was
20 removed after 2 h and fresh medium containing 100 ng/ ml of the HIV coreceptor antagonist AMD3100 was added to prevent further entry events. At 48 h post infection, cells were lysed, and HIV Tat-driven luciferase activity was measured using the Steady-Glo assay (Promega) according to the manufacturer's recommendations.

25 1.20 Data deposition

Crystal structural data for the terminal domain of clathrin respectively complexed with pitstop 1 and pitstop 2 was deposited with the RCSB Protein Data Bank, Rutgers, the State University of New Jersey, Taylor Road, Piscataway, NJ, USA on 25 November 2010 under PDB codes 2xzg (for pitstop 1) and 2xzh (for pitstop 2),
30 reference to which can be had for the design and provision of clathrin inhibitors in accordance with the invention and the contents of all of which is expressly incorporated herein in its entirety by cross-reference. The crystal data for pitstop1 and pitstop 2 complexed with the terminal domain of clathrin is also set out in Figures 13 and 14.

2. Identification and characterisation of the clathrin inhibitors pitstop 1 and pitstop 2 and their binding to the clathrin terminal domain

Clathrin function in mammalian cells has mainly been evaluated via two approaches: knockdown of clathrin or expression of dominant-negatively acting clathrin-binding fragments of endocytic accessory proteins. While both of these strategies have shown that clathrin is essential for CCP formation or stability, the physiological role of endocytic ligand association of its TD remains unclear. This lack of knowledge is surprising as clathrin box-TD interactions form a major hub within the endocytic network. It has been suggested that multiple redundant interactions of clathrin TD with its endocytic ligands may serve to stably recruit clathrin to membranes, thereby facilitating clathrin assembly through local enrichment. Alternatively, clathrin assembled at nascent endocytic sites could serve as an organizing scaffold that regulates CCP dynamics by providing spatially defined binding sites on its TD for accessory proteins that drive CCP maturation and disassembly.

To distinguish between these two models, a chemical biology approach designed to identify inhibitors that selectively interfere with TD complex formation with its endocytic ligands was employed. Specifically, an ELISA-based screen for small molecules that can displace the B/C domain of amphiphysin (an endocytic protein harboring a clathrin box motif (Slepnev et al., 2000; Dell'Angelica, 2001)) from recombinant clathrin TD was carried out. From a library of about 17,000 small molecules two lead compounds that exhibited selective inhibition of clathrin TD-amphiphysin association were identified and validated. Retrosynthetic analysis identified simple approaches to in-house synthesis of two focused compound libraries based on the original leads. This led to the identification of two molecules of distinct chemical scaffolds which specifically disrupt ligand association with the clathrin TD. Based on subsequent studies and their ability to impair CCP function (see below), these compounds were named pitstops 1 and 2 (see Tables 2 and 3 above for the chemical structures of these compounds). Pitstops 1 and 2 selectively inhibited ligand association of clathrin TD with IC₅₀ values of 18 μ M and 12 μ M, respectively. Neither compound affected complex formation between the ear domain of AP-2 α and amphiphysin (a top-site ligand) or stonin 2 (a side-site ligand), or between the AP-1 γ -ear and its accessory binding partner gadkin (Schmidt et al., 2009). The

pitstops also did not influence the *in vitro* GTPase activity of full-length native sheep brain dynamin 1 (not shown). GI_{50} (μM) values for pitstop 2 were determined for the following of cancer cell lines by MTT assay: HT29 (Colon) cells ($6.2 \pm 2.0 \mu M$); A431 (Skin) cells ($38 \pm 9 \mu M$); BE2-C (Neuroblastoma) cells ($5.7 \pm 0.9 \mu M$); U87MG (Glioblastoma) cells ($5.3 \pm 0.9 \mu M$). The GI_{50} value is the concentration of the clathrin inhibitor that inhibits cell growth by 50 % (i.e., the lower the value the greater the growth inhibition). Errors represent SEM (n=3 independent experiments).

3. X-ray crystallography of pitstops in complex with the clathrin TD

The structure of the clathrin TD in complex with pitstops by protein X-ray crystallography was determined to understand the molecular basis of clathrin TD inhibition by pitstops. Clathrin TD-containing crystals diffracted up to a resolution of 1.7 Å and the structure was solved by molecular replacement using the clathrin TD as a search model (see Table 4).

Table 4: X-ray data collection and refinement statistics

	Clathrin TD + Pitstop 1	Clathrin TD + Pitstop 2
Data collection		
Space group	P212121	P212121
Cell dimensions		
a, b, c (Å)	71.34, 73.92, 84.38	70.90, 74.02, 82.12
α, β, γ (°)	90.00, 90.00, 90.00	90.00, 90.00, 90.00
Resolution (Å)	33.85- 1.70	33.7-1.69
R_{sym} or R_{merge}	0.048 (0.243) ^a	0.072 (0.562) ^a
$I / \sigma I$	24.34 (4.19) ^a	19.10 (3.94) ^a
Completeness (%)	95.1 (70.1) ^a	100 (100) ^a
Redundancy	5.9 (2.6) ^a	7.2 (7.1) ^a

Refinement		
Resolution (Å)	33.85- 1.70	33.7-1.69
No. reflections	44946	46615
$R_{\text{work}} / R_{\text{free}}$	15.9 / 20.0	17.1 / 21.4
No. atoms	3341	3431
Protein	2832	2887
Ligand/ion	27	28
Water	458	429
<i>B</i> -factors		
Protein	15.3	17.0
Ligand/ion	26.0	21.6
Water	29.8	29.5
R.m.s. deviations		
Bond lengths (Å)	0.024	0.025
Bond angles (°)	1.892	1.968

^a Highest resolution shell is shown in parentheses

Following refinement, inhibitor molecules in complex with clathrin TD could be identified and modelled into the determined electron densities. This was achieved as follows. The first refinement cycles were run without pitstops (pdb file contained exclusively amino acids from clathrin TD), and the pitstops were then fitted into the electron density map. Further refinement cycles were then run with a pdb file that contained clathrin TD complexed with pitstop 1 or 2.

Ligplots showing the binding interactions of pitstop 1 and pitstop 2 with the clathrin TD are shown in Fig.2 and Fig. 3. The interactions shown are mediated by hydrogen bonds and by hydrophobic contacts. Hydrogen bonds are indicated by dashed lines between the atoms involved, while hydrophobic contacts are represented by an arc with spokes radiating towards the atoms of the relevant pitstop compound that they contact. The contacted atoms are shown with spokes radiating back.

As shown, pitstop 1 makes direct contact with Ile 52, Ile 62, Ile 66, Ile 80, Ile 93, Phe 91, Arg 64, Leu 82 and Lys 96 of the clathrin TD, whilst pitstop 2 makes direct contact with Ile 93, Ile 80, Val 50, Ile 52, Ile 62 and Phe 91.

The overall structures of clathrin TD in complex with either pitstops 1 or 2 are shown in Fig. 4A, C. The clathrin TD adopts a WD40-like structure comprised of a seven-bladed β -propeller each with four antiparallel strands (ter Haar et al., 2000). Both pitstop inhibitors bind the interface between the first and second blades (Fig. 4B, D) at a site overlapping with that used by clathrin box-containing accessory proteins (Fig. 4E-F), which bind to the TD with low affinity (Brodsky et al., 2001; Edeling et al., 2006b).

Pitstop 1 lies in a hydrophobic cavity formed by four isoleucines (52, 62, 80, 93), Leu 82, and Phe 91. Its conformation is stabilized by five hydrogen bonds at both ends of pitstop 1 (see Table 5 below). Only one direct hydrogen bond is formed between the sulfonate group of pitstop 1 and the ϵ -amino group (N ϵ 2 atom) of Gln82. All other hydrogen bonds are mediated by water. Comparison of ligand-free clathrin TD with the pitstop 1-bound form shows that two residues in the clathrin box-binding site undergo major conformational changes upon ligand binding. While the phenyl ring of Phe 91 rotates by 180° around the C α -C β bond and stacks against the non-polar portion of the compound on one side, the guanidinium group of the Arg 64 side chain stacks on pitstop 1 on the opposite side.

Pitstop 2 occupies the identical hydrophobic cavity used by pitstop 1. However, differences in the binding modes of both molecules (Fig. 4D) were noted. In particular, the side chain of Arg 64 is positioned along the central part of pitstop 2 and forms bidentate hydrogen bonds with O and N atoms of the thiazol-4(5*H*)-one ring. The conformational change of Arg 64 allows enough space for the non-polar portion of pitstop 2 to insert deeply into the hydrophobic cavity, towards the first blade. The phenyl ring of Phe 91 stacks against the non-polar edge of the bromobenzene group of pitstop 2 (Fig. 4D), and the conformation is stabilised by 2 two direct hydrogen bonds with Arg 64 (see Table 6). Since the solvent-exposed bromobenzene moiety lies on the second blade of the clathrin TD, the first and the second blades are blocked by pitstop 2.

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Table 5: Interaction of pitstop 1 with clathrin TD via hydrogen bonds

Pitstop 1 (Inhibitor KAM-6-C12)		Dist. Å	Water or Clathrin TD		
Sulfonate group	OAD	2.8	W1	2.6	Ser 67(O)
Sulfonate group	OAE	2.8	W2		
Sulfonate group	OAF	2.9	Gln 89 (NE2)		
Aminobenzene	NAA	3.1	W3	2.9	Val 50(O)
				2.6	Ile 62(O)
				3.4	Arg 64(N)
Aminobenzene	NAA	3.2	W4		

Table 6: Binding interaction of pitstop 2 with clathrin TD - Hydrogen bonds

5

Pitstop 2 (Inhibitor MR1133)		Dist. (Å)	Water or Clathrin TD	
4-keto-thiazol	OAA	2.8	NH ₂	Arg 64
4-keto-thiazol	NAQ	2.9	NE	Arg 64

The analysis reveals that the two pitstops fit into the targeted TD groove in two different poses and induce distinct structural changes in clathrin TD. This provides a molecular basis for the selective displacement of clathrin box ligands from the TD and accounts for the *in vitro* and *in vivo* activities of the pitstops.

10

4. Pitstop 2 selectively inhibits clathrin-mediated endocytosis and HIV entry

To examine whether pitstop 2 can act in living cells the effect of the pitstop on the internalization of transferrin (Tf) and epidermal growth factor (EGF) was analyzed. Receptor-mediated-internalization of both of these ligands is known to largely depend on clathrin function in most cell types (Brodsky et al., 2001). When exposed to Alexa⁵⁶⁸-Tf HeLa cells efficiently internalized Tf into perinuclear recycling endosomes. Preincubation of HeLa cells with pitstop 2 led to a dose-dependent inhibition of Tf uptake with an IC₅₀ value (12-15 μ M) very similar to that measured for blocking clathrin TD function *in vitro* (Fig. 5A, B). Application of 30 μ M pitstop 2 completely blocked Tf endocytosis, similarly to what has been observed in clathrin knockdown cells. Pitstop 2-induced block of Tf endocytosis in HeLa cells was completely reversed within 1-3 h of drug washout. In another cell line, U2OS, the IC₅₀ for Tf uptake was 9.7 $\pm$ 1.5 μ M (n=5 independent experiments, from 3 distinct synthetic batches of pitstop 2). Treatment with similar concentrations of pitstop 2 also blocked Tf uptake in other cell types including Cos7 and BSC1 cells, astrocytes or primary neurons. Pitstop 2 also caused a potent inhibition of EGF uptake (Fig. 5C). This is consistent with the previously suggested notion that the majority of EGF is endocytosed via a clathrin-mediated entry route in most cell types. Pitstop 1 was found to display comparably low cell membrane penetration but exhibited qualitatively similar effects, albeit at higher doses (not shown).

To better understand the mechanism by which pitstop 2 inhibits receptor-mediated endocytosis, Cos7 cells were incubated with Alexa⁴⁸⁸-EGF at 4°C to accumulate ligand-receptor complexes in CCPs (Krauss et al., 2006). A similar although slightly enhanced accumulation of EGF was observed in pitstop 2-treated cells (Fig. 5D, E) suggesting that pitstop 2 does not interfere with cargo recognition or sequestration into CCPs, a step mediated by endocytic adaptors such as AP-2. These data argue against non-specific effects of pitstop 2 on plasma membrane organization and suggests that pitstop 2 blocks CME at a step subsequent to cargo sequestration.

Shiga toxin is known to enter cells via a clathrin-independent glycosphingolipid-dependent route. HeLa cells were found to rapidly internalize Shiga toxin which accumulated in the cis-Golgi area, irrespective of the presence of pitstop 2 (30 μ M). A slight though statistically insignificant delay in Shiga toxin delivery to the cis-Golgi in pitstop 2-treated cells was observed, indicating that clathrin TD-ligand interactions are

not required for retrograde transport of Shiga toxin. This supports the specificity of pitstop 2 for the CME pathway.

Many viruses and pathogens exploit the clathrin-dependent endocytic machinery for cell entry. For example, HIV-1 entry to cells has been shown to occur predominantly or exclusively via CME in studies using dominant negative dynamin or Eps15, or the small molecule dynamin inhibitor dynasore (Miyauchi et al., 2009). This suggests an obligatory role for clathrin in HIV-1 entry. To evaluate this, HeLa reporter cell lines were infected with HIV-1 for 2 h in the presence or absence of pitstop 2. Subsequent fusion events were blocked with an HIV entry inhibitor and infection was scored by luciferase readout after 48 h. Pitstop 2 potently and specifically reduced HIV-1 infectivity in this reporter cell line by about 70% at a concentration of the compound of 5 μ M and >90% at a concentration of 10 μ M (Fig. 5F), confirming that CME is the main route of productive entry in this cell line.

The results demonstrate that pitstop 2 is an efficient and selective inhibitor of CME that acts via blocking ligand access to the clathrin TD.

5. The clathrin TD plays a crucial role in regulating coated pit dynamics

Clathrin dynamics were studied in more detail in view of the lack of knowledge of the physiological role of ligand binding to the clathrin TD in endocytosis. Live Cos7 cells stably expressing eGFP-clathrin light chains (LC) were monitored by total internal reflection microscopy (TIRFM) at 37°C. Cells were treated with DMSO (0.1%) or 30 μ M pitstop 2. Consistent with previously reported data, clathrin LC-containing CCPs formed and disappeared at the plasma membrane with most events exhibiting lifetimes between 26 and 89 s. A smaller fraction of long-lived clathrin-coated structures were detected with lifetimes of more than 90 s. However, a low level of short-lived events were also observed that could not be quantitatively tracked by the imaging software utilised and were therefore excluded from analysis. Treatment of cells with pitstop 2 led to a dramatic shift towards long-lived clathrin-coated structures, many of which exhibited lifetimes well beyond the usual imaging time (limited by the eventual bleaching of clathrin LC-eGFP). Increased CCP lifetimes were paired with a minor decrease in the mean clathrin LC-eGFP intensity per object in TIRFM. A slightly increased dimming of CCPs in confocal was noticed when compared to TIRF imaging, presumably due to increased bleaching of stalled clathrin puncta. Thus,

surprisingly, pitstop 2-mediated interference with clathrin TD-ligand association does not have a strong effect on the stability of membrane-associated clathrin, but dramatically perturbs CCP dynamics.

To corroborate the role of clathrin TD function in CCP dynamics, fluorescence recovery after photobleaching (FRAP) was paired with live cell spinning disc confocal microscopy imaging. Cells were imaged after the addition of 30 μ M pitstop 3 of DMSO (0.1%). Frames were taken at 0.5 Hz, starting 10s before FRAP, followed by imaging for another 2 minutes after photobleaching. Following the bleaching laser pulse, clathrin LC-eGFP fluorescence rapidly recovered to about 80 % of its initial value with a τ of approximately 30 s in control cells, consistent with the mean CCP lifetimes measured by TIRFM-based particle tracking. However, pitstop 2-treated cells did not show recovery of clathrin LC-eGFP fluorescence within 120 s, confirming that clathrin TD function indeed regulates CCP dynamics. Recovery was so slow that a reliable τ value could not be determined under the imaging conditions utilised. These data indicate that pitstop 2 stalls the maturation and/ or consumption of pre-existing CCPs.

The question of whether clathrin TD function may be required for the *de novo* assembly of clathrin-coated structures was then evaluated. For this, use was made of 1-butanol, an alcohol which depletes cellular PI(4,5)P₂ levels causing CCP disassembly and loss of clathrin from the plasma membrane. Clathrin LC-eGFP containing pits rapidly reformed after washout of 1-butanol in control and pitstop 2-treated cells, indicating that TD-ligand interactions are not required for the *de novo* recruitment or assembly of clathrin at the plasma membrane. This conclusion was further supported by ultrastructural analysis (see Fig. 6 and Fig. 7).

If pitstop 2 mainly interferes with CCP dynamics but not with clathrin recruitment relatively subtle effects on the distribution of endogenous endocytic proteins would be expected. No significant changes were observed in the colocalization of clathrin, FCHo 1/2, intersectin, AP-2, or dynamin 2 (Dyn2) by either qualitative or quantitative analysis. A different behavior was seen for endogenous amphiphysin 1, which was partially lost from the plasma membrane of pitstop 2-treated cells. Thus, amphiphysin and possibly other clathrin box ligands require association with the clathrin TD for function, while other components retain their plasma membrane localization, likely via binding to AP-2, to membrane PI(4,5)P₂, or both. However, it

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cannot be ruled out that even those endocytic proteins that appear to be localized correctly at the light microscopic level may exhibit functional defects upon acute inhibition of complex formation with the clathrin TD.

The distribution of various TGN or endosomal proteins including AP-1, EEA1, Gadkin, CD63, TGN46, or the mannose 6-phosphate receptor MPR46 was also determined. None of these factors showed a significant change in localization within 15 min of pitstop 2 application. However, a partial loss of transferrin receptor (TfR) was noted from perinuclear endosomes, which instead appeared to be shifted towards the cell surface of pitstop 2-treated cells, consistent with the observed defects in Tf endocytosis (Fig. 5A, B).

6. Dynamics of FCHo2, AP-2, and dynamin in pitstop 2-treated cells

As pitstop 2 was found to impair clathrin dynamics, the behavior of other key endocytic proteins which act either prior to or concomitant with clathrin assembly was evaluated. FCHo proteins are early-acting factors postulated to facilitate CCP formation by linking PI(4,5)P₂-rich membrane sites to endocytic AP-2 binding scaffold proteins intersectin and Eps15/ Eps15R. TIRFM-based imaging of eGFP-FCHo2 dynamics in Cos7 cells revealed that FCHo2-containing structures were relatively long-lived, with lifetimes between 26 s and over 120 s. The mean lifetimes of FCHo2-eGFP-containing puncta were dramatically increased following application of pitstop 2 with puncta appearing nearly immobile. Consistent with their increased lifetimes, FCHo2-positive spots failed to recover in FRAP experiments. Hence, clathrin TD function is required for the productive consumption of nascent CCPs containing FCHo2.

Surprisingly, pitstop 2 induced a comparatively minor shift in the mean life span of AP-2-eGFP puncta. These results are consistent with our observation that pitstop 2-treated cells remain able to sequester cargo into CCPs, a process largely mediated by AP-2.

Finally, the life span of dynamin.2 (Dyn2) was investigated. Dyn2 is a component required for fission of mature CCPs. Dynamin 2-eGFP displayed a lifetime distribution similar to that observed for AP-2 or clathrin. Application of pitstop 2 shifted this distribution to longer life spans with a behavior that was intermediary between the effects seen for clathrin and AP-2.

7. Morphological analysis of pitstop 2-treated cells

The live imaging data reported herein collectively provides evidence that pitstop 2-mediated inhibition of CME results from defects in endocytic protein dynamics, most strikingly seen for FCHo2 and clathrin, but not in clathrin recruitment. As CME is a constitutive process in non-neuronal cells with CCPs forming and disassembling continuously, it would be expected that acute perturbation of endocytic protein dynamics by pitstop 2-mediated inhibition of clathrin TD function results in acute "trapping" of clathrin-coated intermediates representing multiple stages of the pathway. To analyze whether this is the case, control or pitstop 2-treated Cos7 cells were subjected to electron microscopic and morphometric analyses. Clathrin-coated endocytic intermediates were classified according to their morphological profiles as shallow CCPs, non-constricted u-shaped CCPs, constricted Ω -shaped CCPs corresponding to late fission stages, and structures containing a complete clathrin coat (Fig. 6A). These latter intermediates may comprise free clathrin-coated vesicles as well as late intermediates that remain attached to the plasma membrane via a connection not visible in the plane of the section. mAs predicted, endocytic profiles of control cells and those incubated with pitstop 2 appeared very similar. No major changes in the total number of CCPs (Fig. 6B) or in the representation of various endocytic stages were observed (Fig. 6C). It was, therefore, concluded that pitstop-mediated inhibition of clathrin TD function acutely arrests CME at multiple stages.

8. Pitstops inhibit endocytic recycling of synaptic vesicles in neurons

One of the most prominent functions of CME in vertebrates is the exo-endocytic recycling of synaptic vesicle (SV) membranes at nerve terminals. Unlike CME in non-neuronal cells analyzed thus far, endocytic reformation of SVs in neurons is strictly coupled to stimulation-dependent exocytosis resulting in a wave of endocytic activity. The effects of pitstop 2-mediated clathrin inhibition on SV reformation in cultures of primary hippocampal neurons were investigated using electron microscopy. Chemical stimulation of SV exocytosis resulted in a pronounced depletion of SVs from nerve terminals of pitstop 2-treated neurons, suggesting that SV reformation by endocytosis was inhibited. No significant changes in SV numbers were seen in resting control neurons. Loss of SV membranes in pitstop 2-treated neurons appeared to be counterbalanced partly by presynaptic membrane expansion.

The poor membrane penetration of pitstop 1 prevented analysis of its effects on endocytosis in living cells. However, its high solubility in aqueous media provided ideal conditions for its intracellular application by microinjection. The effect of pitstop 1 on SV endocytosis in the lamprey reticulospinal synapse was therefore assessed (Pechstein et al., 2010), a model perfectly suited to test whether acute inhibition of ligand association with the clathrin TD inhibits CME but permits clathrin recruitment. Pitstop 1 was injected into lamprey reticulospinal axons and stimulated at 5 Hz for 20 min to induce SV recycling at a physiological rate. Analysis of ultrathin sections revealed a profound loss of SVs at active zones in synapses from these axons (Fig. 7A, B, G), compared to control non-injected axons (Fig. 7C). No morphological alterations were observed in axons microinjected with pitstop 1 and kept at rest (Fig. 7E). Loss of SVs in stimulated axons was accompanied by a dramatic expansion of the plasma membrane, which due to anatomical constraints formed membrane pockets around the active zone (Fig. 7A, B, H). A significant accumulation of CCPs was observed within the membrane expansions compared to control synapses (Fig. 7C, D). Consistent with observations in the present study in Cos 7 cells, no dramatic alterations in stage representation of CCPs was observed (Fig. 7J). Analysis of CCPs at high-magnification (Fig. 7D, E) and by tilting in the microscope did not reveal detectable differences in their morphology. It was, therefore, concluded that intra-axonal application of pitstop 1 strongly inhibits SV recycling *in situ* by inhibiting CME at multiple stages.

9. Discussion

Two small molecule inhibitors of clathrin TD function termed pitstops 1 and 2 that competitively interfere with the association of endocytic clathrin box ligands were developed. *In vitro* experiments in conjunction with structural data based on protein X-ray crystallography as well as experiments in living cells clearly demonstrated that these compounds selectively perturb clathrin TD function. Effects of pitstop 2 on CME are evident within 2-5 min of cell treatment, presumably only being limited by the rate of drug diffusion across the cell membrane. Reversible inhibition of CME was observed in a broad variety of cells at concentrations closely matching the IC₅₀ values of the *in vitro* binding experiments. While pitstop 2 readily crosses cell membranes, pitstop 1 has reduced efficacy compared to pitstop 1. The similarity of phenotypes

exhibited by pitstops 1 and 2 in lamprey reticulospinal synapses *in situ* and in Cos 7 cells *in vitro* provides evidence that both compounds share a common clathrin TD-based molecular mechanism of action. In the presence of pitstop 2, clathrin-independent internalization pathways and secretory traffic remain unperturbed (data not shown). As such, pitstops represent novel cellular tools to selectively inhibit CME.

The use of pitstops in living cells revealed a novel and unexpected role for the clathrin TD in endocytic pit dynamics. Although the clathrin TD represents the most frequently used interaction hub within the endocytic network, surprisingly little is known regarding the functional role of TD-clathrin box ligand interactions. The use of pitstop 2 to acutely inhibit ligand access to the clathrin TD provides evidence for a central role for clathrin TD function in regulating CCP dynamics in mammalian cells. Mammalian cell endocytosis differs from yeast where clathrin plays a comparably minor role in endocytosis. While it might be expected that pitstops interfere with clathrin recruitment to the membrane, live cell imaging revealed specific effects on CCP dynamics instead. These data are explained by a model where pitstops interfere with the progression of CCPs at multiple stages. Consistent with this, the accumulation of distinct endocytic intermediates in pitstop 2-treated cells was not observed by electron microscopy.

Proteins that contain clathrin box motifs comprise a diverse set of factors implicated in both early and late stages of endocytosis (Brodsky et al., 2001; Dell'Angelica, 2001). These include adaptors such as AP180/CALM, Dab2 and β -arrestins (ter Haar et al., 2000), membrane deforming proteins (e.g. epsin and amphiphysin; Slepnev et al., 2000), as well as uncoating factors (i.e. auxilin/GAK or the PI(4,5)P₂-hydrolyzing enzymes synaptojanin-p170 and OCRL. This functional diversity of clathrin TD ligands may contribute to the observation that pitstop inhibits CME at multiple stages.

Blocking ligand access to the TD by pitstops may stall productive maturation of CCPs by preventing dynamic exchange of key endocytic factors driving progression of the pathway or monitoring completion of the previous step. The unexpected finding that pitstop-mediated inhibition of ligand binding to the clathrin TD does not cause clathrin dissociation from membranes nor prevents the *de novo* assembly of clathrin-containing structures at the plasma membrane suggests that other interactions may be crucial for clathrin recruitment.

Clathrin is most highly expressed in the brain where it is concentrated at presynaptic nerve terminals. Evidence from morphological, genetic and knockdown studies has suggested that CME may be a key mechanism for the regeneration of SVs during stimulation induced exo-endocytic cycling. A drawback of RNAi and genetic approaches is that changes in clathrin levels occur over several days or even the entire lifespan of the animal, making it difficult to distinguish direct from indirect effects. This is important as synapses can adapt to chronic loss of endocytic proteins (Kim and Ryan, 2009). Using pitstops as reported herein to acutely perturb clathrin TD function provides a direct demonstration for an important role of clathrin in SV recycling in vertebrate neurons under conditions of high activity.

The data reported herein also provides strong evidence that HIV enters cells largely via CME. Hence, the pitstop inhibitors developed herein and their derivatives thereof may serve drugs for blocking the entry of viruses and pathogens which hijack the clathrin machinery, including HIV, hepatitis C virus, ebola virus, and *Listeria monocytogenes* amongst others.

EXAMPLE 2: Inhibition of clathrin function induces aberrant mitotic defects and inhibition of cancer cell division

1. Clathrin function is essential for mitosis

An acute 90 min treatment of HeLa carcinoma cells with pitstop 1 or pitstop 2 caused an increase in mitotic index that is, the percentage of cells in metaphase, in a dose-dependent manner (Fig. 8). The data also suggests that clathrin is required for chromosome segregation. Consistent with this role, clathrin was observed to locate to the mitotic spindle (Fig. 9C). Characterisation of the mitotic spindle revealed that pitstop-treated cells have (1) a thicker metaphase plate, indicating that chromosomes were not aligned properly (Fig. 9C), (2) a reduction in spindle width at the cell equator (Fig. 9A-C), (3) a reduction in spindle microtubules (Fig. 9C), and (4) disrupted HURP staining indicating unattached K-fibres (Fig. 10).

In particular, treatment with pitstops caused the percentage of narrow spindles to increase from 10% and 14% in untreated and DMSO control cells respectively to 24% in pitstop 1 treated cells and 29% in pitstop 2 treated cells. This phenotype was analogous with the phenotype induced in clathrin-depleted cells using specific siRNA,

wherein the percentage of cells with narrow spindles increased to 45%. Further, untreated controls, DMSO and dynole 34-2 treated cells had HURP mis-localisation in 11%, 10%, and 11% of cells respectively. However, pitstop 1 treated cells exhibited HURP mis-localisation in 41% of the cells, whilst in cells treated with pitstop 2, the level was 39% of cells. These levels were comparable to the 42% of cells with HURP mis-localisation seen in clathrin depleted cells after siRNA.

The graph in Fig. 9B also shows that in cells treated with pitstop 1 or pitstop 2, the spindles become significantly ($P>0.01$) narrower with a median ratio of 0.4 (pitstop 1) and 0.35 (pitstop 2) of the total cell width. Depletion of clathrin by siRNA also caused a significant decrease in the median spindle width ratio to 0.36 of the total cell width as compared to untreated control (0.65), DMSO (0.67), and the dynamin (and endocytosis) inhibitor dynole 34-2 (0.63).

In addition to the aberrant mitotic spindles formed when clathrin is inhibited or depleted from cells, an increase in the percentage of cells harboring multipolar spindles was observed (>2 spindle poles; $P>0.01$), an effect also seen in siRNA clathrin depleted cells. Centrioles were identified by Centin2 staining. Spindle microtubules (MTs) originate from the centrosome (spindle pole), which consists of two centrioles surrounded by pericentriolar matrix (PCM). The fidelity of the PCM is important for completion of metaphase in cells undergoing mitosis. The spindle poles of pitstop-treated cells did not affect PCM organization as indicated by γ -tubulin staining. In contrast, a significant number of spindle poles contained less than two centrioles. This phenotype was again analogous to that induced by clathrin siRNA, and suggests that clathrin may participate in centriole cohesion. Untreated, DMSO treated, and dynole 34-2 treated control cells showed a normal count of 2 centriole centrosomes. Dynole 34-2 (2-Cyano-*N*-octyl-3-(1-(3-dimethylaminopropyl)-1*H*-indol-3-yl)-acrylamide (Hill et al., 2009) is a potent inhibitor of dynamin II (Dyn2). Dyn2 plays a central role in endocytosis.

Thus, the action of the pitstop compounds is consistent with targeted inhibition of clathrin. Moreover, these findings indicate that the pitstop compounds prevent mitotic progression by specifically blocking the onset of chromosome segregation and further, that they appear to activate the spindle assembly checkpoint (SAC) in mitosis.

2. Clathrin plays a role in mitotic spindle assembly

Next a MT re-growth assay utilising HeLa cells was employed to determine if clathrin was required for mitotic spindle assembly. This involved allowing microtubules to regrow for 5 min at 37°C following their disassembly by cold treatment (cells were placed on ice for 30 mins). Depletion of clathrin by siRNA prevented MT re-growth from centrosomes in metaphase cells. In contrast, neither pitstop 1 nor pitstop 2 mediated inhibition of clathrin prevented MT regrowth from the mitotic spindle poles. As expected clathrin no longer decorated the mitotic spindle and spindle poles in cells after clathrin knockdown by siRNA.

In particular, in control cells, DMSO, and dynole 34-2 (30 µM) treated cells the MT regrew to median lengths of 3 µM, 3.5 µM, and 3.8 µM respectively. This recovery was not impeded by treatment with the pitstops 1 or 2 (30 µM), with spindle regrowth reaching a median MT length of 4 µM (pitstop 1) or 3.7 µM (pitstop 2). The absence of clathrin protein itself by siRNA treatment caused a significant delay in MT recovery with a median length of 1.3 µM. However, the pitstop compounds do not affect the mitotic spindle localisation of clathrin (Fig. 8C).

The data indicate that mitotic spindle pole localization of clathrin is required for mitotic spindle assembly and that clathrin acts as a scaffold for recruitment of key spindle proteins required for spindle growth. Clathrin forms a complex with TACC3 at the mitotic spindle. Consistent with clathrin being required for TACC3 spindle localization, TACC3 spindle localization was not affected by pitstop-mediated inhibition as clathrin remained on the spindle under these conditions. In contrast, TACC3 spindle localisation was significantly reduced by siRNA-mediated depletion of clathrin by >25% as evidenced by quantitation of TACC3 staining and fluorescence intensity.

3. Pitstops activate the spindle assembly checkpoint

To confirm that pitstops activate the SAC the recruitment of a critical SAC component, MAD2, to the kinetochores was observed. In untreated and DMSO-treated control cells MAD2 did not locate to the kinetochores, as indicated by a lack of co-localisation with the centromere component, CENPB (Fig. 11). This indicates that the SAC has not been activated and cells are able to proceed to segregate their

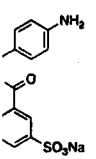
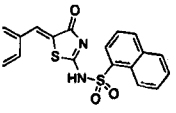
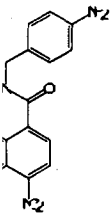
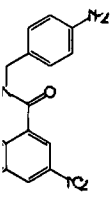
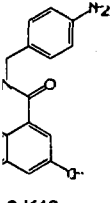
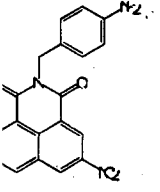
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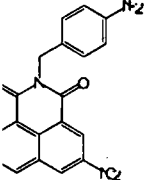
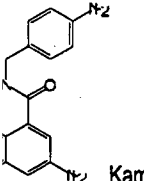
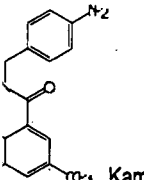
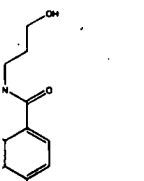
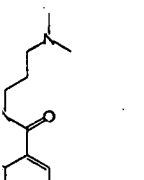
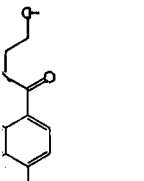
chromosomes. In contrast, MAD2 co-localised or located adjacent to CENPB foci in HeLa cells treated with either pitstop compound and in clathrin-depleted HeLa cells (Fig. 11). Depletion of another endocytic protein, epsin, whose function is also associated with metaphase, did not result in recruitment of MAD2 to the kinetochores and thus the SAC was not active. It was concluded that the pitstops activate the SAC.

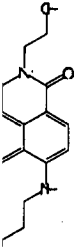
4. Clathrin inhibitors reduce cell growth in a range of cancer cell lines

Several anti-mitotic compounds have anti-cancer properties by causing cell death following activation of the SAC. Aurora A selective inhibitors, such as MLN8237, represent such compounds. As pitstop 1 and pitstop 2 also activate the SAC, the question of whether they can inhibit cell proliferation and induce cell death was assessed. Cell growth was assessed by MTT assay in twelve cancer cell lines derived from different tissues: namely, SMA-560 (mouse glioma), SJ-G2 and U87 (glial), HeLa (cervical), MCF-7 (breast), A2780 (ovary), H460 (lung), A431 (skin), DU145 (prostate), MIA (pancreas), BE(2)-C (neuronal) and HT29 and SW480 (colon). Pitstop 1 had no effect on cell growth after 72 hours of continuous exposure. In contrast, pitstop 2 caused a dose-dependent decline in cell growth in four of the cancer cell lines, namely, HT29, BE2-C and U87 at low concentrations and A431 at a higher concentration (see Table 8). The best (lowest) GI_{50} value (concentration that causes 50% growth inhibition) was $> 5 \mu M$. To improve potency and cell permeability, analogues of pitstop 2 were generated. The structures of these analogues are shown in Table 7. All eleven analogues were significantly more potent at inhibiting growth and reducing viability in all cancer cell lines than the parent pitstop 2. KAM-6-H12 and KAM-6-H53 were the most potent in this series and were also verified to retain inhibition of Amph binding to the clathrin terminal domain (TD). A2780 ovarian cancer cells were the most sensitive cells. Other analogues in the same family as pitstop 1 showed 10 fold more potent inhibition of cancer cell growth at nanomolar concentrations (data not shown). Thus, clathrin inhibitors can prevent cell proliferation and reduce viability in a range of cancer cell lines, favourable properties for an anti-cancer agent.

Table 7: Growth inhibition (GI₅₀) induced by clathrin inhibitors

Compounds	HT29 Colon n=3	SW480 Colon n=3	MCF-7 Breast n=3	A2780 Ovarian n=3	H460 Lung n=3	A431 Skin n=3	DU145 Prostate n=3	BE2-C Neuroblastoma n=3	SJ-G2 Glioblastoma n=3	MIA Pancreas n=3	SMA Glioblastoma (Murine) n=3	U87 Glioblastoma n=3
 1	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
 2	6.2 ± 2.0	>50	>50	>50	>50	38 ± 9	>50	5.7 ± 0.9	>50	>50	>50	5.3 ± 0.9
 6-H12	1.7 ± 0.1	1.0 ± 0.0	1.5 ± 0.2	0.4 ± 0.1	2.3 ± 0.3	1.8 ± 0.3	1.8 ± 0.2	1.5 ± 0.03	0.9 ± 0.2	1.2 ± 0.2	0.4 ± 0.04	2.0 ± 0.1
 6-J12	>50	>50	>50	4 ± 0.2	4 ± 0.3	4.5	tf	3.5	nd	nd	2.8 ± 0.2	>50
 6-K12	29 ± 1.2	29 ± 0.3	35 ± 1.5	14 ± 0.3	19 ± 0.6	19 ± 1.3	20 ± 0.6	24 ± 1.8	14 ± 0.6	24 ± 2.6	8.8 ± 0.3	30 ± 0.0
 6-P12	3.5 ± 0.4	3.2 ± 0.1	20 ± 2.0	2.4 ± 0.3	4.6 ± 0.6	7.5 ± 1.9	6.3 ± 0.2	4.7 ± 0.3	3.3 ± 0.1	3.3 ± 0.5	7.9 ± 0.6	5.3 ± 0.2

 6-Q12	9.8 ± 0.6	8.8 ± 0.3	18 ± 0.9	4.7 ± 0.3	5.9 ± 0.5	8.9 ± 1.1	8.3 ± 0.6	10 ± 10	9.0 ± 0.6	8.2 ± 0.4	4.6 ± 0.3	17 ± 1.2
 Kam-2	17 ± 2.3	16 ± 1.0	20 ± 0.3	6.1 ± 0.4	7.9 ± 0.4	11 ± 1.1	8.0 ± 0.0	8.7 ± 0.3	6.0 ± 0.1	19 ± 2.0	3.9 ± 0.2	16 ± 0.9
 Kam-3	5.0	4.5	>50	3.1 ± 0.1	3.1 ± 0.2	5.0	nd	5.0	nd	nd	2.0 ± 0.0	>50
 6-H2	3.1 ± 0.6	1.4 ± 0.1	3.3 ± 0.2	0.7 ± 0.05	4.6 ± 0.3	2.6 ± 0.2	2.7 ± 0.2	2.3 ± 0.2	1.1 ± 0.1	2.8 ± 0.2	0.7 ± 0.04	3.4 ± 0.3
 6-H53	0.9 ± 0.04	0.9 ± 0.1	1.5 ± 0.1	0.2 ± 0.02	1.5 ± 0.3	1.5 ± 0.3	1.4 ± 0.2	1.1 ± 0.1	0.6 ± 0.1	1.2 ± 0.3	1.3 ± 0.1	1.1 ± 0.1
 6-R32	44 ± 3.0	52 ± 3.9	39 ± 2.1	26 ± 3.6	36 ± 1.2	50 ± 9.0	52 ± 12	44 ± 6.4	15 ± 0.3	50 ± 12	24 ± 2.5	45 ± 10



6-R33	20 ± 1.2	45 ± 2.0	14 ± 0.7	18 ± 1.0	17 ± 2.4	23 ± 2.6	31 ± 2.0	19 ± 2.7	7.8 ± 0.2	18 ± 0.7	20 ± 2.7	35 ± 3.3
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5. Inhibition of clathrin function causes cytokinesis failure

Clathrin has a second role in mitosis at the final stage of mitosis - cytokinesis.

Unlike the role of clathrin at the mitotic spindle, its role in cytokinesis is thought to be dependent on its endocytic function. Consistent with this, inhibition of dynamin II (Dyn2), a key endocytic protein that is required for cytokinesis, utilising dynole 34-2 caused an increase in multinucleated cells, an indicator of cytokinesis failure (Fig. 11). Depletion of clathrin using siRNA and pitstop 1 and pitstop 2-mediated inhibition of clathrin also caused multinucleation (Fig. 12). Hence, the pitstop compounds induce cytokinesis failure consistent with targeted inhibition of clathrin.

6. Discussion

The endocytic protein clathrin plays two roles important for mitotic progression:

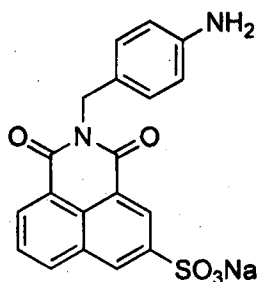
1) at the mitotic spindle to stabilise K-fibres for chromosome segregation; and 2) during cytokinesis for membrane abscission to generate two independent daughter cells. The present study shows that the small molecule inhibitors of clathrin, pitstop 1 and pitstop 2, produce mitotic phenotypes consistent with these two mitotic roles for clathrin. As such, these compounds and their analogues represent extremely valuable tools that can be exploited to characterise the molecular mechanism(s) of action of clathrin during mitosis. More globally, the use of these compounds provide for an improved understanding of the molecular mechanisms of mitotic progression in cells, and hence of the fundamental cell division process.

New classes of potential chemotherapeutics are being developed that specifically target key mitotic proteins to either activate or inhibit the SAC, such as Aurora kinase and kinesin spindle protein. These targeted inhibitors prevent proliferation of most tumour cells *in vitro* and reduce tumour volume *in vivo* by

inhibiting growth and/or triggering cell death following SAC activation/inhibition (Taylor and Peters, 2008). Consequently, many targeted inhibitors, such as the Aurora kinase inhibitor MLN8054 (Millennium, USA), are currently in cancer clinical trials (Ma and Adjei, 2008), and are expected to have a more favourable therapeutic window than currently used chemotherapeutic agents such as taxol, as they would spare non-dividing, non-differentiating cells. The ability of these mitotic inhibitors to be efficacious anti-cancer agents is dependent on their ability to induce apoptosis following mitotic insult as it is predicted to result in tumour regression. However, they do not always result in cell death with varied cellular responses reported which include apoptosis, mitotic catastrophe, mitotic slippage, senescence and reversible mitotic arrest with continued cell growth. Hence, there has been an ongoing need for the identification of new targets and the development of new compounds to broaden the therapeutic index of anti-mitotic agents as anti-cancer chemotherapeutics. The results described herein demonstrate that small molecule inhibitors of clathrin are potent anti-mitotic compounds that activate the SAC and may be exploited as anti-cancer therapeutic agents.

EXAMPLE 3: Synthesis of pitstops

1. *Sodium 2-(4-aminobenzyl)-1,3-dioxo-2,3-dihydro-1H-benzo[de]isoquinoline-5-sulfonate* (Pitstop 1)



- Step 1: 3-sulfo-1,8-naphthalic anhydride, sodium salt

1,8-Naphthalic anhydride (0.990 g, 5.00 mmol) was dissolved in oleum (6 mL). The resulting solution was stirred at 120 °C for 1 hour (until a drop of the mixture, when added to water, did not precipitate), then cooled to room temperature. The cooled

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solution was then poured into water (25 mL). Addition of aqueous saturated sodium chloride (20 mL) resulted in precipitation of the product. The white solid was collected by filtration, washed with water (5 mL), then ethanol (10 mL), and dried under vacuum.

Yield 1.462 g (95.6 %), MP > 300 °C.

5 $^1\text{H-NMR}$ (DMSO- d_6) δ 7.91 (1H, t, $J = 7.4$ Hz), 8.49 (1H, d, $J = 7.5$ Hz), 8.62 (1H, d, $J = 4.7$ Hz), 8.65 (1H, s), 8.73 (1H, s).

$^{13}\text{C-NMR}$ (DMSO- d_6) 119.7, 128.4, 130.0, 130.3, 131.4, 131.6, 133.1, 136.5, 147.7, 161.1, 161.2.

10 Step 2: 2-(4-Aminobenzyl)-1,3-dioxo-2,3-dihydro-1H-benzo[de]isoquinoline-5-sulfonic acid

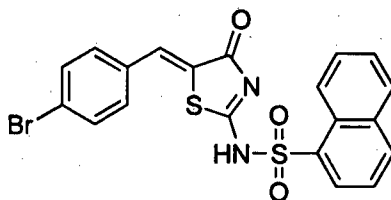
4-Amino(methyl)aniline (0.092 g, 0.75 mmol), DMF (1 mL) and triethylamine (0.5 mL) were added to a suspension of 3-sulfo-1,8-naphthalic anhydride, sodium salt (0.197 g, 0.66 mmol) in toluene (5 mL). The resulting suspension was refluxed at 130 °C for 18 hours. The reaction mixture was then cooled to room temperature and the precipitated product collected by filtration, washed with toluene, and dried under vacuum. A yellow-orange solid was obtained.

Yield 0.167 g (65 %), MP > 300 °C.

20 $^1\text{H-NMR}$ (DMSO- d_6) δ 4.97 (2H, br s, NH_2), 5.07 (2H, s), 6.46 (2H, d, $J = 8.4$ Hz), 7.07 (2H, d, $J = 8.4$ Hz), 7.87 (1H, dd, $J = 7.5, 8.1$ Hz), 8.49 (1H, dd, $J = 0.9, 7.5$ Hz), 8.55 (1H, dd, $J = 0.9, 8.1$ Hz), 8.65 (1H, d, $J = 1.5$ Hz), 8.68 (1H, d, $J = 1.5$ Hz);

$^{13}\text{C-NMR}$ (DMSO- d_6); 42.7, 113.8, 122.1, 122.2, 124.5, 127.3, 127.8, 128.9, 129.2, 130.2, 131.2, 131.3, 135.3, 147.2, 148.0, 163.5, 163.6 ppm.

25 2. (Z)-N-(5-(4-bromobenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl)naphthalene-1-sulfonamide (Pitstop 2)



To 1-naphthalene sulfonyl chloride (420 mg, 1.85 mmol) in dry THF (30 mL) was added pseudothiohydantoin (221 mg, 1.9 mmol) and triethylamine (0.56 mL, 4.0 mmol). The resultant mixture was heated at reflux overnight to produce a brownish precipitate in an orange solution. EtOAc (50 mL) was added and the mixture washed with water (2 x 50 mL), dried (MgSO₄) and evaporated *in vacuo*. An attempt to purify using chromatography failed to yield a clean sample and so the crude material (150 mg, MS 305 [M-H]⁻) was used directly in the next step. This material was dissolved in EtOH (50 mL) and *p*-bromobenzaldehyde (120 mg, 0.65 mmol) and piperidine (2 drops) was then added. After heating at reflux for 3 h the volume of the resultant yellow solution was reduced by half and then cooled. The precipitate formed was filtered off, washed with ether (2 x 10 mL) and dried *in vacuo* to yield the desired product; (132 mg, 15% overall) mp 230 °C (decomp).

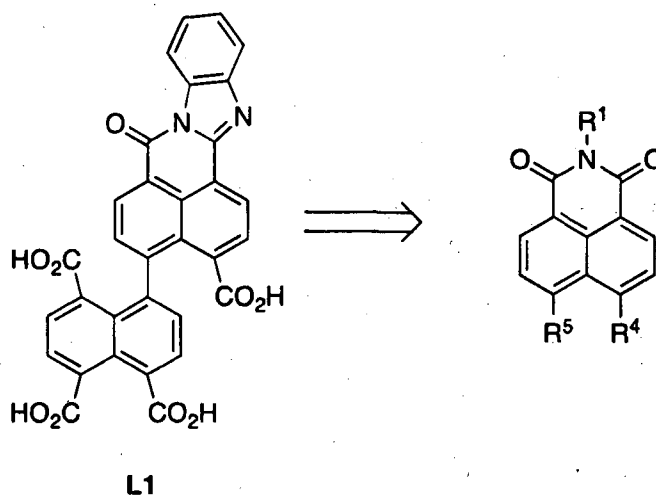
¹H NMR (300 MHz, DMSO-*d*₆): δ 8.69 (d, *J* = 8.4 Hz, 1H), 8.17 (d, *J* = 7.2 Hz, 1H), 8.10 (d, *J* = 8.1 Hz, 1H), 8.00 (d, *J* = 7.8 Hz, 1H), 7.65 (d, *J* = 8.5 Hz, 2H), 7.61 (m, 3H), 7.46 (d, *J* = 8.5 Hz, 2H), 7.29 (s, 1H) ppm.

¹³C NMR (75 MHz, DMSO-*d*₆): 181.0, 174.8, 138.7, 133.9, 133.5, 132.4, 132.0, 131.8, 131.0, 128.3, 127.9, 127.0, 126.9, 126.3, 125.8, 125.1, 124.3, 121.9 ppm. MS: 471 [M-H]⁻.

EXAMPLE 4: Analogues of Sodium 2-(4-aminobenzyl)-1,3-dioxo-2,3-dihydro-1H-benzo[de]isoquinoline-5-sulfonate (Pitstop 1)

The lead compound that allowed development of Pitstop 1 is 8-(3-carboxy-7-oxo-7H-benzo[de]benzo[4,5]imidazo[2,1-*a*]isoquinolin-4-yl)naphthalene-1,4,5-tricarboxylic acid (L1) (see Table 1). Reductive retrosynthetic fragmentation of L1 revealed the compound is essentially a substituted 1,8-naphthalimide derivative, comprised of a 1,8-naphthalic anhydride and a 1,2-phenylenediamine moiety attached to a naphthalimide core. The naphthalimide core was utilised as a minimal pharmaoacophoric entity. The chemistry for preparation of the 1,8-naphthalimide scaffold is well-established and so allows for the rapid generation of analogues, and hence the development of a SAR profile. Scaffold simplification of L1 for the design of inhibitors of clathrin-TD is shown in Scheme 4 and identified inhibitors of clathrin-TD are set out in Tables 8-10.

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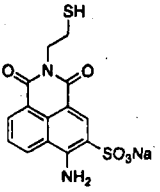
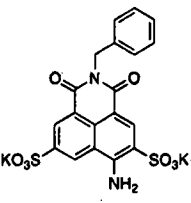
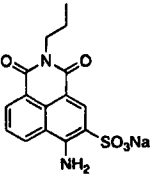
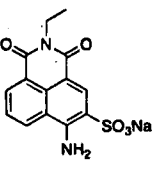
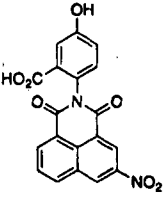
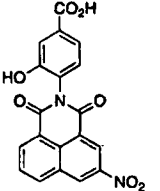


Scheme 4: Scaffold simplification of **L1** reveals a naphthalimide core, and provides a pharmacophoric entity for the rational design of clathrin-TD inhibitors.

Table 8: Compounds identified by an ELISA-based screening strategy as inhibitors of the activity of clathrin. For the purposes of identifying clathrin-selective inhibitors, the dynamin I IC_{50} values are also provided.

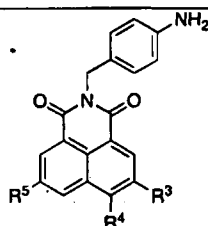
Compound	Structure	Clathrin IC_{50}	Dynamin I IC_{50}	Selectivity*
2 (Pitstop 1)		~10	-	> 30
3		~50	224 ± 42	4.5
4		~10	37 ± 10	3.7

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5		~30	17 ± 3	0.6
6		<10	23 ± 4	2.3
7		~120	193 ± 33	1.6
8		~100	$> 300 \mu\text{M}$	> 3
9		~80	15 ± 4	0.2
10		~100	28 ± 5	0.3

Selectivity was calculated using $[\text{Clathrin IC}_{50}]/[\text{Dynamin I IC}_{50}]$. A higher selectivity value indicates a greater selectivity for clathrin, whilst a selectivity value <1 indicates an inhibitor that is more selective for dynamin I.

Table 9: Inhibition of clathrin and dynamin I GTPase by anhydride modified analogues 2, 11-23.



Compound ID	R ₃	R ₄	R ₅	Clathrin (μM)	IC ₅₀	Dynamin (μM)	IC ₅₀
2	SO ₃ Na	H	H	18	-	-	-
11	H	H	H	-	-	-	-
12	H	SO ₃ K	H	-	-	-	-
13	SO ₃ K	NH ₂	H	~100		123 ± 48	
14	H	Br	H	-		106 ± 12	
15	H	NH ₂	H	-		82	
16	H	NO ₂	H	-		157 ± 35	
17	H	Cl	H	-		125 ± 25	
18	NO ₂	H	H	-		129 ± 53	
19	OH	H	H	-		13 ± 6	
20	NO ₂	H	NO ₂	-		116 ± 60	
21	Br	H	H	-		141 ± 47	
22	NH ₂	H	H	-		22 ± 4	
23	OCH ₃	H	H	-		142 ± 38	

(-) Not Active – No inhibitory activity observed at 100 μM; (~100) ~50 % inhibition at 100 μM.

Table 10: Inhibition of Clathrin-TD and Dynamin I GTPase by compounds in 24-36.

Compound	n	R ₇	R ₈	R ₉	Clathrin IC ₅₀ (μM)	Dynamin IC ₅₀ (μM)
24	0	H	H	NH ₂	-	> 300
25	1	H	H	H	6.9 ± 1.6	-
26	1	H	H	COOH	~100	185 ± 39
27	1	NH ₂	H	H	10 ± 2	108 ± 52
28	1	H	H	Cl	10 ± 1	111
29	1	H	H	OCH ₃	16 ± 3	-
30	1	H	H	Br	15 ± 4	-
31	1	H	H	N(CH ₃) ₂	~100	-
32	1	H	H	NO ₂	-	49 ± 22
33	1	H	NH ₂	H	~100	264
34	1	H	H	OH	22 ± 1	-
35	1	H	H	CH ₃	15 ± 4	-
36	1	H	H	F	12 ± 2	> 300

Compound	n	R ₇	R ₈	R ₉	Clathrin IC ₅₀ (μM)	Dynamin IC ₅₀ (μM)
24	0	H	H	NH ₂	-	> 300
25	1	H	H	H	6.9 ± 1.6	-
26	1	H	H	COOH	~100	185 ± 39
27	1	NH ₂	H	H	10 ± 2	108 ± 52
28	1	H	H	Cl	10 ± 1	111
29	1	H	H	OCH ₃	16 ± 3	-
30	1	H	H	Br	15 ± 4	-

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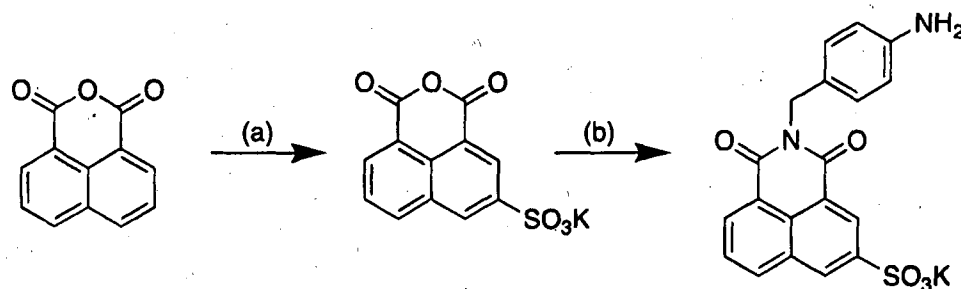
31	1	H	H	N(CH ₃) ₂	~100	-
32	1	H	H	NO ₂	-	49 ± 22
33	1	H	NH ₂	H	~100	264
34	1	H	H	OH	22 ± 1	-
35	1	H	H	CH ₃	15 ± 4	-
36	1	H	H	F	12 ± 2	> 300

(-) Not Active – No inhibition observed at 100 μM (clathrin) or 300 μM (dynamain);

(~100) Approximately 50 % inhibition at a drug concentration of 100 μM.

1. Synthesis of compounds

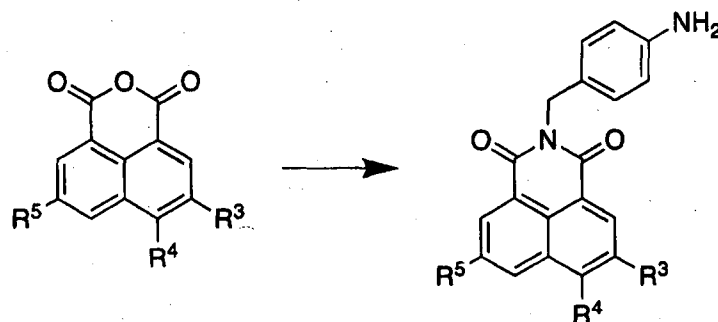
5



Scheme 5: Synthesis of 2 from 1,8-naphthalic anhydride. Reagents and Conditions:

(a) (i) Fuming sulfuric acid (30 % SO₃), 90 °C, 90 min; (ii) Saturated KCl, 4 °C, 18 hr;

10 (b) 4-aminobenzylamine (3 equiv.), ethanol, 100 °C, 18 hr.



Scheme 6: Generic reaction scheme for the synthesis of *N*-4-benzylamino

15 substituted naphthylimides. Reagents and conditions: appropriately substituted naphthylimide, 4-aminobenzylamine (3 equivalents), TEA (3 drops), Ethanol, 100 °C, 18 hr.

1.1 *3-Sulfo-N-(4-aminobenzyl)-1,8-naphthalimide, potassium salt (2)*

To a stirring suspension of 3-sulfo-1,8-naphthalic anhydride (potassium salt) (0.302 g, 0.95 mmol) in ethanol (15 mL), was added TEA (10 drops) and
5 4-aminobenzylamine (350 μ L, 3.09 mmol). An excess of amine was used to minimise formation of the di-imide. The resulting off-white suspension was stirred at 100 °C for 18 hours, then cooled to room temperature with stirring. The cream product was collected by filtration, washed sequentially with ethanol and ether, and dried under vacuum. A cream solid was obtained.

10 Yield 0.401 g (85 %); MP > 250 °C; $^1\text{H-NMR}$ (DMSO- d_6) δ 4.97 (2H, br s, NH_2), 5.07 (2H, s), 6.46 (2H, d, $J = 8.4$ Hz), 7.07 (2H, d, $J = 8.4$ Hz), 7.87 (1H, dd, $J = 7.5, 8.1$ Hz), 8.49 (1H, dd, $J = 0.9, 7.5$ Hz), 8.55 (1H, dd, $J = 0.9, 8.1$ Hz), 8.65 (1H, d, $J = 1.5$ Hz), 8.68 (1H, d, $J = 1.5$ Hz); $^{13}\text{C-NMR}$ (100 MHz) (DMSO- d_6) δ 42.9, 114.0 (2C), 122.3 (2C), 124.7, 127.4, 128.1, 128.9, 129.3 (2C), 130.4, 131.3, 131.6, 135.4,
15 147.0, 148.0, 163.6, 163.7 ppm; MS (ESI-) m/z : 381 (M – K) (100 %); HRMS (ESI-) m/z calculated for $\text{C}_{19}\text{H}_{13}\text{KN}_2\text{O}_5\text{S}$ (M – K) 381.0551

1.2 *3-Sulfo-N-(5-carboxy-2-hydroxyphenyl)-1,8-naphthalimide, sodium salt (3)*

20 Synthesised using the same general procedure as for analogue 2 above, commencing with 3-sulfo-1,8-naphthalic anhydride (sodium salt) and 3-amino-4-hydroxybenzoic acid. Product was isolated as the triethylamine salt. A pale brown solid was obtained.

Yield 0.142 g (38 %); MP > 250 °C; $^1\text{H-NMR}$ (DMSO- d_6) δ 0.99 (9H, Et_3N , t, $J = 7.2$ Hz), 2.60 (6H, Et_3N , q, $J = 7.2$ Hz), 7.00 (1H, d, $J = 8.4$ Hz), 7.82 (1H, d, $J = 2.1$ Hz), 7.86 (1H, dd, $J = 2.1, 8.4$ Hz), 7.90 (1H, dd, $J = 7.2, 8.4$ Hz), 8.48 (1H, dd, $J = 0.9, 7.2$ Hz), 8.60 (1H, dd, $J = 0.9, 8.4$ Hz), 8.65 (1H, d, $J = 1.5$ Hz), 8.70 (1H, d, $J = 1.5$ Hz); $^{13}\text{C-NMR}$ (100 MHz) (DMSO- d_6) δ 10.9 (3C, Et_3N), 45.7 (3C, Et_3N), 116.3, 122.6, 122.9 (2C), 124.4, 127.8, 127.9, 128.6, 130.1, 131.0, 131.3 (2C), 132.3, 135.2,
30 147.1, 157.1, 163.4, 163.6, 167.8 ppm; MS (ESI-) m/z : 412 (M – Na) (100 %); HRMS (ESI-) m/z calculated for $\text{C}_{19}\text{H}_{10}\text{NNaO}_6\text{S}$ (M – Na) 412.0133.

1.3 *4-Amino-3-sulfo-N-benzyl-1,8-naphthalimide, potassium salt (4)*

Synthesised using the same general procedure as for analogue 2, commencing with 4-amino-3-sulfo-1,8-naphthalic anhydride (potassium salt) and benzylamine. A yellow solid was obtained.

Yield 0.148 g (60 %); MP > 250 °C; ¹H-NMR (DMSO-*d*₆) δ 5.31 (2H, s), 7.24 (5H, m), 7.69 (1H, dd, *J* = 7.2, 8.4 Hz), 7.87 (2H, br s), 8.43 (1H, d, *J* = 7.2 Hz), 8.63 (1H, s), 8.71 (1H, d, *J* = 8.4 Hz); ¹³C-NMR (DMSO-*d*₆) δ 42.7, 106.5, 120.8, 121.9, 124.8, 125.2, 127.0, 127.6 (2C), 128.5 (2C), 129.8, 130.2, 131.5, 133.1, 138.1, 148.6, 163.2, 163.9 ppm; MS (ESI-) *m/z*: 381 (M – K) (100 %); HRMS (ESI-) *m/z* calculated for C₁₉H₁₃KN₂O₅S (M – K) 381.0551.

1.4 *4-Amino-3-sulfo-N-(2-mercaptoethyl)-1,8-naphthalimide, potassium salt (5)*

Synthesised using the same general procedure as for analogue 2, commencing with 4-amino-3-sulfo-1,8-naphthalic anhydride (potassium salt) and cysteamine. A yellow solid was obtained.

Yield 0.245 g (82 %); MP > 250 °C; ¹H-NMR (DMSO-*d*₆) δ 2.96 (2H, m), 4.31 (2H, m), 7.69 (1H, dd, *J* = 7.2, 8.4 Hz), 7.84 (2H, br s), 8.43 (1H, d, *J* = 7.2 Hz), 8.61 (1H, s), 8.70 (1H, d, *J* = 8.4 Hz); ¹³C-NMR (DMSO-*d*₆) δ 35.2, 38.2, 106.5, 120.8, 121.8, 124.8, 125.1, 129.7, 130.1, 131.4, 133.0, 148.5, 163.0, 163.9 ppm; MS (ESI-) *m/z*: 351 (M – K); HRMS (ESI-) *m/z* calculated for C₁₄H₁₁KN₂O₅S₂ (M – K) 351.0115

1.5 *4-Amino-3,6-disulfo-N-benzyl-1,8-naphthalimide, dipotassium salt (6)*

25

To a stirred suspension of 4-amino-3,6-sulfo-1,8-naphthalic anhydride, potassium salt (0.6 mmol), in 1 M Li⁺/H⁺ acetate pH 5 buffer (5 mL), was added benzylamine (3.3 mmol). The reaction mixture was stirred at 120 °C for 18 hours, then diluted to 20 mL with hot water. Addition of KCl (~0.5 g), followed by cooling to 4 °C, resulted in precipitation of the product. The resulting suspension was then stored at 4 °C for 24 hours. The solid product was collected by filtration, washed sequentially with water, ethanol and ether, and dried under vacuum. A yellow-grey solid was obtained.

Yield 0.117 g (49 %); MP > 250 °C; ¹H-NMR (DMSO-*d*₆) δ 5.22 (2H, s, H₁), 7.25 (5H, m), 7.99 (2H; br s, NH₂), 8.60 (1H, s), 8.64 (1H, d, *J* = 0.9 Hz), 8.94 (1H, d, *J* = 0.9 Hz); ¹³C-NMR (DMSO-*d*₆) δ 42.7, 106.4, 120.3, 121.6, 125.4, 126.6, 127.1, 127.5 (2C), 128.6 (2C), 129.4, 129.7, 133.3, 138.0, 144.9, 149.3, 163.1, 163.8 ppm; MS (ESI-) *m/z*: 499 (M – K), 461 (M – 2K + H), 230 (M – 2K) (100 %); HRMS (ESI-) *m/z* calculated for C₁₉H₁₂K₂N₂O₈S₂ (M – K) 498.9678.

1.6 4-Amino-3-sulfo-*N*-propyl-1,8-naphthalimide, potassium salt (7)

10 Synthesised using the same general procedure as for 6, commencing with 4-amino-3-sulfo-1,8-naphthalic anhydride (potassium salt) and *n*-propylamine. A yellow solid was obtained.

Yield 0.104 g (48 %); MP > 250 °C; ¹H-NMR (DMSO-*d*₆) δ 0.89 (3H, t, *J* = 7.5 Hz, H₃'), 1.61 (2H, sextet, *J* = 7.5 Hz, H₂'), 3.97 (2H, t, *J* = 7.5 Hz, H₁'), 7.68 (1H, dd, *J* = 7.2, 8.4 Hz, H₆), 7.80 (2H, br s, NH₂), 8.42 (1H, d, *J* = 7.2 Hz, H₇), 8.61 (1H, s, H₂), 8.68 (1H, d, *J* = 8.4 Hz, H₅); ¹³C-NMR (DMSO-*d*₆) δ 11.6 (C₃'), 21.1 (C₂'), 41.0 (C₁'), 106.8 (C₁), 120.8 (C_{4a}), 122.0 (C₈), 124.7 (C₆), 125.2 (C₃), 129.6 (C_{8a}), 129.9 (C₅), 131.2 (C₇), 132.8 (C₂), 148.3 (C₄), 163.1 (C₁₀), 163.9 (C₉) ppm; MS (ESI-) *m/z*: 333 (M – K) (100 %); HRMS (ESI-) *m/z* calculated for C₁₅H₁₃KN₂O₅S (M – K) 333.0551.

20

1.7 4-Amino-3-sulfo-*N*-ethyl-1,8-naphthalimide, potassium salt (8)

25 Synthesised using the same general procedure as for 6, commencing with 4-amino-3-sulfo-1,8-naphthalic anhydride (potassium salt) and ethylamine. A yellow solid was obtained.

Yield 0.187 g (85 %); MP > 250 °C; ¹H-NMR (DMSO-*d*₆) δ 1.17 (3H, t, *J* = 6.9 Hz, H₂'), 4.04 (2H, q, *J* = 6.9 Hz, H₁'), 7.68 (1H, dd, *J* = 7.2, 8.4 Hz, H₆), 7.80 (2H, br s, NH₂), 8.42 (1H, dd, *J* = 0.6, 7.2 Hz, H₇), 8.62 (1H, s, H₂), 8.68 (1H, dd, *J* = 0.6, 8.4 Hz, H₅); ¹³C-NMR (DMSO-*d*₆) δ 13.5 (C₂'), 34.5 (C₁'), 106.8 (C₁), 120.8 (C_{4a}), 122.1 (C₈), 124.7 (C₆), 125.1 (C₃), 129.6 (C_{8a}), 129.9 (C₅), 131.2 (C₇), 132.8 (C₂), 148.3 (C₄), 162.9 (C₁₀), 163.7 (C₉) ppm; MS (ESI-) *m/z*: 319 (M – K) (100 %); HRMS (ESI-) *m/z* calculated for C₁₄H₁₁KN₂O₅S (M – K) 319.0394.

30

1.8 3-Nitro-*N*-(2-carboxy-4-hydroxyphenyl)-1,8-naphthalimide (9)

Synthesised using the same general procedure as for 2, commencing with 3-nitro-1,8-naphthalic anhydride and 2-amino-5-hydroxybenzoic acid. Product was isolated as the triethylamine salt. A yellow-brown solid was obtained.

Yield 0.246 g (64 %); MP 224-226 °C; ¹H-NMR (DMSO-*d*₆) δ 1.00 (9H, Et₃N, t, *J* = 7.2 Hz), 2.59 (6H, Et₃N, q, *J* = 7.2 Hz), 7.32 (1H, d, *J* = 8.1 Hz), 7.47 (1H, dd, *J* = 1.5, 8.1 Hz), 7.55 (1H, d, *J* = 1.5 Hz), 8.09 (1H, dd, *J* = 7.2, 8.4 Hz), 8.70 (1H, dd, *J* = 0.9, 7.2 Hz), 8.83 (1H, dd, *J* = 0.9, 8.1 Hz), 8.98 (1H, d, *J* = 2.4 Hz), 9.53 (1H, d, *J* = 2.4 Hz); ¹³C-NMR (100 MHz) (DMSO-*d*₆) δ 10.3 (3C, Et₃N), 45.5 (3C, Et₃N), 117.4, 120.0, 123.1, 123.3, 124.7, 125.1, 129.5, 129.8, 130.1, 130.2, 131.2, 134.1, 136.7 (2C), 146.1, 153.3, 162.2, 162.7, 168.3 ppm; MS (ESI-) *m/z*: 377 (M – H) (100 %), 755 (2M – H); HRMS (ESI-) *m/z* calculated for C₁₉H₁₀N₂O₇ (M – H) 377.0415

1.9 3-Nitro-*N*-(4-carboxy-2-hydroxyphenyl)-1,8-naphthalimide (10)

Synthesised using the same general procedure as for analogue 2, commencing with 3-nitro-1,8-naphthalic anhydride and 4-amino-3-hydroxybenzoic acid. Product was isolated as the triethylamine salt. A brown solid was obtained.

Yield 0.254 g (64 %); MP 230-232 °C; ¹H-NMR (DMSO-*d*₆) δ 0.87 (9H, Et₃N, t, *J* = 7.2 Hz), 2.58 (6H, q, Et₃N, *J* = 7.2 Hz), 6.93 (1H, dd, *J* = 2.7, 8.4 Hz), 7.12 (1H, d, *J* = 8.4 Hz), 7.44 (1H, d, *J* = 2.7 Hz), 8.06 (1H, dd, *J* = 7.2, 8.4 Hz), 8.64 (1H, dd, *J* = 0.9, 7.2 Hz), 8.79 (1H, dd, *J* = 0.9, 8.4 Hz), 8.92 (1H, d, *J* = 2.4 Hz), 9.49 (1H, d, *J* = 2.4 Hz), 9.83 (1H, br s, OH); ¹³C-NMR (100 MHz) (DMSO-*d*₆) δ 9.2 (3C, Et₃N), 45.1 (3C, Et₃N), 117.5, 117.9, 122.8, 123.5, 125.0, 126.5, 129.4, 129.7, 130.1, 130.9, 131.2, 133.9, 134.9, 136.3, 146.0, 157.3, 162.9, 163.4, 167.5 ppm; MS (ESI-) *m/z*: 377 (M – H) (100 %), 755 (2M – H); HRMS (ESI-) *m/z* calculated for C₁₉H₁₀N₂O₇ (M – H) 377.0415

1.10 *N*-(4-Aminobenzyl)-1,8-naphthalimide (11)

Synthesised using the same general procedure as for analogue 2, commencing with 1,8-naphthalic anhydride and 4-aminobenzylamine. A yellow solid was obtained.

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Yield 0.359 g (95 %); MP > 250 °C; ¹H-NMR (DMSO-*d*₆) δ 4.96 (2H, br s), 5.06 (2H, s), 6.46 (2H, d, *J* = 8.4 Hz), 7.08 (2H, d, *J* = 8.4 Hz), 7.85 (2H, dd, *J* = 7.2, 8.4 Hz), 8.43 (2H, dd, *J* = 1.5, 8.4 Hz), 8.49 (2H, dd, *J* = 1.5, 7.2 Hz); ¹³C-NMR (DMSO-*d*₆) δ 42.7, 113.8 (2C), 122.1 (2C), 124.6, 127.4 (3C), 129.4 (2C), 131.0 (2C), 131.4, 134.5 (2C), 148.0, 163.5 (2C) ppm; MS (ESI+) *m/z*: 303 (M + H) (calc.); HRMS (ESI+) *m/z* calculated for C₁₉H₁₄N₂O₂ (M + H) 303.1128

1.11 4-Sulfo-*N*-(4-aminobenzyl)-1,8-naphthalimide, potassium salt (12)

10 Synthesised using the same general procedure as for analogue 2, commencing with 4-sulfo-1,8-naphthalic anhydride (potassium salt) and 4-aminobenzylamine.

Yield 0.314 g (94 %); MP > 250 °C; ¹H-NMR (DMSO-*d*₆) δ 4.95 (2H, br s), 5.06 (2H, s), 6.46 (2H, d, *J* = 8.4 Hz), 7.07 (2H, d, *J* = 8.4 Hz), 7.87 (1H, dd, *J* = 7.2, 8.4 Hz), 8.21 (1H, d, *J* = 7.5 Hz), 8.47 (1H, d, *J* = 7.5 Hz), 8.49 (1H, dd, *J* = 1.2, 7.2 Hz), 9.25 (1H, dd, *J* = 1.2, 8.4 Hz); ¹³C-NMR (DMSO-*d*₆) δ 42.7, 113.8 (2C), 122.2, 122.9, 124.5, 125.2, 127.0, 127.7, 128.2, 129.3 (2C), 130.5, 130.8, 134.4, 148.0, 150.1, 163.3, 163.7 ppm; MS (ESI-) *m/z*: 381 (M – K) (100 %); HRMS (ESI-) *m/z* calculated for C₁₉H₁₃KN₂O₅S (M – K) 381.0551.

20 1.12. 4-Amino-3-sulfo-*N*-(4-aminobenzyl)-1,8-naphthalimide, potassium salt (13)

To a stirring suspension of 4-amino-3-sulfo-1,8-naphthalic anhydride (potassium salt) (0.200 g, 0.60 mmol) in ethanol (7 mL), was added TEA (3 drops) and 4-aminobenzylamine (0.221 g, 1.81 mmol). A 3-fold excess of 4-aminobenzylamine was used to minimise formation of the di-imide. The resulting yellow-orange suspension was stirred at 100 °C for 18 hours, then cooled to room temperature with stirring. The yellow product was collected by filtration, washed sequentially with ethanol and ether, and dried under vacuum.

Yield 0.245 g (94 %); MP > 250 °C; ¹H-NMR (DMSO-*d*₆) δ 4.92 (2H, br s), 5.02 (2H, s), 6.45 (2H, d, *J* = 8.4 Hz), 7.04 (2H, d, *J* = 8.4 Hz), 7.68 (1H, dd, *J* = 7.2, 8.4 Hz), 7.82 (2H, br s), 8.42 (1H, dd, *J* = 1.2, 7.2 Hz), 8.63 (1H, s), 8.68 (1H, dd, *J* = 1.2, 8.4 Hz); ¹³C-NMR (DMSO-*d*₆) δ 42.3, 106.8, 113.8 (2C), 120.8, 122.1, 124.8, 125.1, 125.2, 129.2 (2C), 129.6, 130.0, 131.4, 133.0, 147.8, 148.4, 163.2, 163.9 ppm;

MS (ESI-) m/z : 396 (M – K) (100 %); HRMS (ESI-) m/z calculated for $C_{19}H_{14}KN_3O_5S$ (M – K) 396.0660.

1.13 *4-Bromo-N-(4-aminobenzyl)-1,8-naphthalimide (14)*

5 Synthesised using the same general procedure as for analogue 2, commencing with 4-bromo-1,8-naphthalic anhydride and 4-aminobenzylamine.

Yield 0.316 g (91 %); MP 238-240 °C; ^1H -NMR (DMSO- d_6) δ 4.97 (2H, br s), 5.04 (2H, s), 6.47 (2H, d, J = 8.4 Hz), 7.08 (2H, d, J = 8.4 Hz), 7.94 (1H, dd, J = 7.2, 8.4 Hz), 8.16 (1H, d, J = 7.8 Hz), 8.30 (1H, d, J = 7.8 Hz), 8.49 (1H, dd, J = 1.2, 8.4 Hz), 8.54 (1H, dd, J = 1.2, 8.4 Hz); ^{13}C -NMR (DMSO- d_6) δ 42.8, 113.7 (2C), 121.9, 122.7, 124.3, 128.2, 128.8, 129.3, 129.6 (2C), 129.8, 131.1, 131.4, 131.8, 132.7, 148.1, 162.8, 162.9 ppm; MS (ESI+) m/z : 381 (M + H) (calc.); HRMS (ESI+) m/z calculated for $C_{19}H_{13}BrN_2O_2$ (M + H) 381.0233.

15 1.14 *4-Amino-N-(4-aminobenzyl)-1,8-naphthalimide (15)*

Synthesised using the same general procedure as for analogue 2, commencing with 4-amino-1,8-naphthalic anhydride and 4-aminobenzylamine.

Yield 0.342 g (91 %); MP > 250 °C; ^1H -NMR (DMSO- d_6) δ 4.92 (2H, br s), 5.01 (2H, s), 6.45 (2H, d, J = 8.4 Hz), 6.83 (1H, d, J = 8.4 Hz), 7.04 (2H, d, J = 8.4 Hz), 7.42 (2H, br s, NH_2), 7.63 (1H, dd, J = 7.2, 8.4 Hz), 8.18 (1H, d, J = 8.4 Hz), 8.41 (1H, dd, J = 1.2, 7.2 Hz), 8.59 (1H, dd, J = 1.2, 8.4 Hz); ^{13}C -NMR (DMSO- d_6) δ 42.2, 107.8, 108.4, 113.7 (2C), 119.6, 122.0, 124.2, 125.3, 129.3 (2C), 129.5, 129.8, 131.3, 134.2, 147.8, 152.9, 163.1, 163.9 ppm; MS (ESI+) m/z : 318 (M + H) (calc.); HRMS (ESI+) m/z calculated for $C_{19}H_{15}N_3O_2$ (M + H) 318.1237

1.15 *4-Nitro-N-(4-aminobenzyl)-1,8-naphthalimide (16)*

30 Synthesised using the same general procedure as for analogue 2, commencing with 4-nitro-1,8-naphthalic anhydride and 4-aminobenzylamine. A 2-fold excess of 4-aminobenzylamine was used to minimise formation of the di-imide and suppress displacement of the 4-nitro group. A pink-brown solid was obtained.

Yield 0.313 g (87 %); MP 218-220 °C; ¹H-NMR (DMSO-*d*₆) δ 4.89 (2H, br s, NH₂), 5.01 (2H, s), 6.47 (2H, d, *J* = 8.4 Hz), 7.06 (2H, d, *J* = 8.4 Hz), 7.97 (1H, dd, *J* = 7.5, 8.7 Hz), 8.42 (1H, d, *J* = 7.8 Hz), 8.52 (1H, d, *J* = 7.8 Hz), 8.53 (1H, dd, *J* = 0.9, 7.5 Hz), 8.58 (1H, dd, *J* = 0.9, 8.7 Hz); ¹³C-NMR (DMSO-*d*₆) δ 43.1, 113.8 (2C), 122.7, 122.8, 124.0, 124.4, 126.5, 128.3, 128.9, 129.5 (2C), 129.9, 130.2, 132.0, 148.2, 149.2, 162.2, 163.0 ppm; MS (ESI+) *m/z*: 348 (M + H) (calc.); HRMS (ESI+) *m/z* calculated for C₁₉H₁₃N₃O₄ (M + H) 348.0979.

1.16 4-Chloro-*N*-(4-aminobenzyl)-1,8-naphthalimide (17)

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Synthesised using the same general procedure as for analogue 2, commencing with 4-chloro-1,8-naphthalic anhydride and 4-aminobenzylamine. A yellow solid was obtained.

Yield 0.325 g (88 %); MP 228-230 °C; ¹H-NMR (DMSO-*d*₆) δ 4.97 (2H, br s), 5.04 (2H, s), 6.47 (2H, d, *J* = 8.4 Hz), 7.08 (2H, d, *J* = 8.4 Hz), 7.93 (1H, dd, *J* = 7.5, 8.4 Hz), 7.96 (1H, d, *J* = 7.8 Hz), 8.38 (1H, d, *J* = 7.8 Hz), 8.52 (1H, dd, *J* = 1.2, 7.5 Hz), 8.53 (1H, dd, *J* = 1.2, 8.4 Hz); ¹³C-NMR (DMSO-*d*₆) δ 42.8, 113.7 (2C), 121.4, 122.7, 124.3, 127.8, 128.3, 128.5, 128.7, 129.5 (2C), 130.2, 131.1, 131.8, 137.7, 148.1, 162.7, 163.0 ppm; MS (ESI+) *m/z*: 337 (M + H) (calc.); HRMS (ESI+) *m/z* calculated for C₁₉H₁₃ClN₂O₂ (M + H) 337.0738

1.17 3-Nitro-*N*-(4-aminobenzyl)-1,8-naphthalimide (18)

Synthesised using the same general procedure as for analogue 2, commencing with 3-nitro-1,8-naphthalic anhydride and 4-aminobenzylamine. A light orange-brown solid was obtained.

Yield 0.296 g (83 %); MP > 250 °C; ¹H-NMR (DMSO-*d*₆) δ 4.99 (2H, br s), 5.05 (2H, s), 6.47 (2H, d, *J* = 8.4 Hz), 7.09 (2H, d, *J* = 8.4 Hz), 7.99 (1H, dd, *J* = 7.2, 8.4 Hz), 8.63 (1H, dd, *J* = 1.2, 7.2 Hz), 8.70 (1H, dd, *J* = 1.2, 8.4 Hz), 8.91 (1H, d, *J* = 2.4 Hz), 9.41 (1H, d, *J* = 2.4 Hz); ¹³C-NMR (DMSO-*d*₆) δ 43.0, 113.7 (2C), 122.5, 123.1, 123.9, 124.0, 129.3, 129.4, 129.5 (2C), 129.8, 130.9, 134.2, 136.4, 145.9, 148.2, 162.3, 162.7 ppm; MS (ESI+) *m/z*: 348 (M + H) (calc.); HRMS (ESI+) *m/z* calculated for C₁₉H₁₃N₃O₄ (M + H) 348.0979.

1.18 3-Hydroxy-*N*-(4-aminobenzyl)-1,8-naphthalimide (19)

Synthesised using the same general procedure as for analogue 2, commencing with 3-hydroxy-1,8-naphthalic anhydride and 4-aminobenzylamine. A yellow solid was obtained.

Yield 0.312 g (83 %); MP > 250 °C; ¹H-NMR (DMSO-*d*₆) δ 4.96 (2H, br s), 5.03 (2H, s), 6.46 (2H, d, *J* = 8.4 Hz), 7.06 (2H, d, *J* = 8.4 Hz), 7.62 (1H, d, *J* = 2.4 Hz), 7.71 (1H, dd, *J* = 7.2, 8.4 Hz), 8.01 (1H, d, *J* = 2.4 Hz), 8.20 (1H, dd, *J* = 1.2, 8.4 Hz), 8.23 (1H, dd, *J* = 1.2, 7.2 Hz), 10.45 (1H, br s, OH); ¹³C-NMR (DMSO-*d*₆) δ 42.7, 113.7 (2C), 116.0, 122.0, 122.1, 122.2, 123.5, 124.6, 127.5, 127.7, 129.3 (2C), 132.8, 133.5, 148.0, 156.4, 163.3, 163.7 ppm; MS (ESI+) *m/z*: 319 (M + H) (calc.); HRMS (ESI+) *m/z* calculated for C₁₉H₁₄N₂O₃ (M + H) 319.1077.

1.19 3,6-Dinitro-*N*-(4-aminobenzyl)-1,8-naphthalimide (20)

Synthesised using the same general procedure as for analogue 2, commencing with 3,6-dinitro-1,8-naphthalic anhydride and 4-aminobenzylamine. A dark brown solid was obtained.

Yield 0.244 g (72 %); MP 218-220 °C; ¹H-NMR (DMSO-*d*₆) δ 4.99 (2H, br s, NH₂), 5.07 (2H, s), 6.46 (2H, d, *J* = 8.4 Hz), 7.09 (2H, d, *J* = 8.4 Hz), 9.04 (2H, d, *J* = 2.1 Hz), 9.71 (2H, d, *J* = 2.1 Hz); ¹³C-NMR (100 MHz) (DMSO-*d*₆) δ 43.4, 113.8 (2C), 123.7, 124.5 (2C), 126.2 (2C), 129.6 (2C), 130.9, 131.6 (2C), 131.7, 147.2 (2C), 148.3, 161.8 (2C) ppm; MS (ESI+) *m/z*: 393 (M + H) (calc.); HRMS (ESI+) *m/z* calculated for C₁₉H₁₂N₄O₆ (M + H) 393.0830.

1.20 3-Bromo-*N*-(4-aminobenzyl)-1,8-naphthalimide (21)

Synthesised using the same general procedure as for analogue 2, commencing with 3-bromo-1,8-naphthalic anhydride and 4-aminobenzylamine. A pale yellow solid was obtained.

Yield 0.316 g (91 %); MP 220-222 °C; ¹H-NMR (DMSO-*d*₆) δ 4.98 (2H, br s), 5.03 (2H, s), 6.46 (2H, d, *J* = 8.4 Hz), 7.07 (2H, d, *J* = 8.4 Hz), 7.86 (1H, dd, *J* = 7.2, 8.4 Hz), 8.35 (1H, dd, *J* = 1.2, 8.4 Hz), 8.41 (1H, d, *J* = 1.8 Hz), 8.47 (1H, dd, *J* = 1.2,

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7.2 Hz), 8.70 (1H, d, $J = 1.8$ Hz); ^{13}C -NMR (100 MHz) (DMSO- d_6) δ 43.0, 113.9 (2C), 120.4, 122.5, 124.2, 124.5, 126.2, 128.8, 129.5 (2C), 131.5, 133.0 (2C), 133.8, 136.1, 148.2, 162.7, 163.3 ppm; MS (ESI+) m/z : 381 (M + H) (calc.); HRMS (ESI+) m/z calculated for $\text{C}_{19}\text{H}_{13}\text{BrN}_2\text{O}_2$ (M + H) 381.0233

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1.21 *3-Amino-N-(4-aminobenzyl)-1,8-naphthalimide (22)*

Synthesised using the same general procedure as for analogue 2, commencing with 3-amino-1,8-naphthalic anhydride and 4-aminobenzylamine. A yellow solid was obtained.

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Yield 0.320 g (86 %); MP 248-250 °C; ^1H -NMR (DMSO- d_6) δ 4.95 (2H, br s), 5.03 (2H, s), 5.97 (2H, br s, NH_2), 6.46 (2H, d, $J = 8.4$ Hz), 7.04 (2H, d, $J = 8.4$ Hz), 7.27 (1H, d, $J = 2.4$ Hz), 7.60 (1H, dd, $J = 7.2, 8.4$ Hz), 7.96 (1H, d, $J = 2.4$ Hz), 8.02 (1H, dd, $J = 1.2, 8.4$ Hz), 8.07 (1H, dd, $J = 1.2, 7.2$ Hz); ^{13}C -NMR (DMSO- d_6) δ 42.5, 112.0, 113.7 (2C), 120.7, 121.9, 122.0, 122.7, 124.7, 125.7, 127.1, 129.2 (2C), 131.7, 133.7, 147.9, 148.1, 163.7, 163.9 ppm; MS (ESI+) m/z : 318 (M + H) (calc.); HRMS (ESI+) m/z calculated for $\text{C}_{19}\text{H}_{15}\text{N}_3\text{O}_2$ (M + H) 318.1237

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1.22 *3-Methoxy-N-(4-aminobenzyl)-1,8-naphthalimide (23)*

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Synthesised using the same general procedure as for analogue 2, commencing with 3-methoxy-1,8-naphthalic anhydride and 4-aminobenzylamine. A pale yellow solid was obtained.

Yield 0.266 g (73 %); MP 238-240 °C; ^1H -NMR (DMSO- d_6) δ 3.97 (3H, s), 4.96 (2H, br s), 5.04 (2H, s), 6.46 (2H, d, $J = 8.4$ Hz), 7.07 (2H, dd, $J = 8.4$ Hz), 7.79 (1H, dd, $J = 7.2, 8.4$ Hz), 7.89 (1H, d, $J = 2.7$ Hz), 8.03 (1H, d, $J = 2.7$ Hz), 8.32 (2H, m); ^{13}C -NMR (DMSO- d_6) δ 42.7, 56.1, 113.5, 113.7 (2C), 121.9, 122.1, 122.9, 123.7, 124.5, 127.9, 128.4, 129.4 (2C), 133.2, 133.3, 148.0, 157.9, 163.2, 163.6 ppm; MS (ESI+) m/z : 333 (M + H) (calc.); HRMS (ESI+) m/z calculated for $\text{C}_{20}\text{H}_{16}\text{N}_2\text{O}_3$ (M + H) 333.1234.

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1.23 3-Sulfo-*N*-(4-aminophenyl)-1,8-naphthalimide, sodium salt (24)

Synthesised using the same general procedure as for analogue 2, commencing with 3-sulfo-1,8-naphthalic anhydride (sodium salt) and 1,4-phenylenediamine. An off-white solid was obtained.

Yield 0.281 g (86 %); MP > 250 °C; ¹H-NMR (DMSO-*d*₆) δ 5.25 (2H, br s), 6.63 (2H, d, *J* = 8.7 Hz), 6.94 (2H, d, *J* = 8.7 Hz), 7.88 (1H, dd, *J* = 7.2, 8.4 Hz), 8.46 (1H, dd, *J* = 0.9, 7.2 Hz), 8.57 (1H, dd, *J* = 0.9, 8.4 Hz), 8.63 (1H, d, *J* = 1.8 Hz), 8.66 (1H, d, *J* = 1.8 Hz); ¹³C-NMR (DMSO-*d*₆) δ 113.9 (2C), 122.8, 122.9, 124.0, 127.7 (2C), 128.6, 129.4 (2C), 130.0, 131.1, 131.2, 135.1, 147.1, 148.8, 164.0, 164.2 ppm; MS (ESI-) *m/z*: 367 (M – Na) (100 %); HRMS (ESI-) *m/z* calculated for C₁₈H₁₁N₂NaO₅S (M – Na) 367.0394.

1.24 3-Sulfo-*N*-(benzyl)-1,8-naphthalimide, potassium salt (25)

To a stirring suspension of 3-sulfo-1,8-naphthalic anhydride (potassium salt) (0.251 g, 0.79 mmol) in 1M Li⁺/H⁺ acetate buffer (pH 5, 5mL) was added benzylamine (173 μL, 1.58 mmol). The resulting cream-brown suspension was then stirred at 130 °C for 18 hours. The reaction mixture was then diluted to 15 mL with water, and KCl (~0.5 g) was added. After cooling to room temperature with stirring, the resulting cream-brown suspension was allowed to stand at 4 °C overnight, to increase product precipitation. The precipitated product was then collected by filtration, washed sequentially with cold water, ethanol and ether, and dried under vacuum. An off-white solid was obtained.

Yield 0.267 g (83 %); MP > 250 °C; ¹H-NMR (DMSO-*d*₆) δ 5.26 (2H, s), 7.28 (5H, m), 7.88 (1H, dd, *J* = 7.2, 8.4 Hz), 8.50 (1H, dd, *J* = 1.2, 7.2, Hz), 8.57 (1H, dd, *J* = 1.2, 8.4 Hz), 8.68 (1H, d, *J* = 1.8 Hz), 8.70 (1H, d, *J* = 1.8 Hz); ¹³C-NMR (DMSO-*d*₆) δ 43.1, 122.1 (2C), 127.2, 127.4, 127.6 (2C), 127.9, 128.6 (2C), 129.0, 130.4, 131.2, 131.4, 135.4, 137.5, 147.2, 163.6, 163.7 ppm; MS (ESI-) *m/z*: 366 (M – K) (100 %); HRMS (ESI-) *m/z* calculated for C₁₉H₁₂KNO₅S (M – K) 366.0442.

1.25 *3-Sulfo-N-(4-carboxybenzyl)-1,8-naphthalimide, potassium salt (26)*

Synthesised using the same general procedure as for analogue 25, commencing with 3-sulfo-1,8-naphthalic anhydride (potassium salt) and 4-(aminomethyl)benzoic acid (1.2 equivalents). A pale yellow solid was obtained.

Yield 0.261 g (91 %); MP > 250 °C; ¹H-NMR (DMSO-*d*₆) δ 5.32 (2H, s), 7.46 (2H, d, *J* = 8.4 Hz), 7.86 (2H, d, *J* = 8.4 Hz), 7.89 (1H, dd, *J* = 7.5, 8.1 Hz), 8.50 (1H, dd, *J* = 1.2, 7.5 Hz), 8.58 (1H, dd, *J* = 1.2, 8.1 Hz), 8.68 (1H, d, *J* = 1.8 Hz), 8.70 (1H, d, *J* = 1.8 Hz); ¹³C-NMR (DMSO-*d*₆) δ 43.1, 122.0, 122.1, 127.5 (3C), 127.8, 129.0, 129.6 (2C), 129.7, 130.4, 131.2, 131.4, 135.4, 142.5, 147.2, 163.6, 163.7, 167.3 ppm; MS (ESI-) *m/z*: 410 (M – K); HRMS (ESI-) *m/z* calculated for C₂₀H₁₂KNO₇S (M – K) 410.0340.

1.26 *3-Sulfo-N-(2-aminobenzyl)-1,8-naphthalimide, potassium salt (27)*

Synthesised using the same general procedure as for analogue 2, commencing with 3-sulfo-1,8-naphthalic anhydride (potassium salt) and 2-aminobenzylamine. A yellow solid was obtained.

Yield 0.292 g (86 %); MP > 250 °C; ¹H-NMR (DMSO-*d*₆) δ 5.02 (2H, s, H_{1'}), 5.15 (2H, br s), 6.43 (1H, m), 6.65 (1H, dd, *J* = 1.2, 8.1 Hz), 6.87 (1H, dd, *J* = 1.5, 7.5 Hz), 6.92 (1H, m), 7.89 (1H, dd, *J* = 7.5, 8.1 Hz), 8.50 (1H, dd, *J* = 1.2, 7.5 Hz), 8.56 (1H, dd, *J* = 1.2, 8.1 Hz), 8.68 (1H, d, *J* = 1.5 Hz), 8.69 (1H, d, *J* = 1.5 Hz); ¹³C-NMR (DMSO-*d*₆) δ 40.3, 115.3, 116.5, 120.0, 122.1, 122.2, 127.5, 127.6, 128.0, 128.1, 129.1, 130.6, 131.3, 131.8, 135.6, 146.3, 147.0, 163.9, 164.0 ppm; MS (ESI-) *m/z*: 381 (M – K) (100 %); HRMS (ESI-) *m/z* calculated for C₁₉H₁₃KN₂O₅S (M – K) 381.0551.

1.27 *3-Sulfo-N-(4-chlorobenzyl)-1,8-naphthalimide, potassium salt (28)*

To a stirring suspension of 3-sulfo-1,8-naphthalic anhydride (potassium salt) (0.251 g, 0.79 mmol) in 1M Li⁺/H⁺ acetate buffer (pH 5, 5mL) was added 4-chlorobenzylamine (173 μL, 1.58 mmol). The resulting cream-brown suspension was then stirred at 130 °C for 18 hours. The reaction mixture was then diluted to 15 mL with water, and KCl (~0.5 g) was added. After cooling to room temperature with

stirring, the resulting cream-brown suspension was allowed to stand at 4 °C overnight, to increase product precipitation. The precipitated product was then collected by filtration, washed sequentially with cold water, ethanol and ether, and dried under vacuum. An off-white solid was obtained.

- 5 Yield 0.057 g (83 %); MP > 250 °C; ¹H-NMR (DMSO-*d*₆) δ 5.24 (2H, s), 7.34 (2H, d, *J* = 8.7 Hz), 7.40 (2H, d, *J* = 8.7 Hz), 7.88 (1H, dd, *J* = 7.2, 8.4 Hz), 8.50 (1H, dd, *J* = 1.2, 7.2 Hz), 8.57 (1H, dd, *J* = 1.2, 8.4 Hz), 8.67 (1H, d, *J* = 1.5 Hz), 8.69 (1H, d, *J* = 1.5 Hz); ¹³C-NMR (DMSO-*d*₆) δ 42.6, 122.0, 122.1, 127.4, 127.8, 128.5 (2C), 129.0, 129.6 (2C), 130.3, 131.2, 131.4, 131.8, 135.4, 136.5, 147.3, 163.6, 163.7 ppm;
- 10 MS (ESI-) *m/z*: 400 (M – K) (100 %); HRMS (ESI-) *m/z* calculated for C₁₉H₁₁ClKNO₅S (M – K) 400.0052.

1.28 3-Sulfo-*N*-(4-methoxybenzyl)-1,8-naphthalimide, potassium salt (29)

- 15 Synthesised using the same general procedure as for analogue 28, commencing with 3-sulfo-1,8-naphthalic anhydride (potassium salt) and 4-methoxybenzylamine (1.05 equivalents). An off-white solid was obtained.

- Yield 0.286 g (83 %); MP > 250 °C; ¹H-NMR (DMSO-*d*₆) δ 3.69 (3H, s), 5.19 (2H, s), 6.84 (2H, d, *J* = 8.7 Hz), 7.32 (2H, d, *J* = 8.7 Hz), 7.87 (1H, dd, *J* = 7.2, 8.1 Hz), 8.49 (1H, dd, *J* = 1.2, 7.2 Hz), 8.55 (1H, dd, *J* = 1.2, 8.1 Hz), 8.66 (1H, d, *J* = 1.8 Hz), 8.69 (1H, d, *J* = 1.8 Hz); ¹³C-NMR (DMSO-*d*₆) δ 42.5, 55.2, 113.9 (2C), 122.0, 122.1, 127.3, 127.8, 128.9, 129.4 (2C), 129.5, 130.3, 131.2, 131.3, 135.3, 147.3, 158.5, 163.5, 163.6 ppm; MS (ESI-) *m/z*: 396 (M – K) (100 %); HRMS (ESI-) *m/z* calculated for C₂₀H₁₄KNO₆S (M – K) 396.0547.
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1.29 3-Sulfo-*N*-(4-bromobenzyl)-1,8-naphthalimide, potassium salt (30)

- Synthesised using the same general procedure as for analogue 28, commencing with 3-sulfo-1,8-naphthalic anhydride (potassium salt) and 4-bromobenzylamine (1.1 equivalents). An off-white solid was obtained.
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Yield 0.037 g (77 %); MP > 250 °C; ¹H-NMR (DMSO-*d*₆) δ 5.22 (2H, s), 7.33 (2H, d, *J* = 8.4 Hz), 7.48 (2H, d, *J* = 8.4 Hz), 7.88 (1H, dd, *J* = 7.2, 8.4 Hz), 8.50 (1H, dd, *J* = 1.2, 7.2 Hz), 8.57 (1H, dd, *J* = 1.2, 8.4 Hz), 8.67 (1H, d, *J* = 1.8 Hz), 8.69 (1H,

d, $J = 1.8$ Hz); ^{13}C -NMR (DMSO- d_6) δ 42.6, 120.3, 122.0, 122.1, 127.4, 127.8, 129.0, 129.9 (2C), 130.3, 131.2, 131.4 (3C), 135.4, 137.0, 147.2, 163.6, 163.7 ppm; MS (ESI-) m/z : 444 (M – K) (100 %); HRMS (ESI-) m/z calculated for $\text{C}_{19}\text{H}_{11}\text{BrKNO}_5\text{S}$ (M – K) 443.9547.

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1.30 *3-Sulfo-N-(4-(dimethylamino)benzyl)-1,8-naphthalimide, potassium salt (31)*

Synthesised using the same general procedure as for analogue 2, commencing with 3-sulfo-1,8-naphthalic anhydride (potassium salt) and 4-

10 (dimethylamino)benzylamine (dihydrochloride salt) (1.2 equivalents). An excess of TEA was added to react with liberated HCl. A pale yellow solid was obtained.

Yield 0.271 g (95 %); MP > 250 °C; ^1H -NMR (DMSO- d_6) δ 2.81 (6H, s), 5.14 (2H, s), 6.63 (2H, d, $J = 8.7$ Hz), 7.23 (2H, d, $J = 8.7$ Hz), 7.87 (1H, dd, $J = 7.5$, 8.1 Hz), 8.49 (1H, dd, $J = 1.2$, 7.5 Hz), 8.55 (1H, dd, $J = 1.2$, 8.1 Hz), 8.65 (1H, d, $J = 1.5$ Hz), 8.69 (1H, d, $J = 1.5$ Hz); ^{13}C -NMR (DMSO- d_6) δ 40.3 (2C), 42.6, 112.4 (2C), 122.1 (2C), 125.1, 127.3, 127.8, 128.9, 129.2 (2C₃), 130.2, 131.2, 131.3, 135.3, 147.2, 149.9, 163.4, 163.5 ppm; MS (ESI-) m/z : 409 (M – K) (100 %); HRMS (ESI-) m/z calculated for $\text{C}_{21}\text{H}_{17}\text{KN}_2\text{O}_5\text{S}$ (M – K) 409.0864.

20 1.31 *3-Sulfo-N-(4-nitrobenzyl)-1,8-naphthalimide, potassium salt (32)*

Synthesised using the same general procedure as for analogue 28, commencing with 3-sulfo-1,8-naphthalic anhydride (potassium salt) and 4-nitrobenzylamine (hydrochloride salt) (1.2 equivalents). A light brown solid was obtained.

25 Yield 0.250 g (88 %); MP > 250 °C; ^1H -NMR (DMSO- d_6) δ 5.37 (2H, s), 7.63 (2H, d, $J = 8.7$ Hz), 7.89 (1H, dd, $J = 7.2$, 8.4 Hz), 8.15 (2H, d, $J = 8.7$ Hz), 8.50 (1H, dd, $J = 1.2$, 7.2 Hz), 8.58 (1H, dd, $J = 1.2$, 8.4 Hz), 8.69 (2H, s); ^{13}C -NMR (DMSO- d_6) δ 43.0, 122.0 (2C), 123.7 (2C), 127.6, 127.9, 128.6 (2C), 129.0, 130.5, 131.2, 131.5, 135.5, 145.4, 146.8, 147.2, 163.6, 163.7 (C₉) ppm; MS (ESI-) m/z : 411 (M – K) (100 %); HRMS (ESI-) m/z calculated for $\text{C}_{19}\text{H}_{11}\text{KN}_2\text{O}_7\text{S}$ (M – K) 411.0292.

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1.32 3-Sulfo-*N*-(3-aminobenzyl)-1,8-naphthalimide, potassium salt (33)

Synthesised using the same general procedure as for analogue 2, commencing with 3-sulfo-1,8-naphthalic anhydride (potassium salt) and 3-aminobenzylamine. A pale yellow solid was obtained.

Yield 0.271 g (83 %); MP > 250 °C; ¹H-NMR (DMSO-*d*₆) δ 4.96 (2H, br s), 5.12 (2H, s), 6.39 (1H, ddd, *J* = 1.2, 2.1, 7.8 Hz), 6.46 (1H, d, *J* = 2.1 Hz), 6.49 (1H, dd, *J* = 1.2, 7.8 Hz), 6.92 (1H, t, *J* = 7.8 Hz), 7.88 (1H, dd, *J* = 7.2, 8.1 Hz), 8.50 (1H, dd, *J* = 1.2, 7.2 Hz), 8.56 (1H, dd, *J* = 1.2, 8.1 Hz), 8.67 (1H, d, *J* = 1.8 Hz), 8.70 (1H, d, *J* = 1.8 Hz); ¹³C-NMR (DMSO-*d*₆) δ 43.2, 112.4, 112.8, 115.0, 122.1 (2C), 127.4, 128.0, 129.0 (2C), 130.4, 131.2, 131.5, 135.4, 138.0, 147.1, 149.0, 163.5 (C₁₀), 163.6 (C₉) ppm; MS (ESI-) *m/z*: 381 (M – K) (100 %); HRMS (ESI-) *m/z* calculated for C₁₉H₁₃KN₂O₅S (M – K) 381.0551.

1.33 3-Sulfo-*N*-(4-hydroxybenzyl)-1,8-naphthalimide, potassium salt (34)

Synthesised using the same general procedure as for analogue 28, commencing with 3-sulfo-1,8-naphthalic anhydride (potassium salt) and 4-hydroxybenzylamine (1.2 equivalents). A cream-brown solid was obtained.

Yield 0.293 g (88 %); MP > 250 °C; ¹H-NMR (DMSO-*d*₆) δ 5.14 (2H, s), 6.67 (2H, d, *J* = 8.7 Hz), 7.20 (2H, d, *J* = 8.7 Hz), 7.87 (1H, dd, *J* = 7.2, 8.1 Hz), 8.49 (1H, dd, *J* = 1.2, 7.2 Hz), 8.55 (1H, dd, *J* = 1.2, 8.1 Hz), 8.65 (1H, d, *J* = 1.5 Hz), 8.69 (1H, d, *J* = 1.5 Hz), 9.27 (1H, br s); ¹³C-NMR (DMSO-*d*₆) δ 42.6, 115.2 (2C), 122.1 (2C), 127.3, 127.8 (2C), 128.9, 129.4 (2C), 130.3, 131.2, 131.3, 135.3, 147.2, 156.6, 163.5, 163.6 ppm; MS (ESI-) *m/z*: 382 (M – K) (100 %); HRMS (ESI-) *m/z* calculated for C₁₉H₁₂KNO₆S (M – K) 382.0391.

1.34 3-Sulfo-*N*-(4-methylbenzyl)-1,8-naphthalimide, potassium salt (35)

Synthesised using the same general procedure as for analogue 28, commencing with 3-sulfo-1,8-naphthalic anhydride (potassium salt) and 4-methylbenzylamine (0.6 equivalents). An off-white solid was obtained.

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Yield 0.365 g (100 %); MP > 250 °C; ¹H-NMR (DMSO-*d*₆) δ 2.23 (3H, s), 5.21 (2H, s), 7.09 (2H, d, *J* = 8.1 Hz), 7.25 (2H, d, *J* = 8.1 Hz), 7.88 (1H, dd, *J* = 7.2, 8.4 Hz), 8.49 (1H, dd, *J* = 1.2, 7.2 Hz), 8.56 (1H, dd, *J* = 1.2, 8.4 Hz), 8.67 (1H, d, *J* = 1.8 Hz), 8.69 (1H, d, *J* = 1.8 Hz); ¹³C-NMR (DMSO-*d*₆) δ 20.8, 42.9, 122.0, 122.1, 127.4, 127.7 (2C), 127.8, 128.9, 129.1 (2C), 130.3, 131.2, 131.4, 134.5, 135.4, 136.3, 147.2, 163.5, 163.6 ppm; MS (ESI-) *m/z*: 380 (M – K) (100 %); HRMS (ESI-) *m/z* calculated for C₂₀H₁₄KNO₅S (M – K) 380.0598.

1.35 3-Sulfo-*N*-(4-fluorobenzyl)-1,8-naphthalimide, potassium salt (36)

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Synthesised using the same general procedure as for analogue 28, commencing with 3-sulfo-1,8-naphthalic anhydride (potassium salt) and 4-fluorobenzylamine (0.1 equivalents). An off-white solid was obtained.

Yield 0.049 g (73 %); MP > 250 °C; ¹H-NMR (DMSO-*d*₆) δ 5.23 (2H, s), 7.10 (2H, t, *J* = 8.7 Hz), 7.42 (2H, dd, *J* = 5.7, 8.7 Hz), 7.88 (1H, dd, *J* = 7.2, 8.4 Hz), 8.50 (1H, dd, *J* = 1.2, 7.2 Hz), 8.57 (1H, dd, *J* = 1.2, 8.4 Hz), 8.67 (1H, d, *J* = 1.5 Hz), 8.69 (1H, d, *J* = 1.5 Hz, H₂); ¹³C-NMR (DMSO-*d*₆) δ 42.5 (C₁), 115.2 (²*J*_{CF} = 21.3 Hz, 2C₄), 122.0, 122.1, 127.4, 127.8, 129.0, 129.9 (³*J*_{CF} = 8.2 Hz, 2C), 130.3, 131.2, 131.4, 133.7 (⁴*J*_{CF} = 2.1 Hz), 135.4, 147.2, 161.4 (¹*J*_{CF} = 242.9 Hz), 163.5, 163.6 ppm; MS (ESI-) *m/z*: 384 (M – K) (100 %); HRMS (ESI-) *m/z* calculated for C₁₉H₁₁FKNO₅S (M – K) 384.0347.

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Although the invention has been described with reference to a number of exemplary embodiments, the skilled addressee will understand that numerous variations and modifications may be made without departing from the scope of the invention. Accordingly, the embodiments described above are not to be taken as being limiting.

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CLAIMS

1. A method for inhibiting clathrin, comprising contacting clathrin with an effective amount of a clathrin inhibitor for forming a complex with a binding site on the the terminal domain (TD) of clathrin for the inhibitor, the binding site of the clathrin TD being defined by at least one or more of amino acids Ile 52, Ile 62, Ile 80, Phe 91 and/or Ile 93 of SEQ ID No. 1, or a prodrug, or physiologically acceptable salt, of the inhibitor.
2. A method according to claim 1 wherein the binding of the inhibitor to the clathrin TD is stabilised by one or more hydrogen bonds between the inhibitor and the clathrin TD.
3. A method according to claim 1 or 2 wherein the binding of the inhibitor is provided by binding interactions of the inhibitor with at least some of amino acids Ile 52, Ile 62, Ile 80, Phe 91 and/or Ile 93 of SEQ. No. 1.
4. A method according to claim 3 wherein the binding of the inhibitor is provided by binding interactions with all of amino acids Ile 52, Ile 62, Ile 80, Phe 91, and Ile 93 of SEQ ID No.1.
5. A method according to claim 3 or 4 wherein the binding site is further defined by one or more of amino acids Ile 66, Arg 64, Leu 82 and/or Lys 96 of SEQ ID No. 1, and the binding of the inhibitor is provided by binding interactions of the inhibitor with the one or more further amino acids.
6. A method according to claim 4 wherein the inhibitor interacts with all of amino Ile 66, Arg 64, Leu 82 and Lys 96 of SEQ ID. No. 1.
7. A method according to claim 3 or 4 wherein the binding site is further defined by amino acid Val 50 of SEQ ID No. 1 and the binding of the inhibitor is provided by binding interaction of the inhibitor with that amino acid.
8. A method according to any one of claims 3 to 7 wherein respective of the binding interactions between the inhibitor with the clathrin TD comprise direct contact of the inhibitor with the clathrin TD.
9. A method according to any one of claims 1 to 6 wherein the binding of the inhibitor to the clathrin TD is stabilised by one or more hydrogen bonds between the inhibitor and amino acids selected from the group consisting of Ser 67, Gln 89, Val 50, Ile 62, and Arg 64 of SEQ ID. No. 1.
10. A method according to claim 9 wherein the binding of the inhibitor to the clathrin TD is stabilised by hydrogen bonds between the inhibitor and amino acids Ser 67, Gln 89, Val 50, Ile 62, and Arg 64 of SEQ ID. No. 1.

11. A method according to any one of claims 1 to 4 wherein the binding of the inhibitor to the clathrin TD is stabilised by one or more hydrogen bonds between the inhibitor and Arg 64 of SEQ ID No. 1.
12. A method according to any one of claims 1 to 11 comprising administering an effective amount of the inhibitor to an animal for inhibiting clathrin in the animal, or a prodrug, or physiologically acceptable salt, of the inhibitor.
13. A method according to claim 12 wherein the inhibitor is administered for the inhibition of mitosis or clathrin-mediated endocytosis (CME).
14. A method for prophylaxis or treatment of a disease or condition in the animal comprising administering an effective amount of a clathrin inhibitor for forming a complex with the terminal domain (TD) of clathrin, the inhibitor binding to a binding site of the terminal domain (TD) of clathrin wherein the binding site of the clathrin TD is defined by at least one or more of amino acids Ile 52, Ile 62, Ile 80, Phe 91 and/or Ile 93 of SEQ ID No. 1, or a prodrug or physiologically acceptable salt of the inhibitor.
15. A method according to claim 14 wherein the binding of the inhibitor to the clathrin TD is stabilised by one or more hydrogen bonds between the inhibitor and the clathrin TD.
16. A method according to claim 14 or 15 wherein the binding of the inhibitor is provided by binding interactions of the inhibitor with at least some of amino acids Ile 52, Ile 62, Ile 80, Phe 91 and/or Ile 93 of SEQ. No. 1.
17. A method according to claim 16 wherein the binding of the inhibitor is provided by binding interactions with all of amino acids Ile 52, Ile 62, Ile 80, Phe 91, and Ile 93 of SEQ ID No.1.
18. A method according to any one of claims 14 to 17 wherein the binding site is further defined by one or more of amino acids Ile 66, Arg 64, Leu 82 and/or Lys 96 of SEQ ID No. 1, and the binding of the inhibitor is provided by binding interactions of the inhibitor with the one or more further amino acids.
19. A method according to claim 18 wherein the binding of the inhibitor is provided by binding interactions with all of amino Ile 66, Arg 64, Leu 82 and Lys 96 of SEQ ID. No. 1.
20. A method according to any one of claims 14 to 17 wherein the binding site is further defined by amino acid Val 50 of SEQ ID No. 1 and the binding of the inhibitor is provided by binding interaction of the inhibitor with that amino acid.

21. A method according to any one of claims 16 to 20 wherein respective of the binding interactions between the inhibitor with the clathrin TD are direct contact of the inhibitor with the clathrin TD.
22. A method according to any one of claims 14 to 18 wherein the binding of the inhibitor to the clathrin TD is stabilised by one or more hydrogen bonds between the inhibitor and amino acids selected from the group consisting of Ser 67, Gln 89, Val 50, Ile 62, and Arg 64 of SEQ ID. No. 1.
23. A method according to claim 22 wherein the binding of the inhibitor to the clathrin TD is stabilised by hydrogen bonds between the inhibitor and amino acids Ser 67, Gln 89, Val 50, Ile 62, and Arg 64 of SEQ ID. No. 1.
24. A method according to claim 20 wherein the binding of the inhibitor to the clathrin TD is stabilised by one or more hydrogen bonds between the inhibitor and Arg 64 of SEQ ID No. 1.
25. A method according to any one of claims 14 to 24 wherein the disease or condition is selected from the group consisting of epilepsy, Alzheimer's disease, Lewy body disease, Lewy body dementia, Parkinson's disease, β -amyloid associated diseases, Dent's disease, pathogenic infections involving clathrin mediated endocytosis (CME) for infection of cells, cancer, cell proliferative disorders, neuropathic pain, multifocal leukoencephalopathy, psychotic disorders, psychosis, bipolar disorders, schizophrenia, aberrant up-regulated neuronal excitation, seizures, migraine, mucopolipidosis, and cell trafficking, neurodegenerative, neuropsychiatric and neurological disease and conditions mediated by, or associated with, synaptic signal transmission or CME.
26. A clathrin inhibitor for use in inhibiting clathrin in a mammal, wherein the inhibitor binding to a binding site of the terminal domain (TD) of clathrin forms a complex with the clathrin TD, the binding site of the clathrin TD being defined by at least one or more of amino acids Ile 52, Ile 62, Ile 80, Phe 91 and/or Ile 93 of SEQ ID No. 1.
27. A clathrin inhibitor wherein the inhibitor binding to a binding site of the terminal domain (TD) of clathrin forms a complex with the clathrin TD, the binding site of the clathrin TD being defined by at least one or more of amino acids Ile 52, Ile 62, Ile 80, Phe 91 and/or Ile 93 of SEQ ID No. 1.
28. An inhibitor according to claim 26 or 27 wherein the binding of the inhibitor is provided by binding interactions of the inhibitor with at least some of amino acids Ile 52, Ile 62, Ile 80, Phe 91 and/or Ile 93 of SEQ. No. 1.
29. An inhibitor according to claim 28 wherein the binding of the inhibitor is provided by binding interactions of the inhibitor with all of amino acids Ile 52, Ile 62, Ile 80, Phe 91, and Ile 93 of SEQ ID No.1.

30. An inhibitor according to claim 28 or 29 wherein the binding site is further defined by one or more of amino acids Ile 66, Arg 64, Leu 82 and/or Lys 96 of SEQ ID No. 1, and the binding of the inhibitor is provided by binding interaction of the inhibitor with the one or more further amino acids.

31. An inhibitor according to claim 30 wherein the binding of the inhibitor is provided by binding interactions of the inhibitor with all of amino Ile 66, Arg 64, Leu 82 and Lys 96 of SEQ ID. No. 1.

32. An inhibitor according to claim 28 or 29 wherein the binding site is further defined by amino acid Val 50 of SEQ ID No. 1 and the binding of the inhibitor is provided by a binding interaction of the inhibitor with that amino acid.

33. An inhibitor according to any one of claims 28 to 32 wherein respective of the binding interactions between the inhibitor with the clathrin TD comprise direct contact of the inhibitor with the clathrin TD.

34. An inhibitor according to any one of claims 28 to 31 wherein the complex is stabilised by one or more hydrogen bonds between the inhibitor and amino acids selected from the group consisting of Ser 67, Gln 89, Val 50, Ile 62, and Arg 64 of SEQ ID. No. 1.

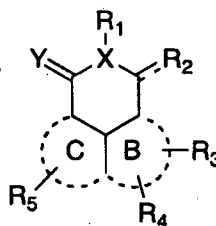
35. An inhibitor according to claim 34 wherein the binding of the inhibitor to the clathrin TD is stabilised by hydrogen bonds between the inhibitor and amino acids Ser 67, Gln 89, Val 50, Ile 62, and Arg 64 of SEQ ID. No. 1.

36. An inhibitor according to claim 32 wherein the binding of the inhibitor to the clathrin TD is stabilised by one or more hydrogen bonds between the inhibitor and Arg 64 of SEQ ID No. 1.

37. A complex of a clathrin inhibitor and the terminal domain (TD) of clathrin or a fragment of the clathrin TD, the inhibitor being as defined in any one of claims 26 to 36 and binding to the binding site of the clathrin TD.

38. A method of screening a putative clathrin inhibitor, comprising:
fitting a model of a putative inhibitor of clathrin to a representation of a binding site for the inhibitor on the terminal domain (TD) of clathrin, the binding site being defined by at least one or more of amino acids Ile 52, Ile 62, Ile 80, Phe 91 and/or Ile 93 of SEQ ID No. 1;
modelling interaction of the inhibitor with the binding site to evaluate whether the inhibitor forms a complex with the binding site of the clathrin TD; and
evaluating whether the inhibitor may inhibit clathrin on the basis of the modelling, the formation of the complex being indicative of capacity of the inhibitor to inhibit clathrin.

39. A method according to claim 38 wherein the binding site for the inhibitor is defined by all of amino acids Ile 52, Ile 62, Ile 80, Phe 91 and Ile 93 of SEQ ID No. 1;
40. A method according to claim 38 or 39 wherein the binding site is further defined by one or more of amino acids Ile 66, Arg 64, Leu 82 and/or Lys 96 of SEQ ID No. 1.
41. A method according to claim 38 or 39 wherein the binding site for the inhibitor is further defined by amino acid Val 50 of SEQ ID No. 1
42. A method according to any one of claims 38 to 41 wherein the modelling comprises evaluating whether the complex is formed by binding interactions between the inhibitor and at least some of the amino acids.
43. A method according to claim 42 wherein respective of the binding interactions comprise direct contact of the inhibitor with the amino acids.
44. A method according to any one of claims 38 to 43 wherein the method further comprises determining whether one or more hydrogen bonds are formed between the inhibitor and the clathrin TD to stabilise the complex, the formation of the hydrogen bond(s) being indicative of the inhibitor to inhibit clathrin.
45. A method for providing a clathrin inhibitor, comprising:
designing the inhibitor to bind to a binding site for the inhibitor on the terminal domain (TD) of clathrin, the binding site of the clathrin TD being defined by at least one or more of amino acids Ile 52, Ile 62, Ile 80, Phe 91 and/or Ile 93 of SEQ ID No. 1;
modelling interaction of the designed inhibitor with the binding site; and
evaluating whether the designed inhibitor forms a complex with the binding site of the clathrin TD on the basis of the modelling, the formation of the complex being indicative of capacity of the inhibitor to inhibit clathrin.
46. A compound of Formula I, or a prodrug or pharmaceutically acceptable salt thereof, wherein:



Formula I

and wherein:

R₁ is alkyl, alkenyl, alkylaryl, or aryl or a ring having from 5 to 7 ring atoms including from 0 to 3 heteroatoms selected from O, N or S, wherein the alkyl, alkenyl, alkylaryl or aryl are optionally substituted; and

R₂ is O, S or NH; or

R₁, X, and R₂ form a ring A, the ring having from 5 to 7 ring atoms including from 0 to 3 heteroatoms selected from O, N or S and being optionally substituted; and

X is N or a carbon atom;

Y is O, S, or NH;

ring B and ring C each independently have 5 to 7 ring atoms including from 0 to 3 heteroatoms selected from O, N or S; and

R₃, R₄ and R₅ are each independently H or an optional substituent.

47. A compound according to claim 46, wherein:

R₁ is alkyl, alkenyl, alkylaryl, or aryl, wherein the alkyl, alkenyl, alkylaryl or aryl is optionally substituted.

48. A compound according to claim 46 or 47, wherein:

R₂ is O; and

rings B and C each have 6 ring atoms.

49. A compound according to any one of claims 46 to 48 wherein R₁ is alkyl.

50. A compound according to claim 49 wherein R₁ is lower alkyl.

51. A compound according to any one of claims 46 to 48 wherein R₁ is alkylaryl.

52. A compound according to claim 51 wherein the lower alkylaryl is an alkylphenyl.

53. A compound according to claim 52 wherein the alkylphenyl is a C₁-C₂ alkylphenyl.

54. A compound according to claim 53 wherein the phenyl ring of the alkylphenyl is selected from the group consisting of 2-substituted phenyl, 3-substituted phenyl and 4-substituted phenyl groups.

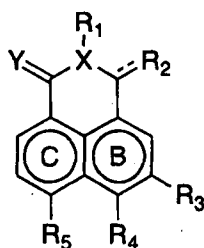
55. A compound according to any one of claims 46 to 48 wherein R₁ is aryl.

56. A compound according to claim 55 wherein R₁ is phenyl.

57. A compound according to claim 56 wherein the phenyl is selected from the group consisting of 2-substituted phenyl, 2,4-substituted phenyl, and 2,5-substituted phenyl groups.

58. A compound according to any one of claims 46 to 57 wherein R₁ is substituted with one or more substituents selected from the group consisting of hydroxyl, sulfhydryl, amino, nitro, halo, cyano, lower alkoxy, CO₂H, SO₃H, PO₄H, phenyl, and aryl.

59. A compound according to claim 58 wherein R_1 is substituted with one or more substituents selected from the group consisting of hydroxyl, sulfhydryl, amino, nitro, halo, cyano, CO_2H , SO_3H , PO_4H , phenyl, and aryl.
60. A compound according to claim 46 wherein R_1 , X and R_2 form ring A.
61. A compound according to claim 60, wherein R_2 is NR_6 and R_6 forms ring A with R_1 and X.
62. A compound according to claim 60 or 61 wherein ring A has 5 ring atoms.
63. A compound according to claim 60 or 61, wherein ring A is substituted with an aryl group, the aryl group being fused with ring A.
64. A compound according to claim 63 wherein the aryl group fused with ring A is phenyl.
65. A compound according to claim 64 wherein ring A and the phenyl group substituent form a benzimidazol group.
66. A compound according to claim 64 or 65 wherein the phenyl fused with ring A is a 4-substituted phenyl.
67. A compound according to any one of claims 64 to 66 wherein the phenyl fused with ring A has one or more substituents selected from the group consisting of hydroxyl, sulfhydryl, amino, nitro, halo, cyano, alkoxy, CO_2H , SO_3H , PO_4H .
68. A compound according to claim 67 wherein the phenyl fused with ring A has one or more substituents selected from the group consisting of hydroxyl, sulfhydryl, amino, nitro, halo, cyano, CO_2H , SO_3H , PO_4H .
69. A compound according to claim any one of claims 43 to 68, wherein rings B and C each have 6 ring atoms.
70. A compound according to any one of claims 46 to 69 being a compound of Formula IIa, wherein:



Formula IIa

and wherein:

R_3 and R_4 are independently selected from H, halo, cyano, hydroxyl, sulfhydryl, nitro, amino, alkoxy, alkyl, alkylaryl, aryl, NHR' , NR'_2 , CO_2H , SO_3H and PO_4H ;

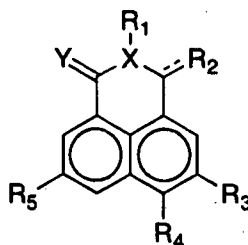
R' is independently H, alkyl, alkenyl, aryl, or alkylaryl, wherein the alkyl, alkenyl, aryl or alkylaryl is optionally substituted; and

R₅ is H, aryl, naphthyl, benzyl or tetralin, wherein the aryl, naphthyl benzyl and tetralin are optionally substituted.

71. A compound according to claim 70 wherein R₃ and R₄ are independently selected from H, halo, cyano, hydroxyl, sulfhydryl, nitro, amino, alkyl, alkylaryl, aryl, NHR', NR'₂, CO₂H, SO₃H and PO₄H.

72. A compound according to claim 70 or 71 wherein at least one of R₃ and R₄ is other than H.

73. A compound according to any one of claims 46 to 69 being a compound of Formula IIb, wherein:



Formula IIb

and wherein:

R₃, R₄ and R₅ are independently selected from H, halo, cyano, hydroxyl, sulfhydryl, nitro, amino, alkoxy, alkyl, alkylaryl, aryl, NHR', NR'₂, CO₂H, SO₃H and PO₄H; and

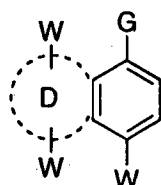
R' is independently H, alkyl, alkenyl, aryl, or alkylaryl, wherein the alkyl, alkenyl, aryl or alkylaryl is optionally substituted.

74. A compound according to claim 73 wherein R₃, R₄ and R₅ are independently selected from H, halo, cyano, hydroxyl, sulfhydryl, nitro, amino, alkyl, alkylaryl, aryl, NHR', NR'₂, CO₂H, SO₃H and PO₄H.

75. A compound according to claim 73 or 74 wherein at least one of R₃, R₄ and R₅ is other than H.

76. A compound according to any one of claims 70 to 72 wherein R₅ is a substituent of Formula III, as follows:

-98-



Formula III

wherein:

G is a bond with ring C;

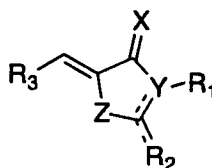
ring D has from 5 to 7 ring atoms optionally including from 0 to 3 heteroatoms selected from N, O and S; and

each W is independently H, halo, nitro, amino, hydroxyl, sulfhydryl, CO₂H, SO₃H, or PO₄H.

77. A compound according to claim 76 wherein at least one W substituent is other than H.

78. A compound according to claim 76 or 77 wherein each W is independently H, CO₂H, SO₃H, or PO₄H.

79. A compound of Formula V, or a prodrug or pharmaceutically acceptable salt thereof, wherein:



Formula V

and wherein:

X is O, S, or NH;

Y is O, N or a carbon atom;

Z is O, S or NH;

R₁ is H, CO₂H, SO₃H, PO₄H, alkyl, alkenyl, alkylcarboxy, or alkylaryl;

R₂ is O, S, NH, NHR', NHS(=O)₂R', S(=O)₂R', sulfonyl, NHC(=O)NH, NHC(=S)NH, or NHC(=O);

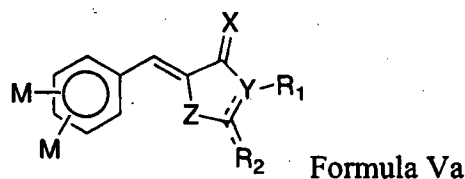
R' is H, alkyl, alkenyl, alkylaryl, aryl, the alkyl, alkenyl, alkylaryl and aryl group being optionally substituted; and

R₃ is aryl or a polycyclic group having at least 2 fused rings each independently having from 5 to 7 ring atoms, the aryl and the polycyclic group being optionally substituted.

80. A compound according to claim 79 wherein R₁ is H or a lower alkyl or lower alkenyl, wherein the lower alkyl or lower alkenyl group is optionally substituted.

81. A compound according to claim 80 wherein R₁ is a lower alkyl and is terminally substituted.

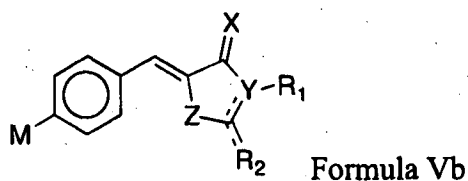
82. A compound according to claim 81 wherein the lower alkyl is a C₁-C₃ alkyl.
83. A compound according to any one of claims 79 to 82 wherein R₂ is S, NH, or HN-S(=O)₂-R', and R' is alkyl, alkenyl or aryl, wherein the alkyl, alkenyl or aryl is optionally substituted.
84. A compound according to claim 83 wherein R₂ is HN-S(=O)₂-R' wherein R' is aryl, and preferably, phenyl or naphthyl.
85. A compound according to any one of claims 79 to 84 wherein R₂ is OR' and R' is a lower alkyl or lower alkenyl, the lower alkyl or alkenyl being optionally terminally substituted with a substituent selected from the group consisting of amino, nitro, halo, cyano, hydroxy, sulfhydryl.
86. A compound according to any one of claims 79 to 85 wherein R₃ is phenyl or an aryl group with 2 fused rings each independently having from 5 to 7 ring atoms including from 0 to 3 heteroatoms selected from O, N and S, the phenyl or aryl group being optionally substituted.
87. A compound according to any one of claims 79 to 86 being a compound of Formula Va as follows:



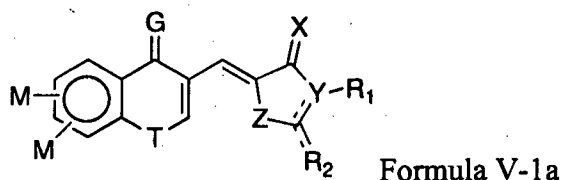
wherein each M is independently selected from H, halo, hydroxy, sulfhydryl, nitro, amino, cyano, alkoxy, CO₂H, SO₃H, PO₄H, OR', NHR', NR'₂, SO₂R', SO₂NH₂; and

R' is independently alkyl, alkenyl, aryl, or alkylaryl, wherein the alkyl, alkenyl, aryl or alkylaryl is optionally substituted

88. A compound according to claim 87 wherein each M is independently selected from H, halo, hydroxy, sulfhydryl, nitro, amino, cyano, alkoxy, CO₂H, SO₃H, PO₄H, OR', NHR', NR'₂, SO₂R', SO₂NH₂.
89. A compound according to claim 87 or 88 wherein at least one M substituent is other than hydrogen.
90. A compound according to any one of claims 87 to 89 being a compound of Formula Vb as follows:



91. A compound according to claim 90 wherein M is halo, nitro or $-OR'NH_2$, wherein R' is a lower alkyl or lower alkenyl.
92. A compound according to claim 91 wherein M is $-OR'NH_2$ and R' is a C_1 - C_4 alkyl and preferably, a C_3 alkyl.
93. A compound according to any one of claims 77 to 86 being a compound of Formula V-1a as follows:



wherein:

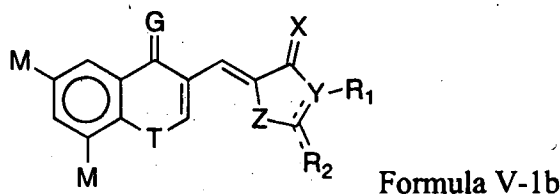
G is O or S;

T is O, S, N or a carbon atom; and

each M is independently selected from H, halo, hydroxy, sulfhydryl, nitro, amino, cyano, CO_2H , SO_3H , PO_4H , OR' , NHR' , NR'_2 , SO_2R' , SO_2NH_2 ; and

R' is independently alkyl, alkenyl, aryl, or alkylaryl, wherein the alkyl, alkenyl, aryl or alkylaryl is optionally substituted

94. A compound according to claim 93 wherein at least one M substituent is other than hydrogen.
95. A compound according to claim 93 or 94 wherein at least one M is halo, nitro or $-OR'NH_2$, wherein R' is a lower alkyl or lower alkenyl.
96. A compound according to claim 95 wherein R' of $-OR'NH_2$ is a C_1 - C_4 alkyl.
97. A compound according to claim 95 wherein R' of $-OR'NH_2$ is a C_3 alkyl.
98. A compound according to any one of claims 93 to 97 being a compound of Formula V-1b as follows:



99. A pharmaceutical composition comprising a compound of any one of claims 46 to 98, or a prodrug or pharmaceutically acceptable salt of the compound, together with a pharmaceutically acceptable carrier.
100. A method for inhibiting clathrin, comprising contacting clathrin with an effective amount of a compound as defined in any one of claims 46 to 98.
101. A method for inhibiting clathrin in an animal, comprising administering to the mammal an effective amount of a compound as defined in any one of claims 46 to 98 to the mammal, or a prodrug or pharmaceutically acceptable salt of the compound.
102. A compound as defined in any one of claims 46 to 98 for use in inhibiting clathrin in a mammal, or a prodrug, or physiologically acceptable salt, of the compound.
103. A compound according to any one of claims 46 to 98 for use in inhibiting clathrin in a mammal, or a prodrug, or physiologically acceptable salt, of the compound.
104. A compound according to any one of claims 46 to 98 for use in prophylaxis or treatment of a disease or condition selected from the group consisting of epilepsy, Alzheimer's disease, Lewy body disease, Lewy body dementia, Parkinson's disease, β -amyloid associated diseases, Dent's disease, pathogenic infections involving clathrin mediated endocytosis (CME) for infection of cells, cancer, cell proliferative disorders, neuropathic pain, multifocal leukoencephalopathy, psychotic disorders, psychosis, bipolar disorders, schizophrenia, aberrant up-regulated neuronal excitation, seizures, migraine, mucopolidosis, and cell trafficking, neurodegenerative, neuropsychiatric and neurological disease and conditions mediated by, or associated with, synaptic signal transmission or CME., or a prodrug, or physiologically acceptable salt, of the compound.

FIGURE 1

MAQILPIRFQEHLLQLNLGINPANIGFSTLTMESDKFICIREKV
GEQAQVVIIDMNDPSNPIRRPISADSAIMNPASKVIALKAGKTLQIFNIEMKSKMKAH
TMTDDVTFWKWISLNTVALVTDNAVYHWSMEGESQPVKMFDRHSSLAGCQIINYRTDA
KQKWLLLTGISAQQNRVVGAMQLYSVDRKVSQPIEGHAASFAQFKMEGNAEESTLFCF
AVRGQAGGKLHIIIEVGTPPTGNQPFPPKAVDVFFPPEAQNDFPVAMQISEKHDVVFLI
TKYGYIHLYDLETGTCTIYMNRISGETIFVTAPHEATAGIIGVNRKGQVLSVCVEEENI
IPYITNVLQNPDLALRMAVRNNLAGAEELFARKFNALFAQGNYSEAAKVAANAPKGIL
RTPDTIRRFQSVPAQPGQTSPLLQYFGILLDQGLNKYESLELCRPVLQQGRKQLLEK
WLKEDKLECSEELGDLVKSVDPTLALSYYLRANVPNKVIQCFAETGQVQKIVLYAKKV
GYTPDWIFLLRNVMRISPDQGGQFAQMLVQDEEPLADITQIVDVFMENLIQQCTAFL
LDALKNNRPSEGPLQTRLLEMNLMHAPQVADAILGNQMFTHYDRAHIAQLCEKAGLLQ
RALEHFTDLYDIKRAVVHTHLLNPEWLVNYFGSLSVEDSLECLRAMLSANIRQNLQIC
VQVASKYHEQLSTQSLIELFESFKSFEGLFYFLGSIVNFSQDPDVHFYKIQAACTGQ
IKEVERICRESNCYDPERVKNFLKEAKLTDQLPLIIVCDRFDFVHDLVLYLYRNNLQK
YIEIYVQKVNPSRLPVVIGGLLDVDCSEDIKNIILVVRGQFSTDELVAEVEKRNRLK
LLLPWLEARIEHGCEEPATHNALAKIYIDSNNNPERFLRENPPYDSRVVGKYCEKRD
HLACVAYERGQCDLELINVCNENSLFKSLSRYLVRKDPPELWGSVLLESNPYRRPLID
QVVQTALSETQDPEEVSVTVKAFMTADLPNELIELLEKIVLDNSVFSEHRNLQNLIL
TAIKADRTRVMEYINRLDNYDAPDIANIAISNELFEEAFAIFRKFDVNTSAVQVLIH
IGNLDRAYEFAERCNEPAVWSQLAKAQLQKGMVKEAIDSYIKADDPSSYMEVVQAANT
SGNWEELVKYLQMARKKARESYVETELIFALAKTNRLAELEEFINGPNNAHIQQVGDR
CYDEKMYDAAKLLYNNVSNFGRLASTLVHLGEYQAAVDGARKANSTRTWKEVCFACVD
GKEFRLAQMCGLHIVVHADELEELINYYQDRGYFEELITMLEAALGLERAHMGMTL
AILYSKFKPQKMREHLELFWSRVNI PKVLRAAEQAHLWAEVLVFLYDKYEEYDNAIITM
MNHPTDAWKEGQFKDIITKVANVELYYRAIQFYLEFKPLLLNDLLMVLSPRLDHTRAV
NYFSKVKQLPLVKPYLRVQNHNNKSVNESLNNLFITEEDYQALRTSIDAYDNFDNIS
LAQRLEKHELIEFRRIAAYLFKGNRWKQSVELCKKDSLYKDAMQYASESKDTELAEE
LLQWFLQEEKRECFCGLFTCYDLLRPDVVLETAWRHNMDFAMPYFIQVMKEYLTKV
DKLDASESLRKEEQATETQPIVYGQPLMLTAGPSVAVPPQAPFGYGYTAPPYGPQ
PGFGYSM

FIGURE 2

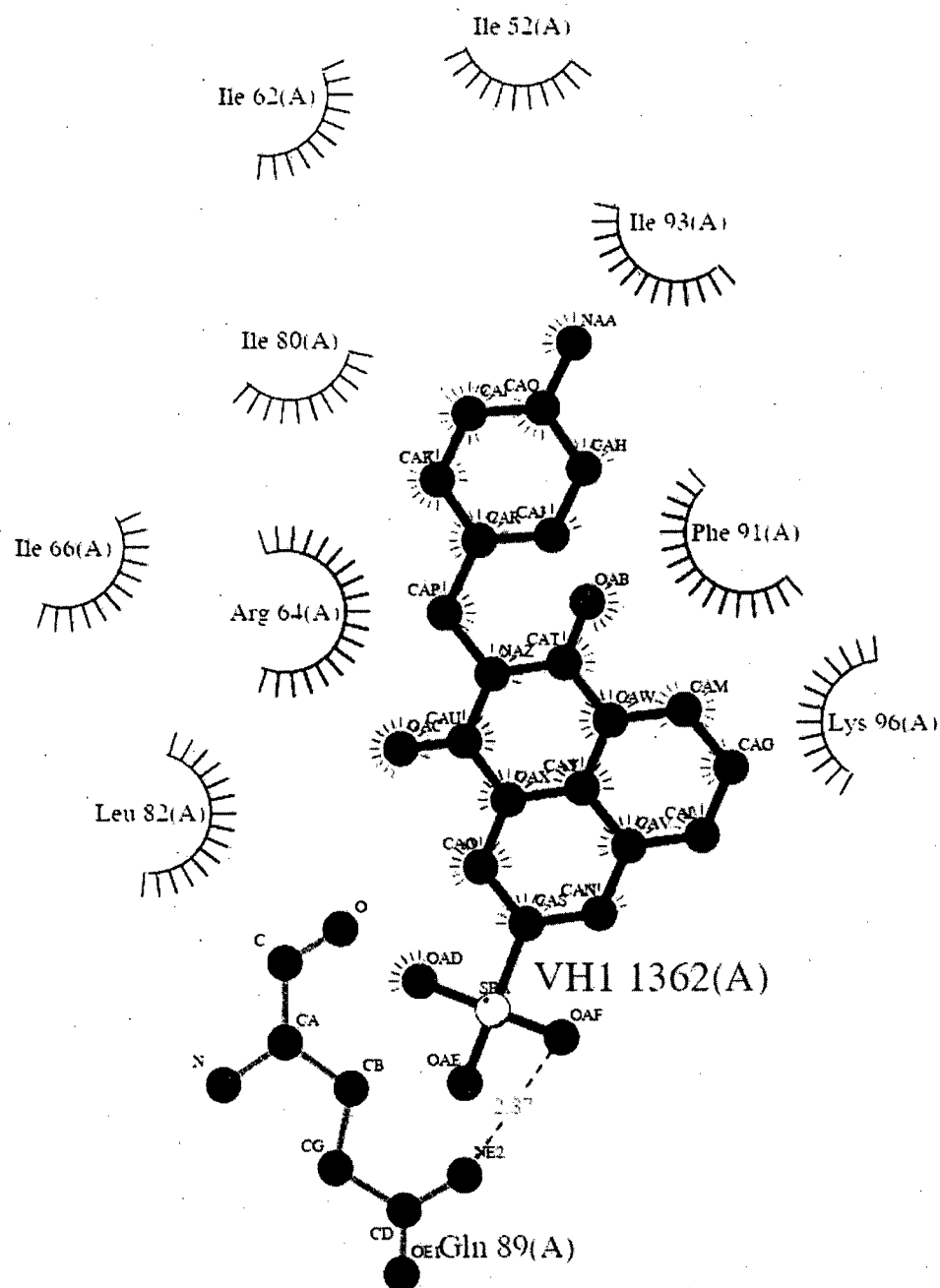


FIGURE 3

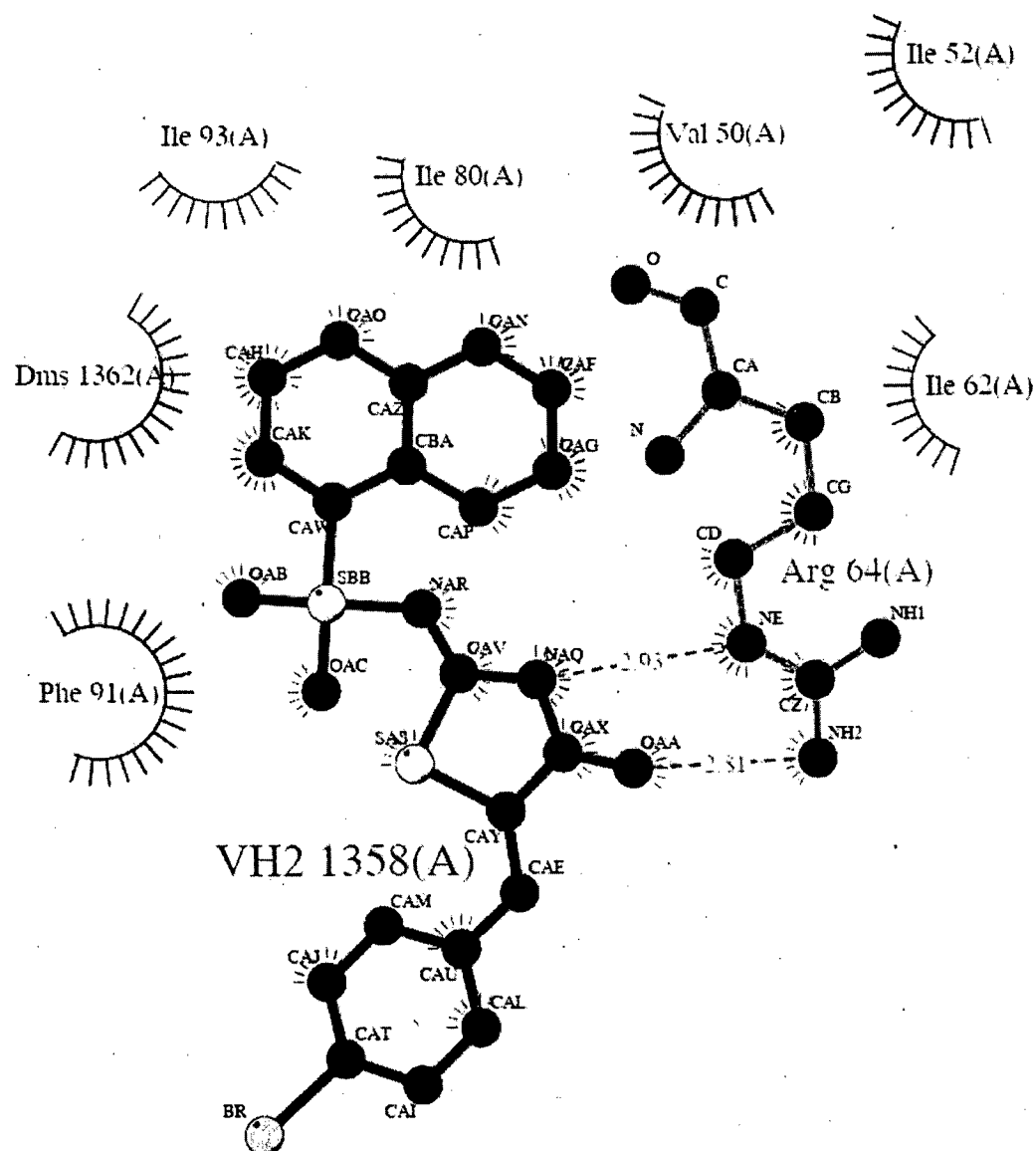


FIGURE 4

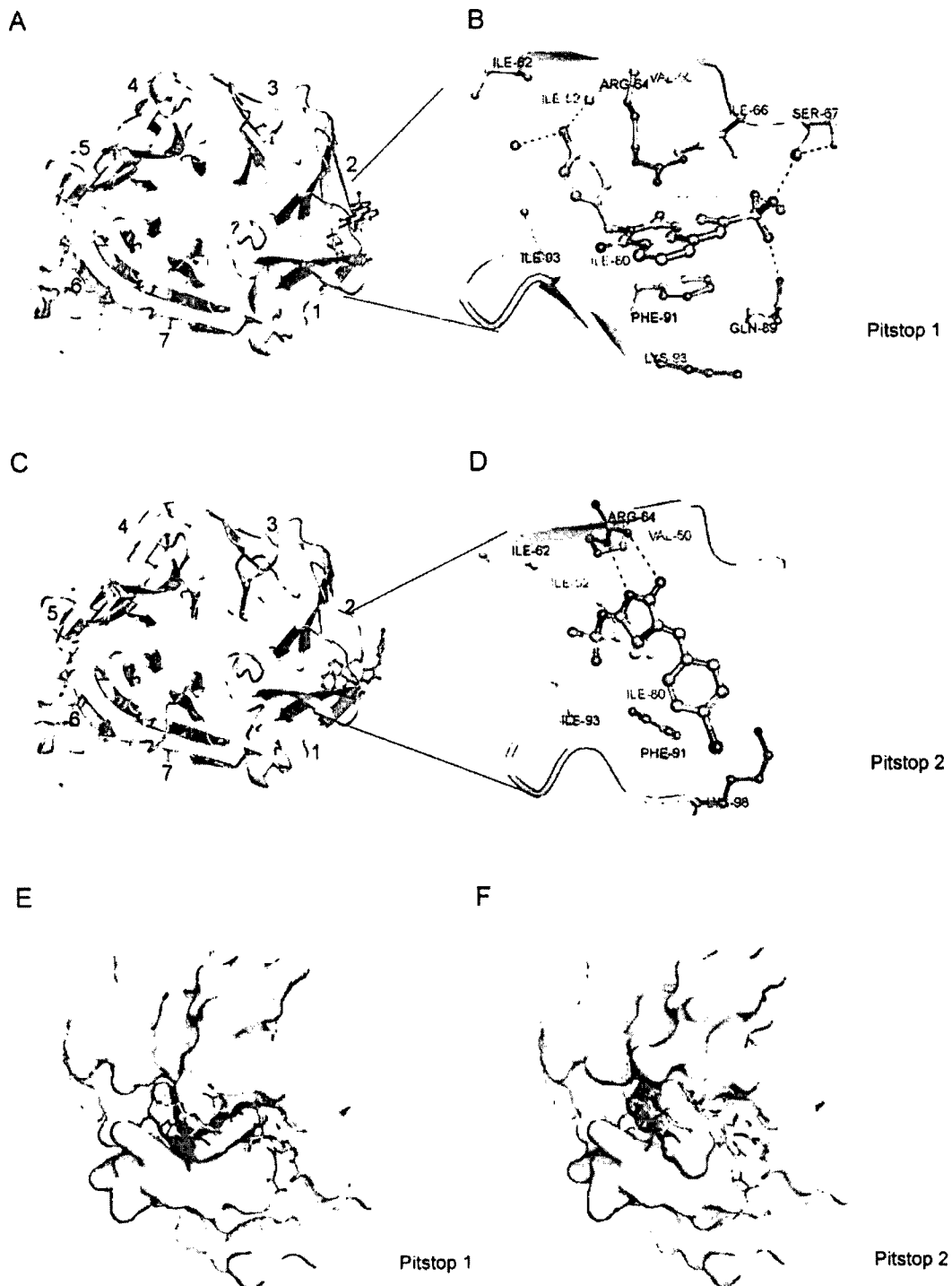


FIGURE 5

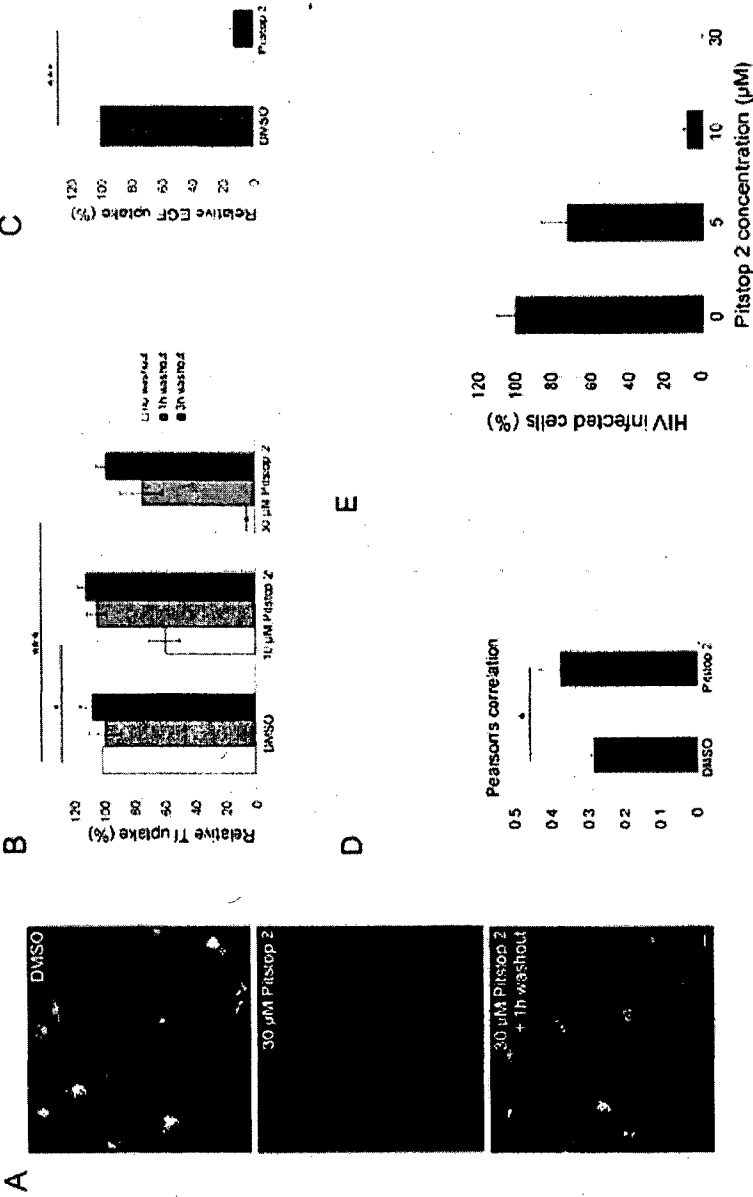


FIGURE 5

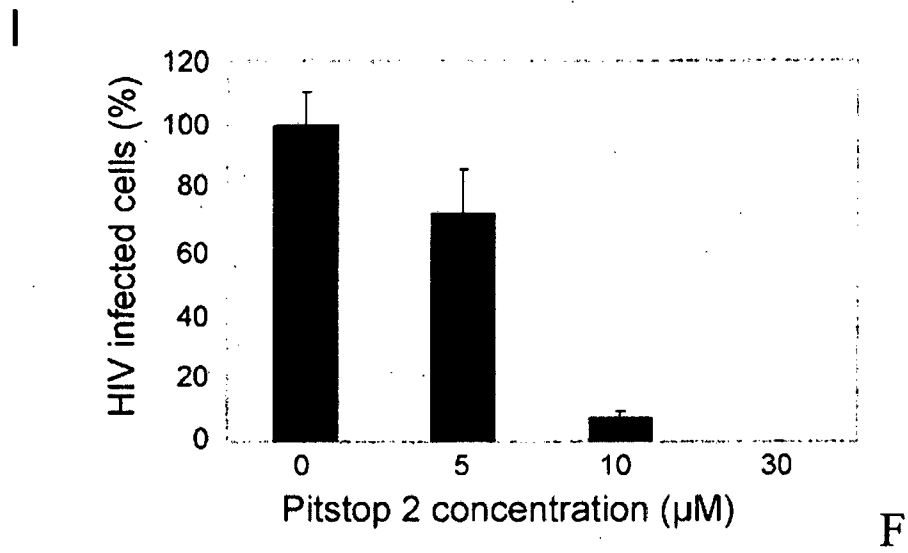
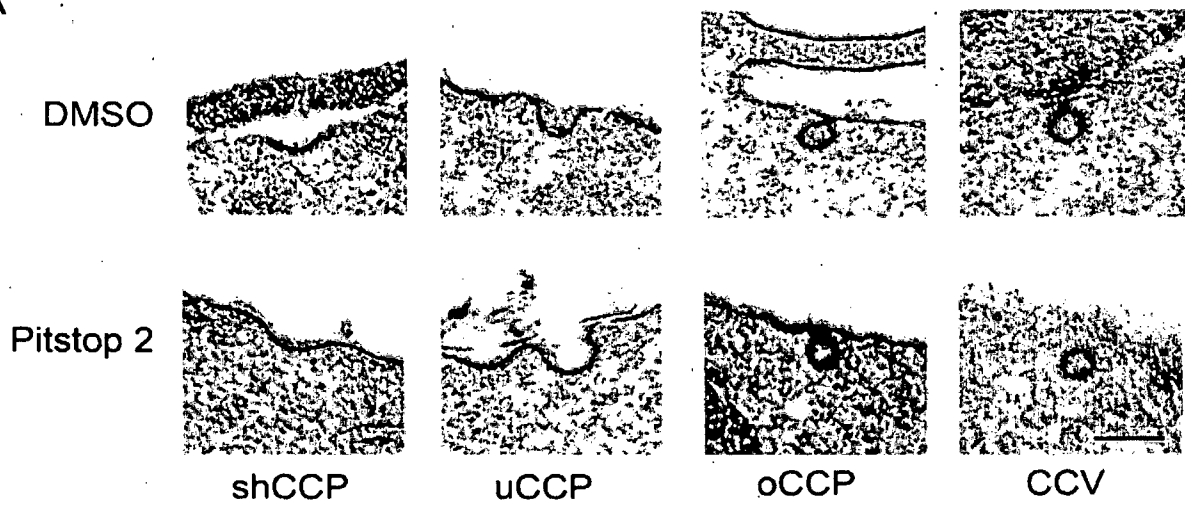
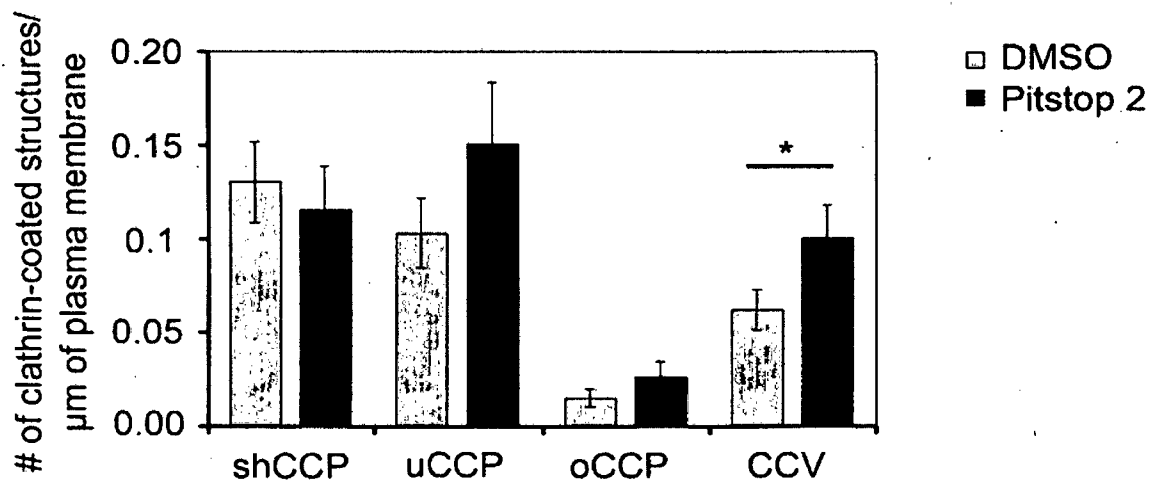


FIGURE 6

A



B



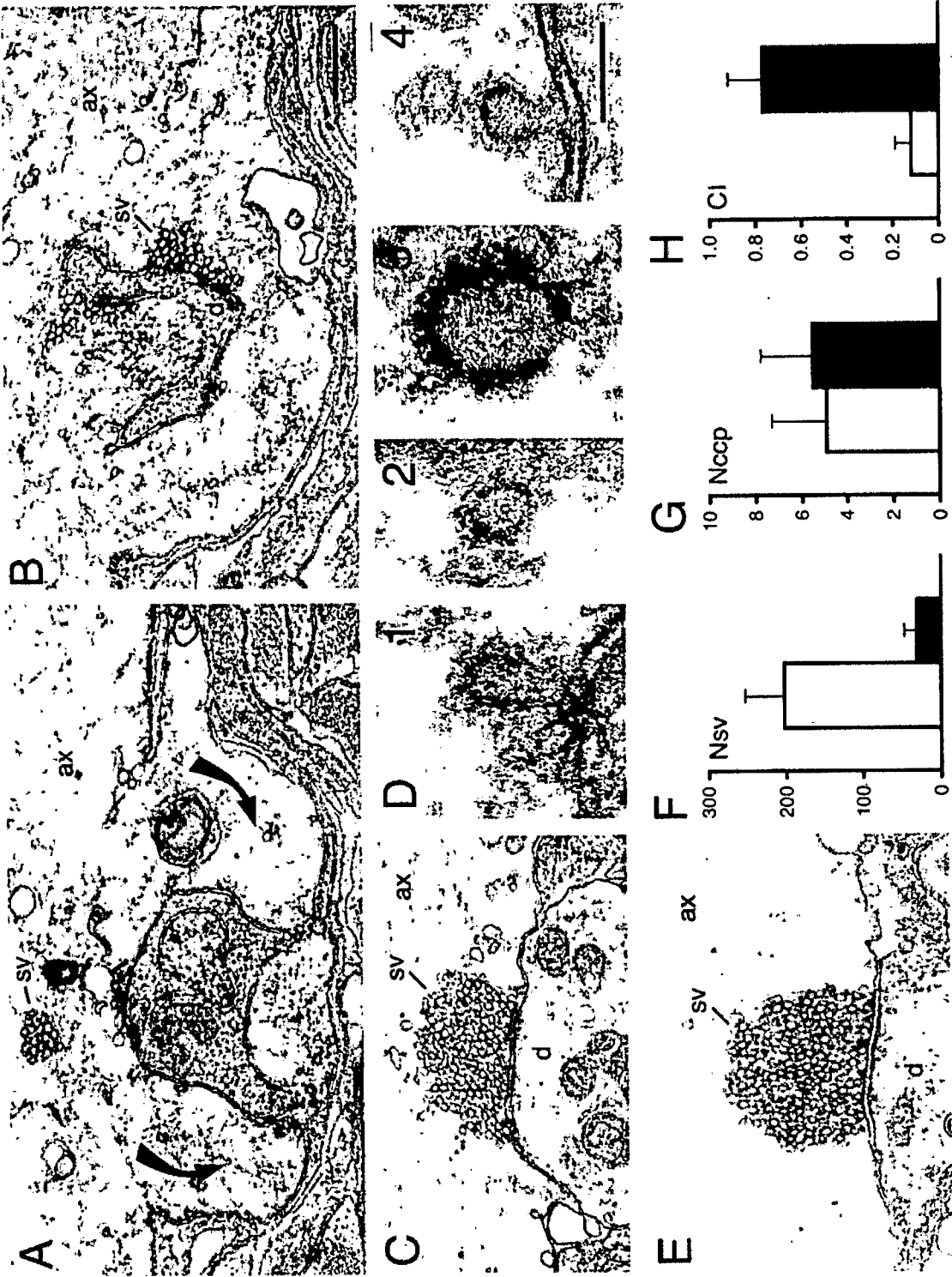


FIGURE 7

FIGURE 8

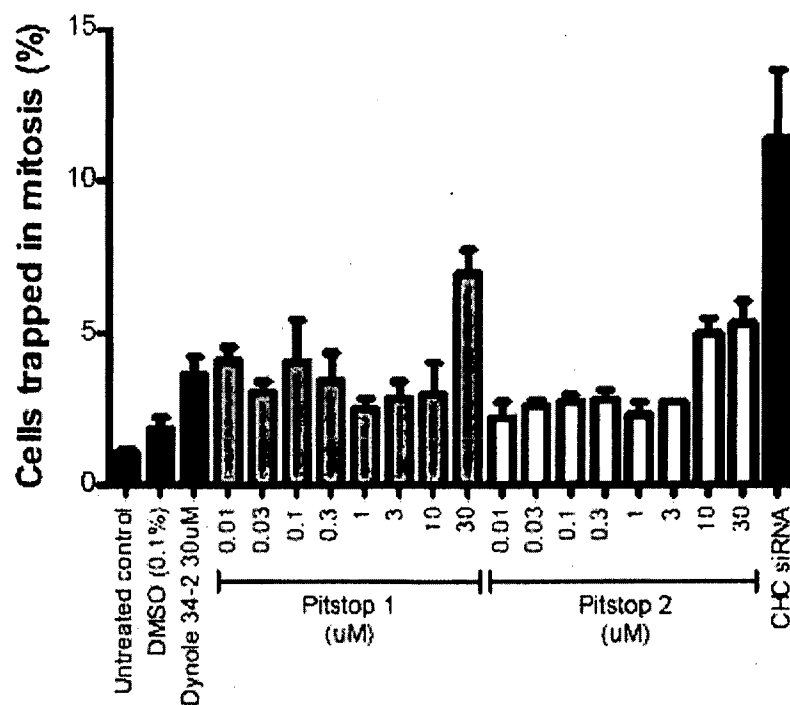


FIGURE 9

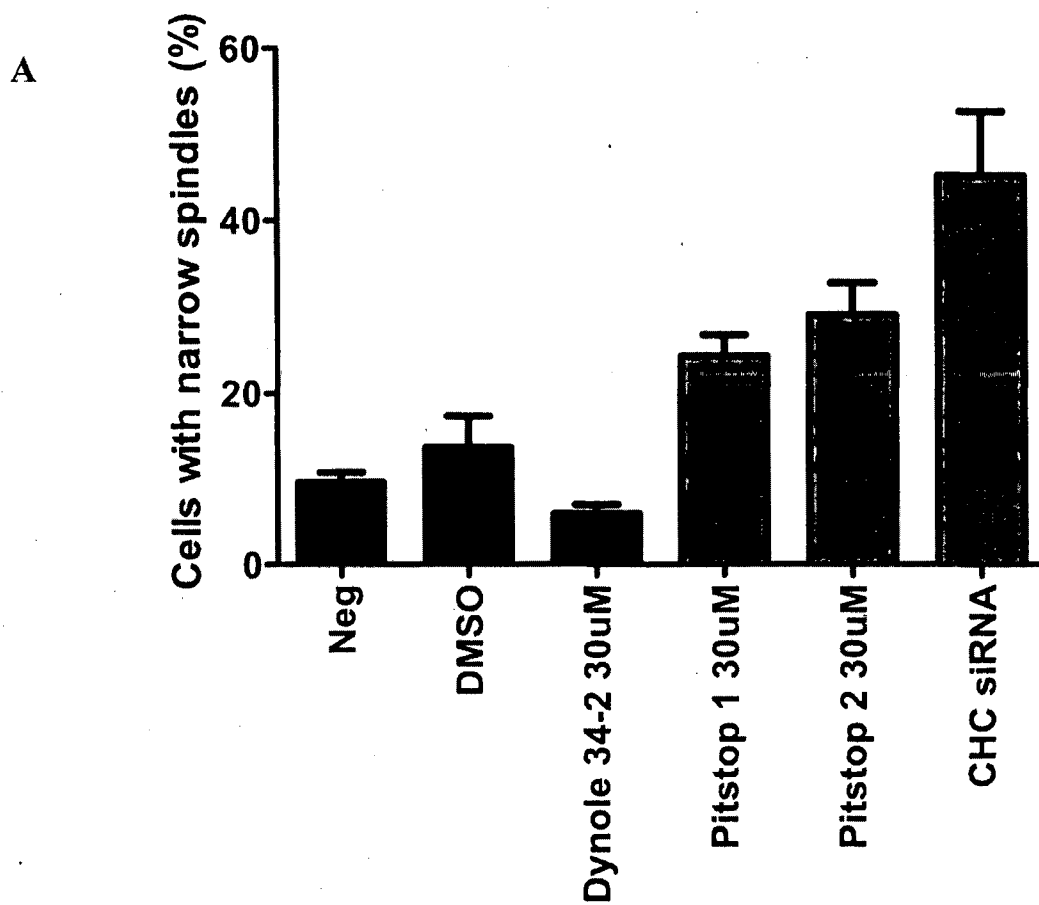
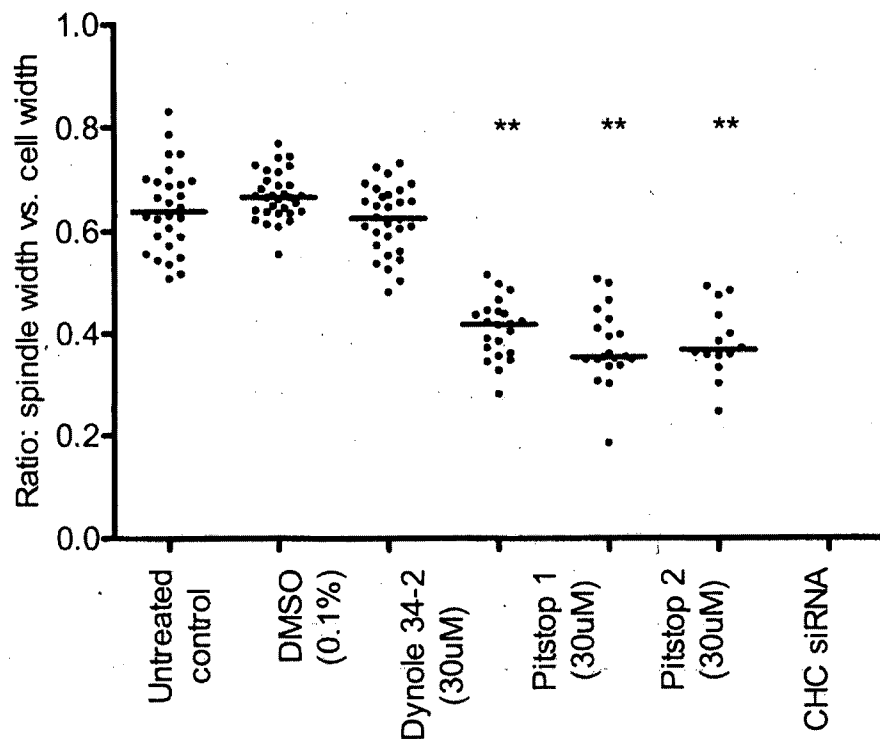


FIGURE 9

B



C

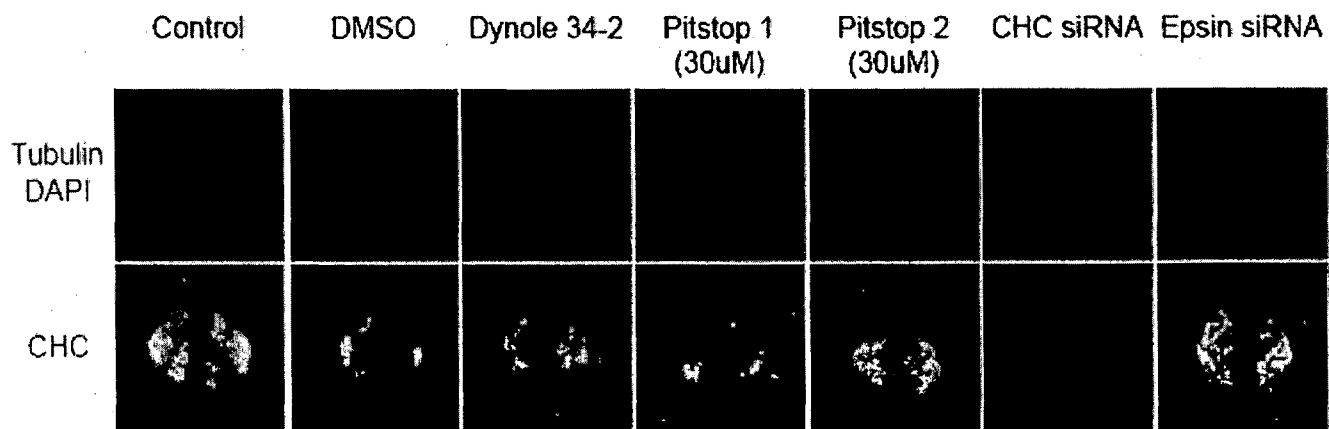


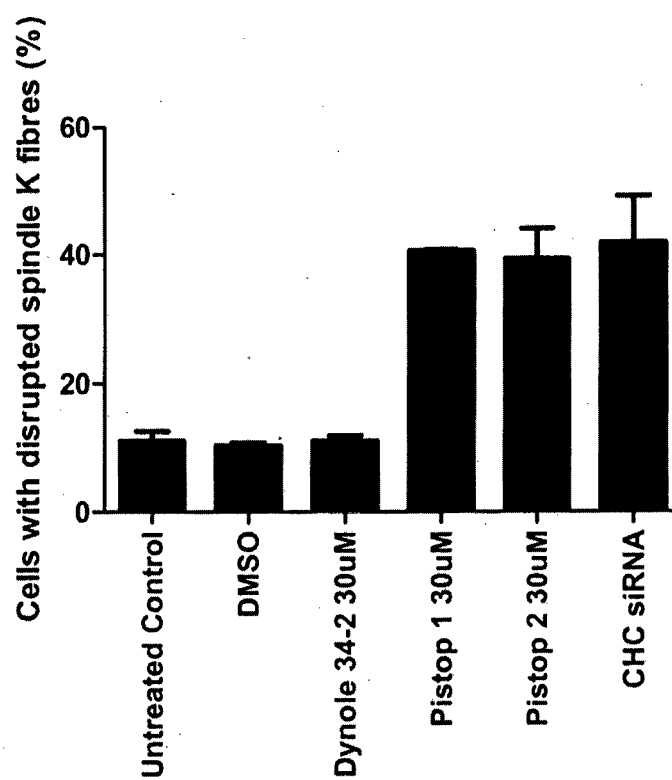
FIGURE 10

FIGURE 11

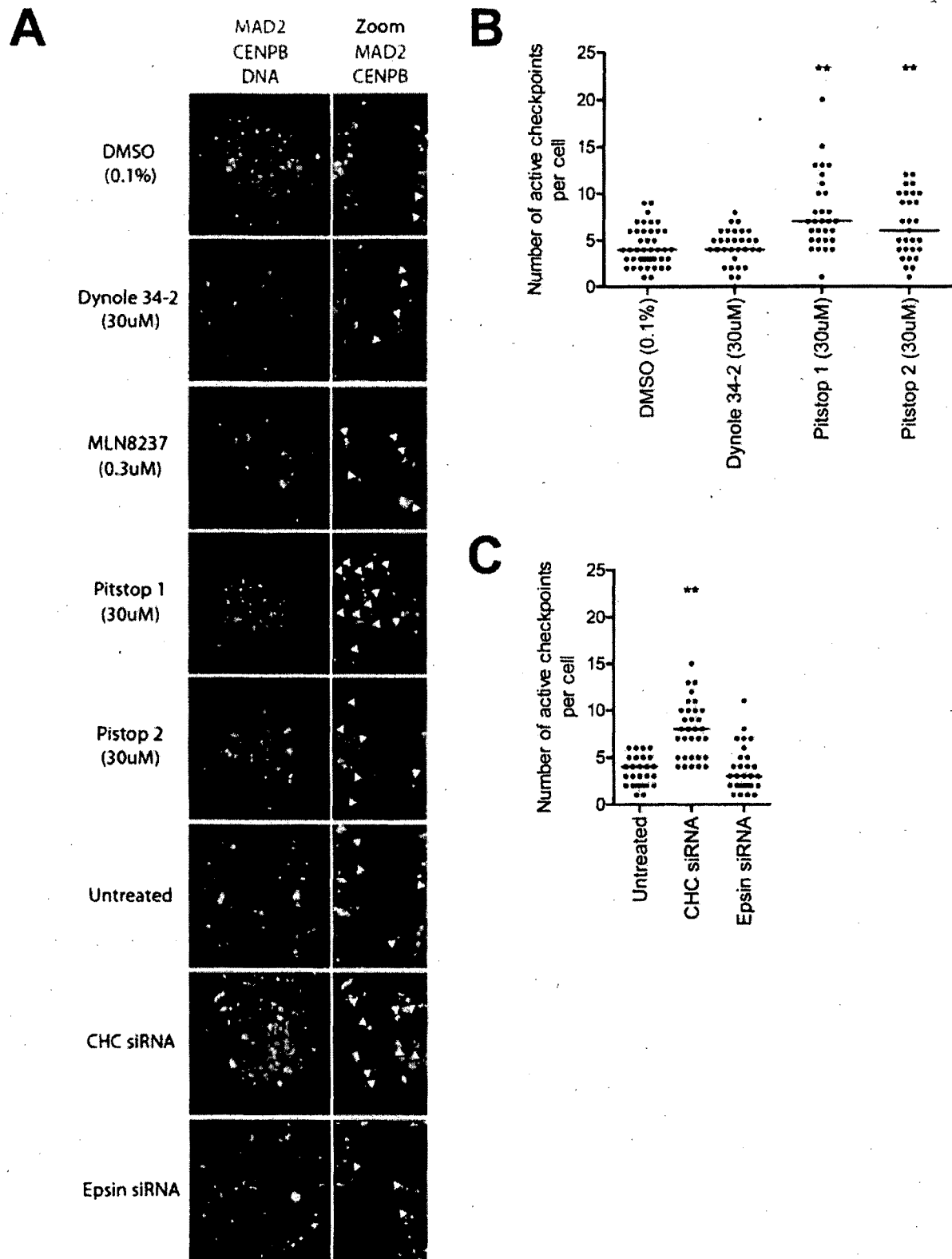
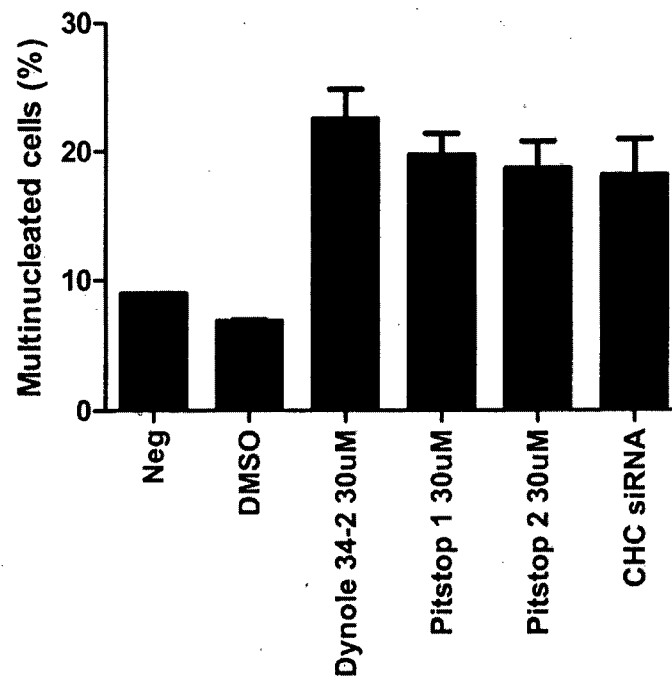


FIGURE 12

CLATHRIN TERMINAL DOMAIN COMPLEXED WITH PITSTOP 1

FIGURE 13

FRAGMENT: 1 AND 2 BLADES, RESIDUES 37-101

RESIDUES IN CLOSE CONTACT WITH PITSTOP1 ($\leq 3.9\text{\AA}$)

Type	A. #	Name	Res. Name	Res. #	X-coor.	Y-coor.	Z-coor.	Occ.	Temp. F	A. Type
ATOM	1	N	PHE A	37	36.966	1.245	19.299	1.00	10.76	N
ATOM	2	CA	PHE A	37	38.395	1.286	19.625	1.00	10.31	C
ATOM	3	C	PHE A	37	39.168	1.988	18.530	1.00	9.47	C
ATOM	4	O	PHE A	37	38.812	1.884	17.343	1.00	9.38	O
ATOM	5	CB	PHE A	37	39.013	-0.152	19.789	1.00	10.56	C
ATOM	6	CG	PHE A	37	38.338	-0.967	20.859	1.00	12.50	C
ATOM	7	CD1	PHE A	37	38.617	-0.695	22.203	1.00	15.46	C
ATOM	8	CD2	PHE A	37	37.432	-1.989	20.503	1.00	14.46	C
ATOM	9	CE1	PHE A	37	38.036	-1.430	23.237	1.00	17.02	C
ATOM	10	CE2	PHE A	37	36.796	-2.713	21.568	1.00	17.00	C
ATOM	11	CZ	PHE A	37	37.135	-2.437	22.898	1.00	16.46	C
ATOM	12	N	ILE A	38	40.241	2.634	18.926	1.00	8.29	N
ATOM	13	CA	ILE A	38	41.325	3.049	18.021	1.00	7.71	C
ATOM	14	C	ILE A	38	42.595	2.332	18.503	1.00	10.08	C
ATOM	15	O	ILE A	38	42.866	2.351	19.688	1.00	11.79	O
ATOM	16	CB	ILE A	38	41.565	4.587	17.950	1.00	8.20	C
ATOM	17	CG1	ILE A	38	40.294	5.178	17.343	1.00	9.79	C
ATOM	18	CG2	ILE A	38	42.787	4.938	17.100	1.00	8.64	C
ATOM	19	CD1	ILE A	38	40.265	6.807	17.499	1.00	10.99	C
ATOM	20	N	CYS A	39	43.324	1.778	17.585	1.00	10.59	N
ATOM	21	CA	CYS A	39	44.564	1.040	17.887	1.00	11.37	C
ATOM	22	C	CYS A	39	45.724	1.673	17.104	1.00	12.32	C
ATOM	23	O	CYS A	39	45.695	1.816	15.853	1.00	10.95	O
ATOM	24	CB	CYS A	39	44.302	-0.397	17.458	1.00	13.37	C
ATOM	25	SG	CYS A	39	45.662	-1.508	17.882	1.00	20.07	S
ATOM	26	N	ILE A	40	46.805	2.008	17.802	1.00	12.03	N
ATOM	27	CA	ILE A	40	47.942	2.652	17.183	1.00	12.38	C
ATOM	28	C	ILE A	40	49.223	1.885	17.515	1.00	12.79	C
ATOM	29	O	ILE A	40	49.407	1.552	18.690	1.00	13.96	O
ATOM	30	CB	ILE A	40	48.124	4.110	17.675	1.00	12.51	C
ATOM	31	CG1	ILE A	40	46.802	4.872	17.591	1.00	11.40	C
ATOM	32	CG2	ILE A	40	49.180	4.844	16.851	1.00	12.58	C
ATOM	33	CD1	ILE A	40	46.227	5.072	18.879	1.00	17.99	C
ATOM	34	N	ARG A	41	49.998	1.605	16.497	1.00	15.03	N
ATOM	35	CA	ARG A	41	51.364	1.064	16.659	1.00	16.16	C
ATOM	36	C	ARG A	41	52.371	2.203	16.744	1.00	17.33	C
ATOM	37	O	ARG A	41	52.461	3.063	15.862	1.00	17.09	O
ATOM	38	CB	ARG A	41	51.691	0.150	15.501	1.00	17.18	C
ATOM	39	CG	ARG A	41	50.869	-1.165	15.451	1.00	17.26	C
ATOM	40	CD	ARG A	41	51.288	-2.108	14.385	1.00	15.94	C
ATOM	41	NE	ARG A	41	51.356	-1.464	13.064	1.00	18.35	N

ATOM	42	CZ	ARG	A	41	52.477	-1.148	12.410	1.00	20.61	C
ATOM	43	NH1	ARG	A	41	53.687	-1.534	12.867	1.00	21.93	N
ATOM	44	NH2	ARG	A	41	52.414	-0.571	11.244	1.00	19.17	N
ATOM	45	N	GLU	A	42	53.194	2.167	17.788	1.00	19.80	N
ATOM	46	CA	GLU	A	42	54.203	3.221	18.023	1.00	21.90	C
ATOM	47	C	GLU	A	42	55.486	2.550	18.510	1.00	24.78	C
ATOM	48	O	GLU	A	42	55.431	1.526	19.166	1.00	25.21	O
ATOM	49	CB	GLU	A	42	53.789	4.175	19.148	1.00	22.96	C
ATOM	50	CG	GLU	A	42	52.433	4.829	19.032	1.00	24.21	C
ATOM	51	CD	GLU	A	42	52.423	6.208	19.696	1.00	27.10	C
ATOM	52	OE1	GLU	A	42	52.432	6.244	20.941	1.00	25.58	O
ATOM	53	OE2	GLU	A	42	52.427	7.240	18.969	1.00	24.35	O
ATOM	54	N	LYS	A	43	56.597	3.197	18.262	1.00	28.15	N
ATOM	55	CA	LYS	A	43	57.907	2.722	18.720	1.00	30.53	C
ATOM	56	C	LYS	A	43	58.468	3.802	19.630	1.00	33.26	C
ATOM	57	O	LYS	A	43	58.687	4.922	19.180	1.00	33.38	O
ATOM	58	CB	LYS	A	43	58.807	2.494	17.517	1.00	30.89	C
ATOM	59	CG	LYS	A	43	58.179	1.741	16.338	0.50	31.83	C
ATOM	60	CD	LYS	A	43	57.488	0.412	16.733	0.50	32.62	C
ATOM	61	CE	LYS	A	43	56.227	0.097	15.878	1.00	32.69	C
ATOM	62	NZ	LYS	A	43	55.091	-0.532	16.697	1.00	33.45	N
ATOM	63	N	VAL	A	44	58.601	3.494	20.922	1.00	36.16	N
ATOM	64	CA	VAL	A	44	59.135	4.430	21.935	1.00	38.56	C
ATOM	65	C	VAL	A	44	60.530	3.951	22.301	1.00	40.09	C
ATOM	66	O	VAL	A	44	60.661	2.897	22.909	1.00	40.69	O
ATOM	67	CB	VAL	A	44	58.293	4.404	23.226	1.00	38.93	C
ATOM	68	CG1	VAL	A	44	59.041	5.132	24.410	1.00	40.82	C
ATOM	69	CG2	VAL	A	44	56.874	4.988	22.976	1.00	39.45	C
ATOM	70	N	GLY	A	45	61.549	4.738	21.960	1.00	42.13	N
ATOM	71	CA	GLY	A	45	62.938	4.267	21.990	1.00	43.09	C
ATOM	72	C	GLY	A	45	63.115	3.145	20.971	1.00	43.60	C
ATOM	73	O	GLY	A	45	62.994	3.378	19.765	1.00	44.45	O
ATOM	74	N	GLU	A	46	63.369	1.926	21.451	1.00	43.53	N
ATOM	75	CA	GLU	A	46	63.554	0.767	20.562	1.00	44.17	C
ATOM	76	C	GLU	A	46	62.437	-0.265	20.722	1.00	42.41	C
ATOM	77	O	GLU	A	46	62.470	-1.333	20.092	1.00	42.95	O
ATOM	78	CB	GLU	A	46	64.918	0.099	20.835	1.00	45.21	C
ATOM	79	CG	GLU	A	46	66.111	1.077	20.794	1.00	47.70	C
ATOM	80	CD	GLU	A	46	66.781	1.164	19.431	1.00	52.17	C
ATOM	81	OE1	GLU	A	46	66.133	1.604	18.443	1.00	53.88	O
ATOM	82	OE2	GLU	A	46	67.979	0.788	19.364	1.00	54.69	O
ATOM	83	N	GLN	A	47	61.462	0.077	21.559	1.00	40.06	N
ATOM	84	CA	GLN	A	47	60.399	-0.813	21.998	1.00	38.35	C
ATOM	85	C	GLN	A	47	59.146	-0.584	21.122	1.00	36.12	C
ATOM	86	O	GLN	A	47	58.606	0.522	21.123	1.00	35.80	O
ATOM	87	CB	GLN	A	47	60.089	-0.458	23.464	1.00	39.12	C
ATOM	88	CG	GLN	A	47	59.258	-1.474	24.279	1.00	40.81	C
ATOM	89	CD	GLN	A	47	58.766	-0.879	25.601	1.00	44.69	C
ATOM	90	NE2	GLN	A	47	59.713	-0.325	26.410	1.00	46.16	N
ATOM	91	OE1	GLN	A	47	57.553	-0.931	25.917	1.00	46.13	O

ATOM	92	N	ALA	A	48	58.735	-1.607	20.368	1.00	32.70	N
ATOM	93	CA	ALA	A	48	57.490	-1.563	19.582	1.00	30.38	C
ATOM	94	C	ALA	A	48	56.278	-1.780	20.502	1.00	28.10	C
ATOM	95	O	ALA	A	48	56.254	-2.719	21.272	1.00	26.98	O
ATOM	96	CB	ALA	A	48	57.536	-2.576	18.513	1.00	30.28	C
ATOM	97	N	GLN	A	49	55.274	-0.890	20.430	1.00	24.85	N
ATOM	98	CA	GLN	A	49	54.158	-0.911	21.350	1.00	22.58	C
ATOM	99	C	GLN	A	49	52.856	-0.826	20.564	1.00	21.39	C
ATOM	100	O	GLN	A	49	52.852	-0.273	19.495	1.00	19.38	O
ATOM	101	CB	GLN	A	49	54.174	0.306	22.287	1.00	23.79	C
ATOM	102	CG	GLN	A	49	55.493	0.502	23.061	1.00	27.03	C
ATOM	103	CD	GLN	A	49	55.503	1.750	23.938	1.00	32.33	C
ATOM	104	NE2	GLN	A	49	56.309	1.702	25.003	1.00	33.55	N
ATOM	105	OE1	GLN	A	49	54.824	2.755	23.657	1.00	36.38	O
ATOM	106	N	VAL	A	50	51.811	-1.374	21.143	1.00	20.76	N
ATOM	107	CA	VAL	A	50	50.432	-1.147	20.674	1.00	20.24	C
ATOM	108	C	VAL	A	50	49.678	-0.325	21.713	1.00	18.69	C
ATOM	109	O	VAL	A	50	49.726	-0.600	22.919	1.00	19.01	O
ATOM	110	CB	VAL	A	50	49.728	-2.448	20.382	1.00	20.96	C
ATOM	111	CG1	VAL	A	50	48.321	-2.176	19.953	1.00	22.50	C
ATOM	112	CG2	VAL	A	50	50.532	-3.226	19.334	1.00	22.84	C
ATOM	113	N	VAL	A	51	48.991	0.735	21.266	1.00	17.15	N
ATOM	114	CA	VAL	A	51	48.197	1.575	22.162	1.00	17.20	C
ATOM	115	C	VAL	A	51	46.749	1.360	21.737	1.00	16.17	C
ATOM	116	O	VAL	A	51	46.461	1.372	20.517	1.00	16.56	O
ATOM	117	CB	VAL	A	51	48.495	3.072	21.993	1.00	17.52	C
ATOM	118	CG1	VAL	A	51	47.726	3.871	23.002	1.00	18.55	C
ATOM	119	CG2	VAL	A	51	49.988	3.339	22.081	1.00	20.67	C
ATOM	120	N	ILE	A	52	45.916	1.062	22.708	1.00	13.85	N
ATOM	121	CA	ILE	A	52	44.488	0.810	22.458	1.00	14.57	C
ATOM	122	C	ILE	A	52	43.727	1.908	23.167	1.00	14.95	C
ATOM	123	O	ILE	A	52	43.754	2.063	24.387	1.00	16.13	O
ATOM	124	CB	ILE	A	52	43.975	-0.577	22.925	1.00	14.98	C
ATOM	125	CG1	ILE	A	52	44.776	-1.688	22.261	1.00	17.11	C
ATOM	126	CG2	ILE	A	52	42.422	-0.699	22.754	1.00	15.44	C
ATOM	127	CD1	ILE	A	52	44.506	-3.083	22.836	1.00	17.71	C
ATOM	128	N	ILE	A	53	42.934	2.645	22.371	1.00	13.57	N
ATOM	129	CA	ILE	A	53	42.071	3.656	22.972	1.00	14.83	C
ATOM	130	C	ILE	A	53	40.645	3.153	22.956	1.00	13.54	C
ATOM	131	O	ILE	A	53	40.082	2.854	21.882	1.00	13.94	O
ATOM	132	CB	ILE	A	53	42.181	5.003	22.202	1.00	15.34	C
ATOM	133	CG1	ILE	A	53	43.631	5.484	22.219	1.00	17.90	C
ATOM	134	CG2	ILE	A	53	41.185	6.076	22.819	1.00	15.02	C
ATOM	135	CD1	ILE	A	53	43.858	6.552	21.266	1.00	19.95	C
ATOM	136	N	ASP	A	54	40.058	3.002	24.140	1.00	14.38	N
ATOM	137	CA	ASP	A	54	38.695	2.565	24.310	1.00	15.18	C
ATOM	138	C	ASP	A	54	37.837	3.817	24.206	1.00	15.24	C
ATOM	139	O	ASP	A	54	38.034	4.754	24.981	1.00	18.09	O
ATOM	140	CB	ASP	A	54	38.515	1.907	25.699	1.00	16.46	C
ATOM	141	CG	ASP	A	54	37.101	1.404	25.944	1.00	20.89	C

ATOM	142	OD1	ASP	A	54	36.127	1.859	25.320	1.00	19.71	O
ATOM	143	OD2	ASP	A	54	36.946	0.432	26.733	1.00	26.52	O
ATOM	144	N	MET	A	55	36.947	3.861	23.229	1.00	16.94	N
ATOM	145	CA	MET	A	55	36.226	5.098	22.944	1.00	17.00	C
ATOM	146	C	MET	A	55	35.169	5.385	24.002	1.00	18.69	C
ATOM	147	O	MET	A	55	34.628	6.464	24.029	1.00	19.04	O
ATOM	148	CB	MET	A	55	35.610	5.051	21.559	1.00	16.64	C
ATOM	149	CG	MET	A	55	36.603	5.015	20.495	1.00	17.52	C
ATOM	150	SD	MET	A	55	37.703	6.436	20.396	1.00	21.20	S
ATOM	151	CE	MET	A	55	36.554	7.486	19.519	1.00	23.19	C
ATOM	152	N	ASN	A	56	34.887	4.412	24.851	1.00	20.98	N
ATOM	153	CA	ASN	A	56	34.080	4.641	26.052	1.00	23.12	C
ATOM	154	C	ASN	A	56	34.870	5.239	27.232	1.00	24.45	C
ATOM	155	O	ASN	A	56	34.271	5.686	28.229	1.00	25.80	O
ATOM	156	CB	ASN	A	56	33.470	3.348	26.579	1.00	23.78	C
ATOM	157	CG	ASN	A	56	32.288	2.871	25.831	0.50	24.13	C
ATOM	158	ND2	ASN	A	56	31.991	1.585	26.032	0.50	26.90	N
ATOM	159	OD1	ASN	A	56	31.601	3.611	25.122	0.50	28.08	O
ATOM	160	N	ASP	A	57	36.191	5.229	27.155	1.00	24.73	N
ATOM	161	CA	ASP	A	57	37.036	5.695	28.251	1.00	25.90	C
ATOM	162	C	ASP	A	57	38.357	6.125	27.687	1.00	24.34	C
ATOM	163	O	ASP	A	57	39.407	5.516	27.974	1.00	24.69	O
ATOM	164	CB	ASP	A	57	37.200	4.579	29.284	1.00	27.52	C
ATOM	165	CG	ASP	A	57	38.025	4.997	30.502	1.00	31.93	C
ATOM	166	OD1	ASP	A	57	37.921	6.170	30.960	1.00	32.07	O
ATOM	167	OD2	ASP	A	57	38.773	4.117	31.011	1.00	40.25	O
ATOM	168	N	PRO	A	58	38.338	7.171	26.866	1.00	23.82	N
ATOM	169	CA	PRO	A	58	39.511	7.454	26.032	1.00	24.29	C
ATOM	170	C	PRO	A	58	40.678	8.084	26.761	1.00	27.16	C
ATOM	171	O	PRO	A	58	41.806	8.014	26.267	1.00	27.08	O
ATOM	172	CB	PRO	A	58	38.964	8.387	24.948	1.00	23.88	C
ATOM	173	CG	PRO	A	58	37.724	8.980	25.548	1.00	24.36	C
ATOM	174	CD	PRO	A	58	37.143	7.921	26.416	1.00	23.25	C
ATOM	175	N	SER	A	59	40.429	8.701	27.928	1.00	29.03	N
ATOM	176	CA	SER	A	59	41.481	9.475	28.566	1.00	30.91	C
ATOM	177	C	SER	A	59	42.572	8.593	29.168	1.00	31.83	C
ATOM	178	O	SER	A	59	43.662	9.119	29.428	1.00	33.16	O
ATOM	179	CB	SER	A	59	40.932	10.421	29.644	1.00	30.90	C
ATOM	180	OG	SER	A	59	40.546	9.642	30.750	1.00	32.40	O
ATOM	181	N	ASN	A	60	42.274	7.293	29.333	1.00	32.13	N
ATOM	182	CA	ASN	A	60	43.169	6.252	29.844	1.00	33.70	C
ATOM	183	C	ASN	A	60	43.541	5.151	28.805	1.00	31.62	C
ATOM	184	O	ASN	A	60	43.094	4.013	28.951	1.00	32.29	O
ATOM	185	CB	ASN	A	60	42.482	5.492	31.001	1.00	33.99	C
ATOM	186	CG	ASN	A	60	41.997	6.400	32.146	1.00	37.66	C
ATOM	187	ND2	ASN	A	60	40.985	5.906	32.872	1.00	39.83	N
ATOM	188	OD1	ASN	A	60	42.496	7.516	32.364	1.00	39.66	O
ATOM	189	N	PRO	A	61	44.316	5.500	27.754	1.00	31.64	N
ATOM	190	CA	PRO	A	61	44.773	4.505	26.781	1.00	31.62	C
ATOM	191	C	PRO	A	61	45.824	3.565	27.368	1.00	32.35	C

ATOM	192	O	PRO	A	61	46.674	4.014	28.162	1.00	32.18	O
ATOM	193	CB	PRO	A	61	45.391	5.344	25.684	1.00	31.74	C
ATOM	194	CG	PRO	A	61	45.692	6.644	26.294	1.00	32.56	C
ATOM	195	CD	PRO	A	61	44.723	6.859	27.373	1.00	31.22	C
ATOM	196	N	ILE	A	62	45.757	2.300	26.939	1.00	31.08	N
ATOM	197	CA	ILE	A	62	46.603	1.236	27.445	1.00	31.10	C
ATOM	198	C	ILE	A	62	47.671	0.930	26.412	1.00	30.97	C
ATOM	199	O	ILE	A	62	47.340	0.762	25.235	1.00	25.68	O
ATOM	200	CB	ILE	A	62	45.754	-0.016	27.813	1.00	31.05	C
ATOM	201	CG1	ILE	A	62	44.793	0.321	28.953	1.00	33.78	C
ATOM	202	CG2	ILE	A	62	46.679	-1.161	28.284	1.00	33.97	C
ATOM	203	CD1	ILE	A	62	43.652	-0.701	29.191	1.00	34.61	C
ATOM	204	N	ARG	A	63	48.954	0.926	26.848	1.00	31.95	N
ATOM	205	CA	ARG	A	63	50.090	0.524	26.006	1.00	33.81	C
ATOM	206	C	ARG	A	63	50.569	-0.882	26.381	1.00	35.07	C
ATOM	207	O	ARG	A	63	50.621	-1.219	27.582	1.00	36.34	O
ATOM	208	CB	ARG	A	63	51.283	1.454	26.168	1.00	35.08	C
ATOM	209	CG	ARG	A	63	51.011	2.939	26.072	1.00	35.77	C
ATOM	210	CD	ARG	A	63	52.286	3.717	25.730	1.00	37.69	C
ATOM	211	NE	ARG	A	63	51.852	4.979	25.153	1.00	37.93	N
ATOM	212	CZ	ARG	A	63	52.152	5.438	23.933	1.00	37.93	C
ATOM	213	NH1	ARG	A	63	53.015	4.808	23.124	1.00	35.84	N
ATOM	214	NH2	ARG	A	63	51.579	6.578	23.535	1.00	35.53	N
ATOM	215	N	ARG	A	64	50.869	-1.718	25.391	1.00	34.17	N
ATOM	216	CA	ARG	A	64	51.452	-3.053	25.655	1.00	33.42	C
ATOM	217	C	ARG	A	64	52.610	-3.202	24.669	1.00	33.67	C
ATOM	218	O	ARG	A	64	52.527	-2.775	23.496	1.00	30.68	O
ATOM	219	CB	ARG	A	64	50.419	-4.224	25.515	1.00	33.63	C
ATOM	220	CG	ARG	A	64	50.937	-5.685	25.990	1.00	33.57	C
ATOM	221	CD	ARG	A	64	50.005	-6.909	25.633	1.00	35.82	C
ATOM	222	NE	ARG	A	64	50.322	-8.166	26.351	1.00	35.13	N
ATOM	223	CZ	ARG	A	64	51.374	-8.969	26.098	1.00	37.14	C
ATOM	224	NH1	ARG	A	64	52.256	-8.689	25.132	1.00	36.95	N
ATOM	225	NH2	ARG	A	64	51.574	-10.070	26.833	1.00	36.56	N
ATOM	226	N	PRO	A	65	53.733	-3.785	25.137	1.00	32.81	N
ATOM	227	CA	PRO	A	65	54.793	-4.118	24.182	1.00	32.99	C
ATOM	228	C	PRO	A	65	54.302	-5.248	23.340	1.00	33.51	C
ATOM	229	O	PRO	A	65	53.758	-6.220	23.874	1.00	35.57	O
ATOM	230	CB	PRO	A	65	55.960	-4.586	25.083	1.00	32.70	C
ATOM	231	CG	PRO	A	65	55.701	-3.950	26.379	1.00	33.10	C
ATOM	232	CD	PRO	A	65	54.182	-3.908	26.530	1.00	33.77	C
ATOM	233	N	ILE	A	66	54.432	-5.099	22.040	1.00	33.08	N
ATOM	234	CA	ILE	A	66	54.027	-6.092	21.105	1.00	34.00	C
ATOM	235	C	ILE	A	66	54.801	-5.745	19.869	1.00	34.22	C
ATOM	236	O	ILE	A	66	54.800	-4.571	19.435	1.00	36.68	O
ATOM	237	CB	ILE	A	66	52.509	-6.011	20.748	1.00	34.45	C
ATOM	238	CG1	ILE	A	66	51.632	-6.421	21.934	1.00	35.53	C
ATOM	239	CG2	ILE	A	66	52.187	-6.911	19.591	1.00	36.05	C
ATOM	240	CD1	ILE	A	66	50.161	-6.595	21.606	1.00	37.92	C
ATOM	241	N	SER	A	67	55.370	-6.767	19.277	1.00	33.26	N

ATOM	242	CA	SER	A	67	55.987	-6.732	17.969	1.00	33.12	C
ATOM	243	C	SER	A	67	54.995	-7.293	16.975	1.00	30.86	C
ATOM	244	O	SER	A	67	54.613	-8.465	17.055	1.00	32.48	O
ATOM	245	CB	SER	A	67	57.236	-7.623	17.992	1.00	34.25	C
ATOM	246	OG	SER	A	67	56.960	-8.794	18.759	0.50	34.68	O
ATOM	247	N	ALA	A	68	54.573	-6.430	16.046	1.00	28.44	N
ATOM	248	CA	ALA	A	68	53.580	-6.735	15.066	1.00	25.22	C
ATOM	249	C	ALA	A	68	53.710	-5.701	14.000	1.00	22.32	C
ATOM	250	O	ALA	A	68	53.951	-4.510	14.278	1.00	23.32	O
ATOM	251	CB	ALA	A	68	52.143	-6.653	15.691	1.00	24.13	C
ATOM	252	N	ASP	A	69	53.505	-6.119	12.780	1.00	20.09	N
ATOM	253	CA	ASP	A	69	53.511	-5.210	11.666	1.00	20.59	C
ATOM	254	C	ASP	A	69	52.137	-4.576	11.471	1.00	17.46	C
ATOM	255	O	ASP	A	69	51.992	-3.645	10.691	1.00	17.61	O
ATOM	256	CB	ASP	A	69	54.072	-5.847	10.370	1.00	20.62	C
ATOM	257	CG	ASP	A	69	53.162	-6.846	9.689	1.00	29.13	C
ATOM	258	OD1	ASP	A	69	51.931	-6.604	9.548	1.00	32.05	O
ATOM	259	OD2	ASP	A	69	53.733	-7.880	9.172	1.00	34.14	O
ATOM	260	N	SER	A	70	51.129	-5.121	12.142	1.00	15.89	N
ATOM	261	CA	SER	A	70	49.738	-4.617	12.028	1.00	13.81	C
ATOM	262	C	SER	A	70	48.943	-5.147	13.201	1.00	12.38	C
ATOM	263	O	SER	A	70	49.139	-6.301	13.678	1.00	13.07	O
ATOM	264	CB	SER	A	70	49.076	-5.073	10.717	1.00	14.11	C
ATOM	265	OG	SER	A	70	47.706	-4.611	10.552	1.00	14.23	O
ATOM	266	N	ALA	A	71	48.012	-4.316	13.697	1.00	12.60	N
ATOM	267	CA	ALA	A	71	47.215	-4.622	14.864	1.00	11.84	C
ATOM	268	C	ALA	A	71	45.800	-4.088	14.595	1.00	11.08	C
ATOM	269	O	ALA	A	71	45.637	-2.901	14.263	1.00	11.98	O
ATOM	270	CB	ALA	A	71	47.823	-3.946	16.158	1.00	13.49	C
ATOM	271	N	ILE	A	72	44.791	-4.973	14.654	1.00	9.96	N
ATOM	272	CA	ILE	A	72	43.413	-4.604	14.362	1.00	8.29	C
ATOM	273	C	ILE	A	72	42.506	-5.116	15.468	1.00	9.78	C
ATOM	274	O	ILE	A	72	42.413	-6.344	15.718	1.00	9.44	O
ATOM	275	CB	ILE	A	72	42.953	-5.034	12.957	1.00	8.74	C
ATOM	276	CG1	ILE	A	72	43.208	-6.545	12.638	1.00	9.66	C
ATOM	277	CG2	ILE	A	72	43.536	-4.009	11.907	1.00	9.41	C
ATOM	278	CD1	ILE	A	72	42.579	-7.025	11.269	1.00	10.31	C
ATOM	279	N	MET	A	73	41.790	-4.197	16.132	1.00	10.15	N
ATOM	280	CA	MET	A	73	40.812	-4.604	17.148	1.00	9.50	C
ATOM	281	C	MET	A	73	39.529	-5.132	16.525	1.00	10.64	C
ATOM	282	O	MET	A	73	39.071	-4.639	15.484	1.00	10.39	O
ATOM	283	CB	MET	A	73	40.454	-3.413	18.078	1.00	9.07	C
ATOM	284	CG	MET	A	73	41.561	-2.876	18.938	1.00	12.17	C
ATOM	285	SD	MET	A	73	41.899	-4.133	20.281	1.00	16.73	S
ATOM	286	CE	MET	A	73	40.336	-4.407	21.043	1.00	11.66	C
ATOM	287	N	ASN	A	74	38.926	-6.149	17.176	1.00	9.55	N
ATOM	288	CA	ASN	A	74	37.641	-6.688	16.798	1.00	9.77	C
ATOM	289	C	ASN	A	74	36.616	-5.548	16.870	1.00	7.94	C
ATOM	290	O	ASN	A	74	36.759	-4.602	17.642	1.00	9.24	O
ATOM	291	CB	ASN	A	74	37.245	-7.773	17.820	1.00	9.97	C

ATOM	292	CG	ASN	A	74	35.958	-8.467	17.514	1.00	9.45	C
ATOM	293	ND2	ASN	A	74	36.071	-9.605	16.888	1.00	11.79	N
ATOM	294	OD1	ASN	A	74	34.861	-8.001	17.852	1.00	10.86	O
ATOM	295	N	PRO	A	75	35.605	-5.626	16.023	1.00	9.56	N
ATOM	296	CA	PRO	A	75	34.613	-4.510	16.019	1.00	11.78	C
ATOM	297	C	PRO	A	75	33.764	-4.407	17.252	1.00	13.82	C
ATOM	298	O	PRO	A	75	33.305	-3.300	17.599	1.00	12.73	O
ATOM	299	CB	PRO	A	75	33.742	-4.832	14.813	1.00	11.80	C
ATOM	300	CG	PRO	A	75	34.518	-5.728	14.019	1.00	13.15	C
ATOM	301	CD	PRO	A	75	35.401	-6.507	14.869	1.00	8.61	C
ATOM	302	N	ALA	A	76	33.626	-5.527	17.971	1.00	16.37	N
ATOM	303	CA	ALA	A	76	32.615	-5.622	19.053	1.00	18.80	C
ATOM	304	C	ALA	A	76	33.252	-5.901	20.400	1.00	19.66	C
ATOM	305	O	ALA	A	76	32.912	-5.318	21.419	1.00	23.48	O
ATOM	306	CB	ALA	A	76	31.580	-6.694	18.672	1.00	20.34	C
ATOM	307	N	SER	A	77	34.222	-6.796	20.425	1.00	17.56	N
ATOM	308	CA	SER	A	77	34.702	-7.356	21.679	1.00	17.62	C
ATOM	309	C	SER	A	77	36.168	-7.021	21.913	1.00	17.42	C
ATOM	310	O	SER	A	77	36.844	-6.512	21.007	1.00	16.62	O
ATOM	311	CB	SER	A	77	34.372	-8.855	21.692	1.00	17.73	C
ATOM	312	OG	SER	A	77	35.120	-9.526	20.756	1.00	19.60	O
ATOM	313	N	LYS	A	78	36.648	-7.232	23.138	1.00	16.65	N
ATOM	314	CA	LYS	A	78	38.014	-6.916	23.508	1.00	17.96	C
ATOM	315	C	LYS	A	78	38.960	-8.011	23.025	1.00	15.54	C
ATOM	316	O	LYS	A	78	39.502	-8.817	23.810	1.00	16.44	O
ATOM	317	CB	LYS	A	78	38.110	-6.685	25.029	1.00	19.33	C
ATOM	318	CG	LYS	A	78	37.421	-5.410	25.461	1.00	22.65	C
ATOM	319	CD	LYS	A	78	37.592	-5.160	26.951	1.00	29.68	C
ATOM	320	CE	LYS	A	78	36.865	-3.909	27.398	0.50	29.69	C
ATOM	321	NZ	LYS	A	78	35.626	-4.256	28.096	0.50	31.88	N
ATOM	322	N	VAL	A	79	39.032	-8.107	21.719	1.00	13.24	N
ATOM	323	CA	VAL	A	79	39.835	-9.140	20.998	1.00	11.28	C
ATOM	324	C	VAL	A	79	40.718	-8.379	20.016	1.00	11.24	C
ATOM	325	O	VAL	A	79	40.250	-7.445	19.423	1.00	10.54	O
ATOM	326	CB	VAL	A	79	38.908	-10.110	20.298	1.00	10.87	C
ATOM	327	CG1	VAL	A	79	39.677	-10.968	19.273	1.00	8.21	C
ATOM	328	CG2	VAL	A	79	38.164	-10.988	21.349	1.00	11.11	C
ATOM	329	N	ILE	A	80	41.990	-8.770	19.838	1.00	10.10	N
ATOM	330	CA	ILE	A	80	42.879	-8.093	18.881	1.00	9.24	C
ATOM	331	C	ILE	A	80	43.544	-9.132	17.976	1.00	10.02	C
ATOM	332	O	ILE	A	80	43.897	-10.227	18.457	1.00	10.54	O
ATOM	333	CB	ILE	A	80	43.886	-7.224	19.610	1.00	8.37	C
ATOM	334	CG1	ILE	A	80	44.663	-6.318	18.710	1.00	10.35	C
ATOM	335	CG2	ILE	A	80	44.875	-8.072	20.569	1.00	12.57	C
ATOM	336	CD1	ILE	A	80	45.447	-5.229	19.513	1.00	8.59	C
ATOM	337	N	ALA	A	81	43.670	-8.801	16.680	1.00	10.13	N
ATOM	338	CA	ALA	A	81	44.428	-9.574	15.767	1.00	10.20	C
ATOM	339	C	ALA	A	81	45.760	-8.886	15.489	1.00	10.65	C
ATOM	340	O	ALA	A	81	45.796	-7.689	15.195	1.00	9.01	O
ATOM	341	CB	ALA	A	81	43.645	-9.777	14.484	1.00	10.33	C

ATOM	342	N	LEU	A	82	46.850	-9.668	15.527	1.00	11.14	N
ATOM	343	CA	LEU	A	82	48.197	-9.168	15.350	1.00	12.53	C
ATOM	344	C	LEU	A	82	48.890	-9.967	14.287	1.00	13.97	C
ATOM	345	O	LEU	A	82	48.804	-11.194	14.226	1.00	14.12	O
ATOM	346	CB	LEU	A	82	48.950	-9.317	16.680	1.00	14.32	C
ATOM	347	CG	LEU	A	82	48.415	-8.573	17.888	1.00	16.99	C
ATOM	348	CD1	LEU	A	82	49.197	-9.000	19.129	1.00	16.28	C
ATOM	349	CD2	LEU	A	82	48.515	-7.035	17.632	1.00	16.08	C
ATOM	350	N	LYS	A	83	49.616	-9.288	13.428	1.00	13.35	N
ATOM	351	CA	LYS	A	83	50.403	-10.065	12.485	1.00	16.86	C
ATOM	352	C	LYS	A	83	51.831	-9.637	12.464	1.00	17.22	C
ATOM	353	O	LYS	A	83	52.124	-8.456	12.664	1.00	16.15	O
ATOM	354	CB	LYS	A	83	49.728	-10.075	11.152	1.00	18.96	C
ATOM	355	CG	LYS	A	83	49.767	-8.903	10.344	1.00	22.69	C
ATOM	356	CD	LYS	A	83	49.215	-9.278	8.965	1.00	26.83	C
ATOM	357	CE	LYS	A	83	49.053	-8.067	8.080	1.00	24.86	C
ATOM	358	NZ	LYS	A	83	50.441	-7.575	7.669	1.00	29.62	N
ATOM	359	N	ALA	A	84	52.724	-10.616	12.301	1.00	18.24	N
ATOM	360	CA	ALA	A	84	54.186	-10.412	12.279	1.00	21.08	C
ATOM	361	C	ALA	A	84	54.680	-11.234	11.127	1.00	22.26	C
ATOM	362	O	ALA	A	84	54.773	-12.452	11.227	1.00	22.42	O
ATOM	363	CB	ALA	A	84	54.796	-10.911	13.554	1.00	21.11	C
ATOM	364	N	GLY	A	85	54.847	-10.590	10.003	1.00	24.62	N
ATOM	365	CA	GLY	A	85	55.088	-11.274	8.730	1.00	25.32	C
ATOM	366	C	GLY	A	85	53.958	-12.247	8.381	1.00	24.04	C
ATOM	367	O	GLY	A	85	52.796	-11.872	8.337	1.00	25.46	O
ATOM	368	N	LYS	A	86	54.292	-13.521	8.181	1.00	21.50	N
ATOM	369	CA	LYS	A	86	53.277	-14.468	7.728	1.00	19.35	C
ATOM	370	C	LYS	A	86	52.519	-15.079	8.893	1.00	17.67	C
ATOM	371	O	LYS	A	86	51.569	-15.852	8.641	1.00	17.11	O
ATOM	372	CB	LYS	A	86	53.937	-15.586	6.872	1.00	19.66	C
ATOM	373	CG	LYS	A	86	54.459	-15.093	5.516	1.00	22.19	C
ATOM	374	CD	LYS	A	86	55.199	-16.172	4.722	1.00	26.28	C
ATOM	375	CE	LYS	A	86	55.697	-15.675	3.384	1.00	29.54	C
ATOM	376	NZ	LYS	A	86	56.583	-14.544	3.578	1.00	31.87	N
ATOM	377	N	THR	A	87	52.901	-14.763	10.141	1.00	16.69	N
ATOM	378	CA	THR	A	87	52.253	-15.362	11.303	1.00	17.31	C
ATOM	379	C	THR	A	87	51.153	-14.447	11.841	1.00	15.11	C
ATOM	380	O	THR	A	87	51.385	-13.295	12.157	1.00	16.50	O
ATOM	381	CB	THR	A	87	53.210	-15.667	12.450	1.00	19.78	C
ATOM	382	CG2	THR	A	87	52.476	-16.404	13.602	1.00	21.61	C
ATOM	383	OG1	THR	A	87	54.229	-16.539	11.936	1.00	25.53	O
ATOM	384	N	LEU	A	88	49.985	-15.025	11.981	1.00	13.61	N
ATOM	385	CA	LEU	A	88	48.779	-14.276	12.437	1.00	13.03	C
ATOM	386	C	LEU	A	88	48.384	-14.803	13.788	1.00	11.19	C
ATOM	387	O	LEU	A	88	48.388	-16.008	13.989	1.00	13.57	O
ATOM	388	CB	LEU	A	88	47.651	-14.497	11.432	1.00	13.64	C
ATOM	389	CG	LEU	A	88	47.907	-14.413	9.906	1.00	13.51	C
ATOM	390	CD1	LEU	A	88	46.609	-14.732	9.162	1.00	16.02	C
ATOM	391	CD2	LEU	A	88	48.502	-13.077	9.506	1.00	15.18	C

ATOM	392	N	GLN	A	89	48.005	-13.948	14.742	1.00	10.61	N
ATOM	393	CA	GLN	A	89	47.501	-14.381	15.999	1.00	11.17	C
ATOM	394	C	GLN	A	89	46.298	-13.545	16.366	1.00	11.64	C
ATOM	395	O	GLN	A	89	46.208	-12.356	16.018	1.00	13.08	O
ATOM	396	CB	GLN	A	89	48.564	-14.249	17.085	1.00	13.19	C
ATOM	397	CG	GLN	A	89	49.810	-15.094	16.808	1.00	15.83	C
ATOM	398	CD	GLN	A	89	50.749	-15.031	18.014	1.00	24.48	C
ATOM	399	NE2	GLN	A	89	51.475	-13.930	18.143	1.00	26.63	N
ATOM	400	OE1	GLN	A	89	50.773	-15.930	18.825	1.00	29.59	O
ATOM	401	N	ILE	A	90	45.376	-14.148	17.087	1.00	11.30	N
ATOM	402	CA	ILE	A	90	44.257	-13.412	17.658	1.00	10.86	C
ATOM	403	C	ILE	A	90	44.293	-13.673	19.173	1.00	12.18	C
ATOM	404	O	ILE	A	90	44.453	-14.817	19.601	1.00	12.01	O
ATOM	405	CB	ILE	A	90	42.951	-13.937	17.063	1.00	9.91	C
ATOM	406	CG1	ILE	A	90	42.994	-13.868	15.538	1.00	10.93	C
ATOM	407	CG2	ILE	A	90	41.752	-13.173	17.672	1.00	8.39	C
ATOM	408	CD1	ILE	A	90	41.689	-14.531	14.826	1.00	12.30	C
ATOM	409	N	PHE	A	91	44.187	-12.609	19.979	1.00	11.33	N
ATOM	410	CA	PHE	A	91	44.182	-12.653	21.436	1.00	11.82	C
ATOM	411	C	PHE	A	91	42.946	-12.106	22.080	1.00	14.39	C
ATOM	412	O	PHE	A	91	42.402	-11.092	21.653	1.00	13.16	O
ATOM	413	CB	PHE	A	91	45.289	-11.822	21.975	1.00	13.35	C
ATOM	414	CG	PHE	A	91	46.575	-12.395	21.752	1.00	16.16	C
ATOM	415	CD1	PHE	A	91	47.245	-12.140	20.591	1.00	18.73	C
ATOM	416	CD2	PHE	A	91	47.145	-13.253	22.725	1.00	18.04	C
ATOM	417	CE1	PHE	A	91	48.549	-12.688	20.354	1.00	20.46	C
ATOM	418	CE2	PHE	A	91	48.403	-13.843	22.472	1.00	23.63	C
ATOM	419	CZ	PHE	A	91	49.084	-13.540	21.290	1.00	23.16	C
ATOM	420	N	ASN	A	92	42.571	-12.767	23.154	1.00	12.67	N
ATOM	421	CA	ASN	A	92	41.521	-12.359	24.018	1.00	12.14	C
ATOM	422	C	ASN	A	92	42.152	-11.435	25.102	1.00	13.70	C
ATOM	423	O	ASN	A	92	42.849	-11.894	25.990	1.00	11.95	O
ATOM	424	CB	ASN	A	92	40.866	-13.557	24.689	1.00	11.38	C
ATOM	425	CG	ASN	A	92	39.657	-13.145	25.521	1.00	13.86	C
ATOM	426	ND2	ASN	A	92	38.620	-13.965	25.520	1.00	17.37	N
ATOM	427	OD1	ASN	A	92	39.675	-12.103	26.185	1.00	13.44	O
ATOM	428	N	ILE	A	93	41.897	-10.136	24.997	1.00	12.66	N
ATOM	429	CA	ILE	A	93	42.519	-9.159	25.899	1.00	13.91	C
ATOM	430	C	ILE	A	93	42.106	-9.307	27.366	1.00	13.34	C
ATOM	431	O	ILE	A	93	42.966	-9.264	28.250	1.00	12.95	O
ATOM	432	CB	ILE	A	93	42.221	-7.682	25.396	1.00	13.53	C
ATOM	433	CG1	ILE	A	93	42.918	-7.413	24.076	1.00	16.05	C
ATOM	434	CG2	ILE	A	93	42.700	-6.646	26.481	1.00	16.28	C
ATOM	435	CD1	ILE	A	93	42.332	-6.157	23.403	1.00	17.94	C
ATOM	436	N	GLU	A	94	40.801	-9.459	27.594	1.00	13.03	N
ATOM	437	CA	GLU	A	94	40.208	-9.528	28.900	1.00	15.01	C
ATOM	438	C	GLU	A	94	40.803	-10.740	29.637	1.00	13.02	C
ATOM	439	O	GLU	A	94	41.110	-10.640	30.845	1.00	13.76	O
ATOM	440	CB	GLU	A	94	38.700	-9.690	28.826	1.00	15.74	C
ATOM	441	CG	GLU	A	94	38.033	-9.799	30.204	1.00	21.81	C

ATOM	442	CD	GLU	A	94	36.508	-9.927	30.142	0.50	23.32	C
ATOM	443	OE1	GLU	A	94	35.915	-9.820	29.055	0.50	24.55	O
ATOM	444	OE2	GLU	A	94	35.907	-10.148	31.213	0.50	26.13	O
ATOM	445	N	MET	A	95	40.994	-11.847	28.915	1.00	11.90	N
ATOM	446	CA	MET	A	95	41.468	-13.098	29.527	1.00	10.79	C
ATOM	447	C	MET	A	95	42.943	-13.306	29.363	1.00	10.65	C
ATOM	448	O	MET	A	95	43.483	-14.338	29.773	1.00	9.50	O
ATOM	449	CB	MET	A	95	40.721	-14.314	28.935	1.00	11.22	C
ATOM	450	CG	MET	A	95	39.230	-14.176	29.008	1.00	11.84	C
ATOM	451	SD	MET	A	95	38.587	-13.868	30.670	1.00	15.49	S
ATOM	452	CE	MET	A	95	38.754	-15.488	31.432	1.00	17.04	C
ATOM	453	N	LYS	A	96	43.590	-12.327	28.747	1.00	10.71	N
ATOM	454	CA	LYS	A	96	45.016	-12.290	28.554	1.00	11.04	C
ATOM	455	C	LYS	A	96	45.559	-13.592	27.981	1.00	10.35	C
ATOM	456	O	LYS	A	96	46.611	-14.108	28.484	1.00	12.40	O
ATOM	457	CB	LYS	A	96	45.705	-11.936	29.869	1.00	10.99	C
ATOM	458	CG	LYS	A	96	45.291	-10.539	30.468	1.00	12.25	C
ATOM	459	CD	LYS	A	96	45.893	-10.400	31.864	1.00	18.38	C
ATOM	460	CE	LYS	A	96	45.585	-9.061	32.540	1.00	21.55	C
ATOM	461	NZ	LYS	A	96	46.118	-8.976	33.938	1.00	23.90	N
ATOM	462	N	SER	A	97	44.907	-14.080	26.905	1.00	8.70	N
ATOM	463	CA	SER	A	97	45.167	-15.385	26.362	1.00	9.06	C
ATOM	464	C	SER	A	97	45.057	-15.419	24.866	1.00	10.27	C
ATOM	465	O	SER	A	97	44.166	-14.777	24.275	1.00	9.05	O
ATOM	466	CB	SER	A	97	44.149	-16.396	26.945	1.00	8.63	C
ATOM	467	OG	SER	A	97	44.349	-16.585	28.374	1.00	9.66	O
ATOM	468	N	LYS	A	98	45.955	-16.180	24.271	1.00	9.58	N
ATOM	469	CA	LYS	A	98	45.905	-16.470	22.851	1.00	12.34	C
ATOM	470	C	LYS	A	98	44.619	-17.243	22.513	1.00	12.71	C
ATOM	471	O	LYS	A	98	44.252	-18.186	23.225	1.00	12.32	O
ATOM	472	CB	LYS	A	98	47.126	-17.279	22.439	1.00	13.14	C
ATOM	473	CG	LYS	A	98	47.151	-17.518	20.916	1.00	17.23	C
ATOM	474	CD	LYS	A	98	48.510	-18.055	20.395	1.00	22.42	C
ATOM	475	CE	LYS	A	98	48.587	-19.530	20.716	1.00	27.89	C
ATOM	476	NZ	LYS	A	98	49.800	-20.231	20.143	0.50	25.71	N
ATOM	477	N	MET	A	99	43.904	-16.828	21.468	1.00	10.99	N
ATOM	478	CA	MET	A	99	42.752	-17.554	20.959	1.00	11.09	C
ATOM	479	C	MET	A	99	43.136	-18.494	19.820	1.00	12.42	C
ATOM	480	O	MET	A	99	42.816	-19.673	19.879	1.00	14.20	O
ATOM	481	CB	MET	A	99	41.656	-16.596	20.480	1.00	10.49	C
ATOM	482	CG	MET	A	99	41.092	-15.776	21.588	1.00	11.32	C
ATOM	483	SD	MET	A	99	40.196	-14.290	21.064	1.00	11.24	S
ATOM	484	CE	MET	A	99	38.833	-15.048	20.147	1.00	12.11	C
ATOM	485	N	LYS	A	100	43.789	-17.983	18.771	1.00	11.30	N
ATOM	486	CA	LYS	A	100	44.097	-18.742	17.588	1.00	12.76	C
ATOM	487	C	LYS	A	100	45.322	-18.207	16.973	1.00	12.59	C
ATOM	488	O	LYS	A	100	45.689	-17.066	17.202	1.00	10.73	O
ATOM	489	CB	LYS	A	100	42.961	-18.626	16.498	1.00	14.08	C
ATOM	490	CG	LYS	A	100	41.728	-19.162	16.910	1.00	17.66	C
ATOM	491	CD	LYS	A	100	40.738	-19.125	15.715	1.00	15.32	C

ATOM	492	CE	LYS	A	100	40.912	-20.102	14.630	1.00	14.27	C
ATOM	493	NZ	LYS	A	100	39.631	-20.213	13.751	1.00	10.12	N
ATOM	494	N	ALA	A	101	45.972	-19.027	16.146	1.00	13.10	N
ATOM	495	CA	ALA	A	101	47.121	-18.610	15.381	1.00	12.51	C
ATOM	496	C	ALA	A	101	47.132	-19.344	14.041	1.00	13.84	C
ATOM	497	O	ALA	A	101	46.627	-20.474	13.942	1.00	14.55	O
ATOM	498	CB	ALA	A	101	48.484	-18.875	16.107	1.00	14.11	C
ATOM	499	N	HIS	A	102	47.753	-18.724	13.064	1.00	13.05	N
ATOM	500	CA	HIS	A	102	47.925	-19.326	11.728	1.00	13.59	C
ATOM	501	C	HIS	A	102	49.134	-18.766	11.057	1.00	15.57	C
ATOM	502	O	HIS	A	102	49.380	-17.541	11.083	1.00	15.80	O
ATOM	503	CB	HIS	A	102	46.689	-19.101	10.840	1.00	13.94	C
ATOM	504	CG	HIS	A	102	46.761	-19.792	9.530	1.00	14.45	C
ATOM	505	CD2	HIS	A	102	46.941	-19.311	8.275	1.00	14.89	C
ATOM	506	ND1	HIS	A	102	46.672	-21.167	9.409	1.00	14.15	N
ATOM	507	CE1	HIS	A	102	46.801	-21.488	8.135	1.00	14.40	C
ATOM	508	NE2	HIS	A	102	46.957	-20.387	7.429	1.00	15.29	N
TER	509		HIS	A	102						
HETATM	510	CAG	VH1	A1362		50.186	-13.396	26.460	0.80	30.10	C
HETATM	511	CAH	VH1	A1362		46.175	-6.267	26.783	0.80	28.62	C
HETATM	512	CAI	VH1	A1362		46.792	-5.412	24.605	0.80	26.42	C
HETATM	513	CAJ	VH1	A1362		46.083	-7.562	26.251	0.80	28.51	C
HETATM	514	CAK	VH1	A1362		46.726	-6.707	24.082	0.80	25.54	C
HETATM	515	CAL	VH1	A1362		51.120	-13.250	25.426	0.80	29.27	C
HETATM	516	CAM	VH1	A1362		49.023	-12.595	26.420	0.80	30.35	C
HETATM	517	CAN	VH1	A1362		51.843	-12.177	23.342	0.80	24.10	C
HETATM	518	CAO	VH1	A1362		50.505	-10.467	22.275	0.80	22.60	C
HETATM	519	CAP	VH1	A1362		46.263	-9.156	24.281	0.80	22.66	C
HETATM	520	CAQ	VH1	A1362		46.519	-5.194	25.959	0.80	29.60	C
HETATM	521	CAR	VH1	A1362		46.368	-7.776	24.912	0.80	21.90	C
HETATM	522	CAS	VH1	A1362		51.640	-11.256	22.308	0.80	24.56	C
HETATM	523	CAT	VH1	A1362		47.653	-10.887	25.330	0.80	24.94	C
HETATM	524	CAU	VH1	A1362		48.410	-9.827	23.298	0.80	20.53	C
HETATM	525	CAV	VH1	A1362		50.909	-12.340	24.374	0.80	25.77	C
HETATM	526	CAW	VH1	A1362		48.809	-11.669	25.368	0.80	25.65	C
HETATM	527	CAX	VH1	A1362		49.574	-10.597	23.292	0.80	20.71	C
HETATM	528	CAY	VH1	A1362		49.759	-11.529	24.349	0.80	23.66	C
HETATM	529	NAA	VH1	A1362		46.591	-3.940	26.474	0.80	27.32	N
HETATM	530	NAZ	VH1	A1362		47.490	-9.962	24.313	0.80	20.26	N
HETATM	531	OAB	VH1	A1362		46.796	-10.934	26.195	0.80	25.17	O
HETATM	532	OAC	VH1	A1362		48.157	-9.013	22.429	0.80	18.57	O
HETATM	533	OAD	VH1	A1362		52.809	-9.895	20.252	0.80	31.87	O
HETATM	534	OAE	VH1	A1362		54.156	-11.415	21.663	0.80	31.97	O
HETATM	535	OAF	VH1	A1362		52.613	-12.271	20.183	0.80	32.82	O
HETATM	536	SBA	VH1	A1362		52.845	-11.172	21.020	0.80	29.41	S
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CLATHRIN TERMINAL DOMAIN COMPLEXED WITH PITSTOP 2

FIGURE 14

FRAGMENT: 1 AND 2 BLADES, RESIDUES 37-101

RESIDUES IN CLOSE CONTACT WITH PITSTOP2 ($\leq 3.9\text{\AA}$)

Type	A. #	Name	Res. Name	Res. #	X-coor.	Y-coor.	Z-coor.	Occ.	Temp. F	A. Type
ATOM	1	N	PHE A	37	36.766	1.228	18.851	1.00	10.73	N
ATOM	2	CA	PHE A	37	38.185	1.273	19.125	1.00	9.63	C
ATOM	3	C	PHE A	37	38.956	1.961	18.023	1.00	10.00	C
ATOM	4	O	PHE A	37	38.591	1.882	16.858	1.00	9.67	O
ATOM	5	CB	PHE A	37	38.820	-0.149	19.283	1.00	10.20	C
ATOM	6	CG	PHE A	37	38.217	-1.015	20.347	1.00	9.95	C
ATOM	7	CD1	PHE A	37	38.496	-0.777	21.707	1.00	13.78	C
ATOM	8	CD2	PHE A	37	37.370	-2.041	20.008	1.00	13.38	C
ATOM	9	CE1	PHE A	37	37.896	-1.542	22.617	1.00	16.57	C
ATOM	10	CE2	PHE A	37	36.798	-2.809	20.953	1.00	16.03	C
ATOM	11	CZ	PHE A	37	37.084	-2.574	22.247	1.00	15.17	C
ATOM	12	N	ILE A	38	40.058	2.610	18.421	1.00	10.11	N
ATOM	13	CA	ILE A	38	41.154	3.008	17.498	1.00	10.49	C
ATOM	14	C	ILE A	38	42.446	2.324	17.894	1.00	12.02	C
ATOM	15	O	ILE A	38	42.809	2.354	19.068	1.00	13.06	O
ATOM	16	CB	ILE A	38	41.325	4.561	17.377	1.00	9.36	C
ATOM	17	CG1	ILE A	38	40.065	5.142	16.792	1.00	10.67	C
ATOM	18	CG2	ILE A	38	42.561	4.981	16.536	1.00	10.38	C
ATOM	19	CD1	ILE A	38	39.946	6.706	17.018	1.00	12.34	C
ATOM	20	N	CYS A	39	43.170	1.769	16.905	1.00	11.47	N
ATOM	21	CA	CYS A	39	44.400	1.088	17.222	1.00	10.18	C
ATOM	22	C	CYS A	39	45.561	1.739	16.422	1.00	10.54	C
ATOM	23	O	CYS A	39	45.470	1.895	15.204	1.00	10.46	O
ATOM	24	CB	CYS A	39	44.216	-0.393	16.931	1.00	10.68	C
ATOM	25	SG	CYS A	39	45.736	-1.331	17.222	1.00	15.01	S
ATOM	26	N	ILE A	40	46.647	2.144	17.095	1.00	11.49	N
ATOM	27	CA	ILE A	40	47.744	2.853	16.491	1.00	11.01	C
ATOM	28	C	ILE A	40	49.057	2.099	16.766	1.00	12.86	C
ATOM	29	O	ILE A	40	49.355	1.774	17.908	1.00	12.32	O
ATOM	30	CB	ILE A	40	47.911	4.290	16.995	1.00	11.04	C
ATOM	31	CG1	ILE A	40	46.537	5.020	16.993	1.00	12.48	C
ATOM	32	CG2	ILE A	40	48.879	5.062	16.147	1.00	11.56	C
ATOM	33	CD1	ILE A	40	45.829	4.935	18.363	1.00	15.65	C
ATOM	34	N	ARG A	41	49.796	1.883	15.723	1.00	13.18	N
ATOM	35	CA	ARG A	41	51.194	1.320	15.830	1.00	13.56	C
ATOM	36	C	ARG A	41	52.217	2.440	15.852	1.00	14.27	C
ATOM	37	O	ARG A	41	52.259	3.285	14.943	1.00	15.22	O
ATOM	38	CB	ARG A	41	51.483	0.402	14.641	1.00	13.52	C
ATOM	39	CG	ARG A	41	50.678	-0.860	14.669	1.00	12.64	C
ATOM	40	CD	ARG A	41	51.043	-1.849	13.649	1.00	12.74	C
ATOM	41	NE	ARG A	41	51.139	-1.255	12.340	1.00	15.60	N

ATOM	42	CZ	ARG	A	41	52.262	-0.979	11.676	1.00	13.94	C
ATOM	43	NH1	ARG	A	41	53.437	-1.448	12.053	1.00	16.85	N
ATOM	44	NH2	ARG	A	41	52.228	-0.334	10.529	1.00	19.00	N
ATOM	45	N	GLU	A	42	53.053	2.493	16.898	1.00	15.10	N
ATOM	46	CA	GLU	A	42	54.009	3.585	17.023	1.00	16.45	C
ATOM	47	C	GLU	A	42	55.410	3.021	17.313	1.00	20.30	C
ATOM	48	O	GLU	A	42	55.530	2.070	18.064	1.00	21.23	O
ATOM	49	CB	GLU	A	42	53.681	4.579	18.130	1.00	17.31	C
ATOM	50	CG	GLU	A	42	52.196	5.120	18.160	1.00	19.89	C
ATOM	51	CD	GLU	A	42	52.171	6.520	18.769	1.00	20.44	C
ATOM	52	OE1	GLU	A	42	52.301	6.620	20.009	1.00	20.86	O
ATOM	53	OE2	GLU	A	42	52.111	7.513	18.027	1.00	16.76	O
ATOM	54	N	LYS	A	43	56.446	3.618	16.754	1.00	22.01	N
ATOM	55	CA	LYS	A	43	57.810	3.150	17.083	1.00	25.35	C
ATOM	56	C	LYS	A	43	58.368	4.261	17.925	1.00	27.76	C
ATOM	57	O	LYS	A	43	58.510	5.385	17.406	1.00	27.46	O
ATOM	58	CB	LYS	A	43	58.652	2.935	15.840	1.00	26.10	C
ATOM	59	CG	LYS	A	43	60.135	2.490	16.146	1.00	29.78	C
ATOM	60	CD	LYS	A	43	60.541	1.281	15.320	1.00	37.68	C
ATOM	61	CE	LYS	A	43	59.938	-0.069	15.881	1.00	38.50	C
ATOM	62	NZ	LYS	A	43	60.254	-1.271	14.997	1.00	41.43	N
ATOM	63	N	VAL	A	44	58.579	4.000	19.217	1.00	31.09	N
ATOM	64	CA	VAL	A	44	59.140	5.020	20.152	1.00	32.37	C
ATOM	65	C	VAL	A	44	60.537	4.594	20.595	1.00	33.73	C
ATOM	66	O	VAL	A	44	60.724	3.504	21.137	1.00	32.74	O
ATOM	67	CB	VAL	A	44	58.243	5.294	21.369	1.00	33.01	C
ATOM	68	CG1	VAL	A	44	56.817	5.536	20.915	1.00	33.32	C
ATOM	69	CG2	VAL	A	44	58.335	4.147	22.405	1.00	36.14	C
ATOM	70	N	GLY	A	45	61.531	5.435	20.301	1.00	35.07	N
ATOM	71	CA	GLY	A	45	62.902	4.946	20.279	1.00	35.90	C
ATOM	72	C	GLY	A	45	63.101	3.743	19.349	1.00	36.87	C
ATOM	73	O	GLY	A	45	62.850	3.812	18.143	1.00	37.49	O
ATOM	74	N	GLU	A	46	63.547	2.610	19.890	1.00	37.53	N
ATOM	75	CA	GLU	A	46	63.710	1.433	19.034	1.00	37.71	C
ATOM	76	C	GLU	A	46	62.595	0.438	19.295	1.00	35.62	C
ATOM	77	O	GLU	A	46	62.603	-0.666	18.751	1.00	36.98	O
ATOM	78	CB	GLU	A	46	65.094	0.779	19.223	1.00	38.86	C
ATOM	79	CG	GLU	A	46	66.280	1.568	18.597	1.00	41.68	C
ATOM	80	CD	GLU	A	46	66.059	1.876	17.126	1.00	45.89	C
ATOM	81	OE1	GLU	A	46	65.774	0.931	16.352	1.00	48.59	O
ATOM	82	OE2	GLU	A	46	66.148	3.064	16.742	1.00	50.29	O
ATOM	83	N	GLN	A	47	61.598	0.868	20.065	1.00	32.74	N
ATOM	84	CA	GLN	A	47	60.574	-0.028	20.552	1.00	30.13	C
ATOM	85	C	GLN	A	47	59.264	0.070	19.759	1.00	27.49	C
ATOM	86	O	GLN	A	47	58.640	1.134	19.778	1.00	24.95	O
ATOM	87	CB	GLN	A	47	60.249	0.332	21.962	1.00	30.56	C
ATOM	88	CG	GLN	A	47	59.543	-0.759	22.676	1.00	32.91	C
ATOM	89	CD	GLN	A	47	59.310	-0.375	24.111	1.00	34.29	C
ATOM	90	NE2	GLN	A	47	60.406	-0.334	24.920	1.00	37.38	N
ATOM	91	OE1	GLN	A	47	58.190	-0.048	24.492	1.00	34.57	O

ATOM	92	N	ALA	A	48	58.837	-1.037	19.136	1.00	24.90	N
ATOM	93	CA	ALA	A	48	57.486	-1.081	18.517	1.00	24.04	C
ATOM	94	C	ALA	A	48	56.435	-1.117	19.591	1.00	23.41	C
ATOM	95	O	ALA	A	48	56.502	-1.937	20.496	1.00	24.25	O
ATOM	96	CB	ALA	A	48	57.310	-2.269	17.563	1.00	22.88	C
ATOM	97	N	GLN	A	49	55.435	-0.231	19.504	1.00	20.98	N
ATOM	98	CA	GLN	A	49	54.372	-0.254	20.447	1.00	20.24	C
ATOM	99	C	GLN	A	49	53.046	-0.199	19.737	1.00	19.59	C
ATOM	100	O	GLN	A	49	52.983	0.278	18.604	1.00	20.68	O
ATOM	101	CB	GLN	A	49	54.418	0.936	21.379	1.00	20.99	C
ATOM	102	CG	GLN	A	49	55.763	1.120	22.092	1.00	23.25	C
ATOM	103	CD	GLN	A	49	55.829	2.348	22.967	1.00	24.11	C
ATOM	104	NE2	GLN	A	49	56.707	2.291	23.979	1.00	31.71	N
ATOM	105	OE1	GLN	A	49	55.128	3.345	22.761	1.00	22.97	O
ATOM	106	N	VAL	A	50	52.043	-0.739	20.384	1.00	17.98	N
ATOM	107	CA	VAL	A	50	50.643	-0.700	19.870	1.00	18.61	C
ATOM	108	C	VAL	A	50	49.779	-0.043	20.942	1.00	16.71	C
ATOM	109	O	VAL	A	50	49.853	-0.381	22.159	1.00	17.03	O
ATOM	110	CB	VAL	A	50	50.162	-2.029	19.496	1.00	17.99	C
ATOM	111	CG1	VAL	A	50	48.747	-1.957	18.924	1.00	21.60	C
ATOM	112	CG2	VAL	A	50	51.106	-2.668	18.480	1.00	18.77	C
ATOM	113	N	VAL	A	51	48.983	0.934	20.546	1.00	14.03	N
ATOM	114	CA	VAL	A	51	48.137	1.674	21.439	1.00	13.92	C
ATOM	115	C	VAL	A	51	46.700	1.321	21.020	1.00	14.32	C
ATOM	116	O	VAL	A	51	46.392	1.368	19.820	1.00	14.13	O
ATOM	117	CB	VAL	A	51	48.291	3.201	21.290	1.00	14.32	C
ATOM	118	CG1	VAL	A	51	47.459	3.918	22.236	1.00	14.96	C
ATOM	119	CG2	VAL	A	51	49.742	3.665	21.466	1.00	19.47	C
ATOM	120	N	ILE	A	52	45.899	0.970	21.999	1.00	12.92	N
ATOM	121	CA	ILE	A	52	44.425	0.763	21.834	1.00	13.58	C
ATOM	122	C	ILE	A	52	43.643	1.851	22.592	1.00	15.99	C
ATOM	123	O	ILE	A	52	43.754	1.998	23.830	1.00	18.98	O
ATOM	124	CB	ILE	A	52	43.980	-0.641	22.280	1.00	14.38	C
ATOM	125	CG1	ILE	A	52	44.772	-1.706	21.505	1.00	15.39	C
ATOM	126	CG2	ILE	A	52	42.507	-0.792	22.033	1.00	12.30	C
ATOM	127	CD1	ILE	A	52	44.673	-3.120	22.122	1.00	18.71	C
ATOM	128	N	ILE	A	53	42.883	2.633	21.814	1.00	13.92	N
ATOM	129	CA	ILE	A	53	41.995	3.605	22.368	1.00	14.96	C
ATOM	130	C	ILE	A	53	40.588	3.019	22.381	1.00	14.39	C
ATOM	131	O	ILE	A	53	40.042	2.659	21.337	1.00	14.64	O
ATOM	132	CB	ILE	A	53	42.047	4.916	21.559	1.00	13.47	C
ATOM	133	CG1	ILE	A	53	43.456	5.451	21.569	1.00	18.71	C
ATOM	134	CG2	ILE	A	53	41.033	5.877	22.140	1.00	14.76	C
ATOM	135	CD1	ILE	A	53	43.666	6.635	20.682	1.00	23.00	C
ATOM	136	N	ASP	A	54	40.038	2.859	23.582	1.00	15.32	N
ATOM	137	CA	ASP	A	54	38.667	2.456	23.802	1.00	15.57	C
ATOM	138	C	ASP	A	54	37.854	3.702	23.748	1.00	16.57	C
ATOM	139	O	ASP	A	54	38.060	4.596	24.557	1.00	17.42	O
ATOM	140	CB	ASP	A	54	38.521	1.850	25.193	1.00	17.18	C
ATOM	141	CG	ASP	A	54	37.105	1.363	25.464	1.00	20.39	C

ATOM	142	OD1	ASP	A	54	36.115	1.833	24.867	1.00	17.11	O
ATOM	143	OD2	ASP	A	54	36.990	0.448	26.317	1.00	24.32	O
ATOM	144	N	MET	A	55	36.954	3.810	22.772	1.00	16.69	N
ATOM	145	CA	MET	A	55	36.204	5.057	22.556	1.00	17.21	C
ATOM	146	C	MET	A	55	35.223	5.358	23.703	1.00	19.54	C
ATOM	147	O	MET	A	55	34.788	6.473	23.840	1.00	21.25	O
ATOM	148	CB	MET	A	55	35.465	4.999	21.225	1.00	15.24	C
ATOM	149	CG	MET	A	55	36.373	4.854	20.110	1.00	17.76	C
ATOM	150	SD	MET	A	55	37.453	6.258	19.894	1.00	22.55	S
ATOM	151	CE	MET	A	55	36.356	7.383	19.090	1.00	20.53	C
ATOM	152	N	ASN	A	56	34.882	4.348	24.496	1.00	21.29	N
ATOM	153	CA	ASN	A	56	34.188	4.556	25.761	1.00	24.84	C
ATOM	154	C	ASN	A	56	35.032	5.001	26.941	1.00	24.86	C
ATOM	155	O	ASN	A	56	34.491	5.316	28.010	1.00	24.62	O
ATOM	156	CB	ASN	A	56	33.531	3.236	26.188	1.00	26.26	C
ATOM	157	CG	ASN	A	56	32.288	2.947	25.464	1.00	29.73	C
ATOM	158	ND2	ASN	A	56	31.879	1.674	25.537	1.00	40.47	N
ATOM	159	OD1	ASN	A	56	31.658	3.816	24.862	1.00	36.35	O
ATOM	160	N	ASP	A	57	36.342	4.967	26.813	1.00	23.67	N
ATOM	161	CA	ASP	A	57	37.243	5.356	27.879	1.00	24.96	C
ATOM	162	C	ASP	A	57	38.518	5.908	27.258	1.00	23.56	C
ATOM	163	O	ASP	A	57	39.627	5.380	27.473	1.00	21.10	O
ATOM	164	CB	ASP	A	57	37.557	4.136	28.759	1.00	26.20	C
ATOM	165	CG	ASP	A	57	38.366	4.496	29.995	1.00	28.57	C
ATOM	166	OD1	ASP	A	57	38.302	5.674	30.408	1.00	30.05	O
ATOM	167	OD2	ASP	A	57	39.062	3.574	30.523	1.00	35.49	O
ATOM	168	N	PRO	A	58	38.375	6.957	26.425	1.00	24.35	N
ATOM	169	CA	PRO	A	58	39.527	7.334	25.580	1.00	25.78	C
ATOM	170	C	PRO	A	58	40.668	7.981	26.298	1.00	28.87	C
ATOM	171	O	PRO	A	58	41.831	7.976	25.807	1.00	29.52	O
ATOM	172	CB	PRO	A	58	38.919	8.310	24.578	1.00	25.58	C
ATOM	173	CG	PRO	A	58	37.664	8.837	25.266	1.00	26.20	C
ATOM	174	CD	PRO	A	58	37.156	7.715	26.082	1.00	24.70	C
ATOM	175	N	SER	A	59	40.396	8.542	27.469	1.00	30.60	N
ATOM	176	CA	SER	A	59	41.454	9.284	28.126	1.00	33.49	C
ATOM	177	C	SER	A	59	42.479	8.320	28.725	1.00	33.91	C
ATOM	178	O	SER	A	59	43.593	8.743	29.116	1.00	35.45	O
ATOM	179	CB	SER	A	59	40.867	10.248	29.165	1.00	33.56	C
ATOM	180	OG	SER	A	59	40.373	9.512	30.266	1.00	38.56	O
ATOM	181	N	ASN	A	60	42.122	7.031	28.762	1.00	33.18	N
ATOM	182	CA	ASN	A	60	42.988	5.961	29.251	1.00	32.57	C
ATOM	183	C	ASN	A	60	43.421	4.944	28.136	1.00	30.70	C
ATOM	184	O	ASN	A	60	43.081	3.765	28.206	1.00	29.77	O
ATOM	185	CB	ASN	A	60	42.292	5.227	30.406	1.00	33.32	C
ATOM	186	CG	ASN	A	60	41.960	6.168	31.580	1.00	36.87	C
ATOM	187	ND2	ASN	A	60	40.676	6.520	31.723	1.00	39.12	N
ATOM	188	OD1	ASN	A	60	42.852	6.587	32.328	1.00	42.08	O
ATOM	189	N	PRO	A	61	44.210	5.409	27.154	1.00	29.02	N
ATOM	190	CA	PRO	A	61	44.669	4.445	26.125	1.00	28.87	C
ATOM	191	C	PRO	A	61	45.543	3.379	26.711	1.00	28.88	C

ATOM	192	O	PRO	A	61	46.212	3.615	27.727	1.00	29.01	O
ATOM	193	CB	PRO	A	61	45.455	5.297	25.154	1.00	28.91	C
ATOM	194	CG	PRO	A	61	45.712	6.644	25.891	1.00	29.73	C
ATOM	195	CD	PRO	A	61	44.706	6.779	26.939	1.00	28.27	C
ATOM	196	N	ILE	A	62	45.537	2.202	26.121	1.00	28.15	N
ATOM	197	CA	ILE	A	62	46.441	1.153	26.585	1.00	26.83	C
ATOM	198	C	ILE	A	62	47.557	0.980	25.600	1.00	24.36	C
ATOM	199	O	ILE	A	62	47.317	0.689	24.433	1.00	20.85	O
ATOM	200	CB	ILE	A	62	45.708	-0.155	26.761	1.00	27.96	C
ATOM	201	CG1	ILE	A	62	44.544	0.027	27.752	1.00	32.63	C
ATOM	202	CG2	ILE	A	62	46.679	-1.239	27.268	1.00	27.57	C
ATOM	203	CD1	ILE	A	62	44.939	0.558	29.155	1.00	32.97	C
ATOM	204	N	ARG	A	63	48.793	1.086	26.091	1.00	22.60	N
ATOM	205	CA	ARG	A	63	49.963	1.098	25.252	1.00	21.51	C
ATOM	206	C	ARG	A	63	50.897	-0.031	25.668	1.00	21.58	C
ATOM	207	O	ARG	A	63	51.236	-0.120	26.880	1.00	21.49	O
ATOM	208	CB	ARG	A	63	50.697	2.437	25.395	1.00	21.58	C
ATOM	209	CG	ARG	A	63	52.020	2.540	24.595	1.00	21.70	C
ATOM	210	CD	ARG	A	63	52.657	3.880	24.732	1.00	24.76	C
ATOM	211	NE	ARG	A	63	51.836	4.961	24.207	1.00	22.89	N
ATOM	212	CZ	ARG	A	63	51.976	5.494	23.002	1.00	22.79	C
ATOM	213	NH1	ARG	A	63	52.909	5.012	22.185	1.00	18.63	N
ATOM	214	NH2	ARG	A	63	51.191	6.530	22.637	1.00	20.81	N
ATOM	215	N	ARG	A	64	51.321	-0.854	24.702	1.00	20.93	N
ATOM	216	CA	ARG	A	64	52.191	-2.002	24.998	1.00	20.86	C
ATOM	217	C	ARG	A	64	53.230	-2.243	23.950	1.00	21.22	C
ATOM	218	O	ARG	A	64	53.050	-1.912	22.759	1.00	19.81	O
ATOM	219	CB	ARG	A	64	51.354	-3.264	25.234	1.00	20.33	C
ATOM	220	CG	ARG	A	64	50.461	-3.276	26.497	1.00	25.97	C
ATOM	221	CD	ARG	A	64	51.264	-3.250	27.862	1.00	29.48	C
ATOM	222	NE	ARG	A	64	52.194	-4.386	27.966	1.00	30.21	N
ATOM	223	CZ	ARG	A	64	53.380	-4.386	28.577	1.00	33.57	C
ATOM	224	NH1	ARG	A	64	53.850	-3.269	29.137	1.00	34.03	N
ATOM	225	NH2	ARG	A	64	54.139	-5.496	28.558	1.00	30.11	N
ATOM	226	N	PRO	A	65	54.353	-2.879	24.346	1.00	21.89	N
ATOM	227	CA	PRO	A	65	55.361	-3.213	23.396	1.00	22.20	C
ATOM	228	C	PRO	A	65	54.959	-4.471	22.695	1.00	22.72	C
ATOM	229	O	PRO	A	65	55.048	-5.566	23.243	1.00	25.02	O
ATOM	230	CB	PRO	A	65	56.637	-3.380	24.247	1.00	23.37	C
ATOM	231	CG	PRO	A	65	56.109	-3.735	25.562	1.00	23.89	C
ATOM	232	CD	PRO	A	65	54.828	-3.021	25.727	1.00	23.22	C
ATOM	233	N	ILE	A	66	54.563	-4.322	21.450	1.00	20.97	N
ATOM	234	CA	ILE	A	66	54.064	-5.399	20.647	1.00	20.70	C
ATOM	235	C	ILE	A	66	54.567	-5.165	19.251	1.00	22.64	C
ATOM	236	O	ILE	A	66	54.405	-4.050	18.733	1.00	21.48	O
ATOM	237	CB	ILE	A	66	52.541	-5.305	20.562	1.00	20.68	C
ATOM	238	CG1	ILE	A	66	51.861	-5.487	21.933	1.00	22.08	C
ATOM	239	CG2	ILE	A	66	52.010	-6.208	19.512	1.00	22.41	C
ATOM	240	CD1	ILE	A	66	52.129	-6.850	22.564	1.00	27.27	C
ATOM	241	N	SER	A	67	55.175	-6.194	18.665	1.00	22.35	N

ATOM	242	CA	SER	A	67	55.751	-6.056	17.360	1.00	24.34	C
ATOM	243	C	SER	A	67	54.755	-6.622	16.325	1.00	23.66	C
ATOM	244	O	SER	A	67	54.391	-7.773	16.376	1.00	26.12	O
ATOM	245	CB	SER	A	67	57.132	-6.731	17.308	1.00	26.45	C
ATOM	246	OG	SER	A	67	56.990	-8.110	17.542	0.50	26.53	O
ATOM	247	N	ALA	A	68	54.238	-5.774	15.451	1.00	20.32	N
ATOM	248	CA	ALA	A	68	53.365	-6.241	14.398	1.00	17.48	C
ATOM	249	C	ALA	A	68	53.487	-5.293	13.205	1.00	16.02	C
ATOM	250	O	ALA	A	68	53.706	-4.099	13.401	1.00	16.43	O
ATOM	251	CB	ALA	A	68	51.903	-6.222	14.944	1.00	17.77	C
ATOM	252	N	ASP	A	69	53.353	-5.822	11.998	1.00	15.15	N
ATOM	253	CA	ASP	A	69	53.319	-5.054	10.804	1.00	14.44	C
ATOM	254	C	ASP	A	69	51.977	-4.322	10.670	1.00	14.02	C
ATOM	255	O	ASP	A	69	51.816	-3.430	9.865	1.00	14.19	O
ATOM	256	CB	ASP	A	69	53.393	-5.949	9.553	1.00	17.85	C
ATOM	257	CG	ASP	A	69	54.620	-6.821	9.484	0.50	20.31	C
ATOM	258	OD1	ASP	A	69	55.653	-6.455	10.035	0.50	19.57	O
ATOM	259	OD2	ASP	A	69	54.540	-7.847	8.750	1.00	29.58	O
ATOM	260	N	SER	A	70	50.965	-4.876	11.338	1.00	13.72	N
ATOM	261	CA	SER	A	70	49.588	-4.377	11.176	1.00	12.96	C
ATOM	262	C	SER	A	70	48.773	-4.898	12.306	1.00	12.06	C
ATOM	263	O	SER	A	70	49.031	-5.986	12.783	1.00	12.71	O
ATOM	264	CB	SER	A	70	48.990	-4.853	9.866	1.00	14.45	C
ATOM	265	OG	SER	A	70	47.704	-4.267	9.645	1.00	13.40	O
ATOM	266	N	ALA	A	71	47.782	-4.133	12.770	1.00	12.59	N
ATOM	267	CA	ALA	A	71	47.013	-4.488	13.980	1.00	10.66	C
ATOM	268	C	ALA	A	71	45.597	-3.975	13.768	1.00	10.37	C
ATOM	269	O	ALA	A	71	45.428	-2.756	13.536	1.00	11.15	O
ATOM	270	CB	ALA	A	71	47.553	-3.823	15.215	1.00	11.56	C
ATOM	271	N	ILE	A	72	44.604	-4.846	13.903	1.00	7.90	N
ATOM	272	CA	ILE	A	72	43.194	-4.498	13.715	1.00	8.90	C
ATOM	273	C	ILE	A	72	42.366	-5.074	14.830	1.00	9.32	C
ATOM	274	O	ILE	A	72	42.242	-6.271	14.996	1.00	9.86	O
ATOM	275	CB	ILE	A	72	42.674	-4.892	12.329	1.00	8.48	C
ATOM	276	CG1	ILE	A	72	42.980	-6.342	11.934	1.00	11.22	C
ATOM	277	CG2	ILE	A	72	43.186	-3.906	11.349	1.00	9.97	C
ATOM	278	CD1	ILE	A	72	42.253	-6.806	10.651	1.00	9.93	C
ATOM	279	N	MET	A	73	41.728	-4.181	15.572	1.00	9.68	N
ATOM	280	CA	MET	A	73	40.743	-4.553	16.586	1.00	9.93	C
ATOM	281	C	MET	A	73	39.457	-5.118	15.989	1.00	10.51	C
ATOM	282	O	MET	A	73	38.962	-4.686	14.959	1.00	10.08	O
ATOM	283	CB	MET	A	73	40.436	-3.371	17.547	1.00	10.25	C
ATOM	284	CG	MET	A	73	41.462	-2.906	18.453	1.00	10.66	C
ATOM	285	SD	MET	A	73	41.853	-4.123	19.750	1.00	13.41	S
ATOM	286	CE	MET	A	73	40.304	-4.323	20.624	1.00	14.42	C
ATOM	287	N	ASN	A	74	38.932	-6.147	16.627	1.00	9.89	N
ATOM	288	CA	ASN	A	74	37.591	-6.643	16.391	1.00	9.06	C
ATOM	289	C	ASN	A	74	36.545	-5.489	16.456	1.00	9.40	C
ATOM	290	O	ASN	A	74	36.713	-4.553	17.206	1.00	9.60	O
ATOM	291	CB	ASN	A	74	37.232	-7.751	17.411	1.00	9.24	C

ATOM	292	CG	ASN	A	74	35.919	-8.463	17.109	1.00	9.05	C
ATOM	293	ND2	ASN	A	74	36.028	-9.620	16.418	1.00	10.92	N
ATOM	294	OD1	ASN	A	74	34.805	-8.029	17.519	1.00	14.09	O
ATOM	295	N	PRO	A	75	35.469	-5.590	15.643	1.00	10.28	N
ATOM	296	CA	PRO	A	75	34.472	-4.498	15.684	1.00	11.37	C
ATOM	297	C	PRO	A	75	33.779	-4.351	16.997	1.00	13.34	C
ATOM	298	O	PRO	A	75	33.244	-3.234	17.284	1.00	11.74	O
ATOM	299	CB	PRO	A	75	33.462	-4.876	14.608	1.00	12.61	C
ATOM	300	CG	PRO	A	75	34.262	-5.667	13.619	1.00	14.04	C
ATOM	301	CD	PRO	A	75	35.224	-6.470	14.478	1.00	12.61	C
ATOM	302	N	ALA	A	76	33.707	-5.432	17.773	1.00	13.81	N
ATOM	303	CA	ALA	A	76	32.914	-5.371	19.029	1.00	15.32	C
ATOM	304	C	ALA	A	76	33.548	-5.874	20.293	1.00	17.14	C
ATOM	305	O	ALA	A	76	33.246	-5.383	21.374	1.00	19.69	O
ATOM	306	CB	ALA	A	76	31.680	-6.141	18.793	1.00	17.16	C
ATOM	307	N	SER	A	77	34.295	-6.958	20.176	1.00	14.24	N
ATOM	308	CA	SER	A	77	34.987	-7.622	21.271	1.00	15.04	C
ATOM	309	C	SER	A	77	36.423	-7.126	21.500	1.00	14.58	C
ATOM	310	O	SER	A	77	37.068	-6.511	20.649	1.00	13.73	O
ATOM	311	CB	SER	A	77	35.026	-9.160	20.973	1.00	15.02	C
ATOM	312	OG	SER	A	77	33.714	-9.675	20.643	1.00	25.82	O
ATOM	313	N	LYS	A	78	36.909	-7.380	22.700	1.00	15.31	N
ATOM	314	CA	LYS	A	78	38.290	-7.098	23.034	1.00	15.66	C
ATOM	315	C	LYS	A	78	39.202	-8.205	22.513	1.00	13.68	C
ATOM	316	O	LYS	A	78	39.777	-8.981	23.285	1.00	16.02	O
ATOM	317	CB	LYS	A	78	38.490	-6.878	24.534	1.00	17.05	C
ATOM	318	CG	LYS	A	78	37.543	-5.847	25.156	1.00	22.60	C
ATOM	319	CD	LYS	A	78	37.232	-6.038	26.691	1.00	29.54	C
ATOM	320	CE	LYS	A	78	38.352	-5.531	27.570	0.50	28.62	C
ATOM	321	NZ	LYS	A	78	38.150	-5.981	28.999	0.50	32.05	N
ATOM	322	N	VAL	A	79	39.254	-8.294	21.190	1.00	12.13	N
ATOM	323	CA	VAL	A	79	39.982	-9.257	20.433	1.00	11.08	C
ATOM	324	C	VAL	A	79	40.752	-8.406	19.385	1.00	10.84	C
ATOM	325	O	VAL	A	79	40.204	-7.431	18.832	1.00	10.06	O
ATOM	326	CB	VAL	A	79	39.036	-10.246	19.727	1.00	11.08	C
ATOM	327	CG1	VAL	A	79	39.737	-11.098	18.649	1.00	9.28	C
ATOM	328	CG2	VAL	A	79	38.301	-11.138	20.792	1.00	12.22	C
ATOM	329	N	ILE	A	80	41.983	-8.788	19.124	1.00	9.85	N
ATOM	330	CA	ILE	A	80	42.840	-8.038	18.134	1.00	11.16	C
ATOM	331	C	ILE	A	80	43.490	-9.025	17.175	1.00	11.45	C
ATOM	332	O	ILE	A	80	43.846	-10.146	17.603	1.00	11.66	O
ATOM	333	CB	ILE	A	80	43.878	-7.156	18.850	1.00	11.02	C
ATOM	334	CG1	ILE	A	80	44.529	-6.227	17.839	1.00	13.79	C
ATOM	335	CG2	ILE	A	80	44.932	-7.985	19.614	1.00	12.94	C
ATOM	336	CD1	ILE	A	80	45.279	-5.076	18.572	1.00	13.72	C
ATOM	337	N	ALA	A	81	43.536	-8.708	15.878	1.00	10.49	N
ATOM	338	CA	ALA	A	81	44.317	-9.484	14.880	1.00	9.83	C
ATOM	339	C	ALA	A	81	45.636	-8.731	14.632	1.00	11.77	C
ATOM	340	O	ALA	A	81	45.645	-7.515	14.490	1.00	11.06	O
ATOM	341	CB	ALA	A	81	43.505	-9.768	13.591	1.00	9.98	C

ATOM	342	N	LEU	A	82	46.769	-9.495	14.707	1.00	11.12	N
ATOM	343	CA	LEU	A	82	48.121	-8.998	14.557	1.00	11.99	C
ATOM	344	C	LEU	A	82	48.818	-9.757	13.438	1.00	12.73	C
ATOM	345	O	LEU	A	82	48.659	-10.976	13.338	1.00	13.50	O
ATOM	346	CB	LEU	A	82	48.904	-9.213	15.848	1.00	13.89	C
ATOM	347	CG	LEU	A	82	48.337	-8.479	17.028	1.00	12.88	C
ATOM	348	CD1	LEU	A	82	48.926	-8.964	18.353	1.00	19.31	C
ATOM	349	CD2	LEU	A	82	48.527	-6.953	16.858	1.00	15.84	C
ATOM	350	N	LYS	A	83	49.625	-9.084	12.630	1.00	12.79	N
ATOM	351	CA	LYS	A	83	50.335	-9.773	11.569	1.00	15.30	C
ATOM	352	C	LYS	A	83	51.791	-9.430	11.682	1.00	15.84	C
ATOM	353	O	LYS	A	83	52.126	-8.318	12.042	1.00	16.78	O
ATOM	354	CB	LYS	A	83	49.734	-9.382	10.197	1.00	17.45	C
ATOM	355	CG	LYS	A	83	50.659	-9.296	8.989	1.00	24.18	C
ATOM	356	CD	LYS	A	83	49.780	-8.891	7.757	1.00	31.13	C
ATOM	357	CE	LYS	A	83	50.500	-7.898	6.931	1.00	31.49	C
ATOM	358	NZ	LYS	A	83	52.002	-8.251	6.990	1.00	36.94	N
ATOM	359	N	ALA	A	84	52.625	-10.419	11.416	1.00	16.93	N
ATOM	360	CA	ALA	A	84	54.063	-10.218	11.284	1.00	20.14	C
ATOM	361	C	ALA	A	84	54.585	-11.130	10.166	1.00	20.07	C
ATOM	362	O	ALA	A	84	54.499	-12.343	10.266	1.00	21.20	O
ATOM	363	CB	ALA	A	84	54.684	-10.551	12.606	1.00	21.06	C
ATOM	364	N	GLY	A	85	55.060	-10.567	9.076	1.00	22.51	N
ATOM	365	CA	GLY	A	85	55.406	-11.385	7.892	1.00	23.58	C
ATOM	366	C	GLY	A	85	54.159	-12.087	7.406	1.00	22.87	C
ATOM	367	O	GLY	A	85	53.103	-11.499	7.269	1.00	21.67	O
ATOM	368	N	LYS	A	86	54.252	-13.380	7.212	1.00	20.80	N
ATOM	369	CA	LYS	A	86	53.139	-14.101	6.625	1.00	20.24	C
ATOM	370	C	LYS	A	86	52.351	-14.739	7.753	1.00	17.64	C
ATOM	371	O	LYS	A	86	51.435	-15.527	7.494	1.00	16.21	O
ATOM	372	CB	LYS	A	86	53.680	-15.172	5.668	1.00	22.14	C
ATOM	373	CG	LYS	A	86	54.250	-14.544	4.336	1.00	24.63	C
ATOM	374	CD	LYS	A	86	55.134	-15.465	3.546	1.00	28.50	C
ATOM	375	CE	LYS	A	86	55.280	-15.076	2.097	1.00	31.39	C
ATOM	376	NZ	LYS	A	86	55.784	-13.737	1.937	1.00	35.28	N
ATOM	377	N	THR	A	87	52.779	-14.489	8.986	1.00	17.02	N
ATOM	378	CA	THR	A	87	52.116	-15.054	10.156	1.00	17.19	C
ATOM	379	C	THR	A	87	51.067	-14.107	10.779	1.00	16.09	C
ATOM	380	O	THR	A	87	51.322	-12.983	11.024	1.00	15.50	O
ATOM	381	CB	THR	A	87	53.147	-15.557	11.134	1.00	20.49	C
ATOM	382	CG2	THR	A	87	52.540	-16.009	12.460	1.00	21.33	C
ATOM	383	OG1	THR	A	87	53.757	-16.724	10.504	1.00	22.24	O
ATOM	384	N	LEU	A	88	49.877	-14.647	10.926	1.00	14.47	N
ATOM	385	CA	LEU	A	88	48.706	-13.961	11.437	1.00	14.00	C
ATOM	386	C	LEU	A	88	48.338	-14.556	12.809	1.00	13.65	C
ATOM	387	O	LEU	A	88	48.380	-15.766	12.995	1.00	14.63	O
ATOM	388	CB	LEU	A	88	47.562	-14.144	10.475	1.00	14.28	C
ATOM	389	CG	LEU	A	88	47.911	-13.863	9.003	1.00	17.01	C
ATOM	390	CD1	LEU	A	88	46.559	-14.216	8.140	1.00	18.26	C
ATOM	391	CD2	LEU	A	88	48.444	-12.506	8.774	1.00	17.69	C

ATOM	392	N	GLN	A	89	47.993	-13.712	13.778	1.00	13.71	N
ATOM	393	CA	GLN	A	89	47.471	-14.173	15.029	1.00	14.79	C
ATOM	394	C	GLN	A	89	46.208	-13.432	15.419	1.00	12.93	C
ATOM	395	O	GLN	A	89	46.130	-12.206	15.225	1.00	13.48	O
ATOM	396	CB	GLN	A	89	48.555	-14.022	16.117	1.00	16.02	C
ATOM	397	CG	GLN	A	89	48.148	-14.379	17.538	1.00	24.83	C
ATOM	398	CD	GLN	A	89	48.991	-13.740	18.622	1.00	28.90	C
ATOM	399	NE2	GLN	A	89	48.656	-14.079	19.872	0.50	28.13	N
ATOM	400	OE1	GLN	A	89	49.907	-12.949	18.366	0.50	28.63	O
ATOM	401	N	ILE	A	90	45.315	-14.100	16.107	1.00	10.75	N
ATOM	402	CA	ILE	A	90	44.151	-13.474	16.678	1.00	10.02	C
ATOM	403	C	ILE	A	90	44.280	-13.687	18.171	1.00	11.98	C
ATOM	404	O	ILE	A	90	44.470	-14.804	18.599	1.00	13.13	O
ATOM	405	CB	ILE	A	90	42.827	-14.018	16.132	1.00	10.03	C
ATOM	406	CG1	ILE	A	90	42.733	-13.960	14.606	1.00	12.46	C
ATOM	407	CG2	ILE	A	90	41.661	-13.296	16.779	1.00	12.18	C
ATOM	408	CD1	ILE	A	90	41.528	-14.524	13.959	1.00	13.11	C
ATOM	409	N	PHE	A	91	44.172	-12.625	18.947	1.00	10.83	N
ATOM	410	CA	PHE	A	91	44.415	-12.658	20.379	1.00	11.04	C
ATOM	411	C	PHE	A	91	43.230	-12.114	21.149	1.00	13.67	C
ATOM	412	O	PHE	A	91	42.637	-11.108	20.792	1.00	13.42	O
ATOM	413	CB	PHE	A	91	45.757	-11.866	20.616	1.00	11.64	C
ATOM	414	CG	PHE	A	91	46.291	-11.936	21.985	1.00	14.30	C
ATOM	415	CD1	PHE	A	91	47.113	-12.970	22.335	1.00	14.61	C
ATOM	416	CD2	PHE	A	91	45.942	-10.967	22.913	1.00	15.12	C
ATOM	417	CE1	PHE	A	91	47.622	-13.056	23.600	1.00	14.77	C
ATOM	418	CE2	PHE	A	91	46.430	-10.991	24.242	1.00	14.15	C
ATOM	419	CZ	PHE	A	91	47.341	-12.109	24.562	1.00	14.61	C
ATOM	420	N	ASN	A	92	42.896	-12.801	22.233	1.00	11.71	N
ATOM	421	CA	ASN	A	92	41.841	-12.419	23.159	1.00	12.72	C
ATOM	422	C	ASN	A	92	42.429	-11.566	24.254	1.00	13.22	C
ATOM	423	O	ASN	A	92	43.119	-12.059	25.115	1.00	12.20	O
ATOM	424	CB	ASN	A	92	41.221	-13.675	23.766	1.00	13.00	C
ATOM	425	CG	ASN	A	92	39.997	-13.396	24.638	1.00	14.45	C
ATOM	426	ND2	ASN	A	92	39.125	-14.435	24.753	1.00	24.57	N
ATOM	427	OD1	ASN	A	92	39.825	-12.309	25.247	1.00	16.24	O
ATOM	428	N	ILE	A	93	42.133	-10.259	24.237	1.00	12.75	N
ATOM	429	CA	ILE	A	93	42.815	-9.321	25.092	1.00	13.12	C
ATOM	430	C	ILE	A	93	42.312	-9.481	26.488	1.00	13.08	C
ATOM	431	O	ILE	A	93	43.073	-9.440	27.442	1.00	14.28	O
ATOM	432	CB	ILE	A	93	42.672	-7.844	24.646	1.00	12.73	C
ATOM	433	CG1	ILE	A	93	43.276	-7.703	23.277	1.00	17.14	C
ATOM	434	CG2	ILE	A	93	43.436	-6.908	25.733	1.00	15.09	C
ATOM	435	CD1	ILE	A	93	43.023	-6.280	22.671	1.00	20.82	C
ATOM	436	N	GLU	A	94	41.015	-9.622	26.604	1.00	14.11	N
ATOM	437	CA	GLU	A	94	40.393	-9.723	27.896	1.00	16.79	C
ATOM	438	C	GLU	A	94	40.901	-10.946	28.690	1.00	15.36	C
ATOM	439	O	GLU	A	94	41.176	-10.872	29.881	1.00	18.22	O
ATOM	440	CB	GLU	A	94	38.885	-9.817	27.706	1.00	17.39	C
ATOM	441	CG	GLU	A	94	38.052	-9.793	28.988	1.00	25.54	C

ATOM	442	CD	GLU	A	94	36.534	-9.839	28.705	1.00	33.27	C
ATOM	443	OE1	GLU	A	94	36.063	-9.271	27.678	0.50	33.43	O
ATOM	444	OE2	GLU	A	94	35.818	-10.491	29.502	0.50	34.15	O
ATOM	445	N	MET	A	95	41.101	-12.059	28.004	1.00	14.84	N
ATOM	446	CA	MET	A	95	41.607	-13.265	28.631	1.00	15.35	C
ATOM	447	C	MET	A	95	43.107	-13.485	28.440	1.00	13.84	C
ATOM	448	O	MET	A	95	43.658	-14.538	28.794	1.00	14.74	O
ATOM	449	CB	MET	A	95	40.798	-14.491	28.123	1.00	15.39	C
ATOM	450	CG	MET	A	95	39.334	-14.340	28.087	1.00	16.35	C
ATOM	451	SD	MET	A	95	38.749	-14.072	29.827	1.00	23.66	S
ATOM	452	CE	MET	A	95	38.779	-15.741	30.555	1.00	21.28	C
ATOM	453	N	LYS	A	96	43.774	-12.459	27.861	1.00	12.38	N
ATOM	454	CA	LYS	A	96	45.234	-12.459	27.641	1.00	12.99	C
ATOM	455	C	LYS	A	96	45.713	-13.815	27.049	1.00	14.03	C
ATOM	456	O	LYS	A	96	46.717	-14.394	27.473	1.00	15.03	O
ATOM	457	CB	LYS	A	96	45.973	-12.115	28.946	1.00	15.45	C
ATOM	458	CG	LYS	A	96	45.585	-10.745	29.517	0.50	11.48	C
ATOM	459	CD	LYS	A	96	45.995	-10.612	30.992	1.00	25.51	C
ATOM	460	CE	LYS	A	96	45.489	-9.316	31.625	0.50	23.75	C
ATOM	461	NZ	LYS	A	96	46.096	-9.128	32.982	1.00	30.62	N
ATOM	462	N	SER	A	97	45.045	-14.234	25.997	1.00	12.27	N
ATOM	463	CA	SER	A	97	45.223	-15.547	25.431	1.00	11.62	C
ATOM	464	C	SER	A	97	45.175	-15.557	23.956	1.00	12.36	C
ATOM	465	O	SER	A	97	44.316	-14.924	23.372	1.00	12.26	O
ATOM	466	CB	SER	A	97	44.168	-16.548	25.950	1.00	12.13	C
ATOM	467	OG	SER	A	97	44.350	-16.812	27.309	1.00	11.98	O
ATOM	468	N	ALYS	A	98	46.076	-16.325	23.351	0.50	10.95	N
ATOM	469	N	BLYS	A	98	46.088	-16.295	23.350	0.50	11.55	N
ATOM	470	CA	ALYS	A	98	46.077	-16.593	21.899	0.50	11.80	C
ATOM	471	CA	BLYS	A	98	46.031	-16.529	21.916	0.50	12.98	C
ATOM	472	C	ALYS	A	98	44.863	-17.401	21.459	0.50	12.94	C
ATOM	473	C	BLYS	A	98	44.740	-17.250	21.591	0.50	13.46	C
ATOM	474	O	ALYS	A	98	44.615	-18.519	21.959	0.50	10.97	O
ATOM	475	O	BLYS	A	98	44.306	-18.128	22.346	0.50	12.69	O
ATOM	476	CB	ALYS	A	98	47.350	-17.322	21.439	0.50	13.09	C
ATOM	477	CB	BLYS	A	98	47.215	-17.339	21.400	0.50	14.33	C
ATOM	478	CG	ALYS	A	98	48.620	-16.562	21.686	0.50	11.84	C
ATOM	479	CG	BLYS	A	98	47.269	-17.369	19.897	0.50	15.80	C
ATOM	480	CD	ALYS	A	98	49.821	-17.243	21.027	0.50	16.75	C
ATOM	481	CD	BLYS	A	98	48.508	-18.097	19.404	0.50	19.85	C
ATOM	482	CE	ALYS	A	98	51.094	-16.526	21.284	0.50	16.63	C
ATOM	483	CE	BLYS	A	98	48.106	-19.479	19.011	0.50	19.33	C
ATOM	484	NZ	ALYS	A	98	50.935	-15.047	21.237	0.50	21.61	N
ATOM	485	NZ	BLYS	A	98	49.128	-20.508	19.372	0.50	23.41	N
ATOM	486	N	MET	A	99	44.146	-16.889	20.453	1.00	12.52	N
ATOM	487	CA	MET	A	99	42.964	-17.576	19.929	1.00	11.96	C
ATOM	488	C	MET	A	99	43.283	-18.519	18.809	1.00	14.42	C
ATOM	489	O	MET	A	99	43.054	-19.725	18.891	1.00	14.87	O
ATOM	490	CB	MET	A	99	41.825	-16.606	19.540	1.00	12.68	C
ATOM	491	CG	MET	A	99	41.208	-15.892	20.709	1.00	10.60	C

ATOM	492	SD	MET	A	99	40.326	-14.383	20.305	1.00	13.39	S
ATOM	493	CE	MET	A	99	39.087	-15.029	19.161	1.00	13.94	C
ATOM	494	N	LYS	A	100	43.855	-17.985	17.753	1.00	13.41	N
ATOM	495	CA	LYS	A	100	44.306	-18.768	16.640	1.00	15.27	C
ATOM	496	C	LYS	A	100	45.428	-18.077	15.895	1.00	14.58	C
ATOM	497	O	LYS	A	100	45.625	-16.875	16.083	1.00	13.43	O
ATOM	498	CB	LYS	A	100	43.185	-19.214	15.766	1.00	18.53	C
ATOM	499	CG	LYS	A	100	42.250	-18.298	15.289	1.00	20.11	C
ATOM	500	CD	LYS	A	100	40.773	-19.028	15.092	1.00	19.65	C
ATOM	501	CE	LYS	A	100	40.716	-19.954	13.933	1.00	18.50	C
ATOM	502	NZ	LYS	A	100	39.430	-20.046	13.162	1.00	14.20	N
ATOM	503	N	ALA	A	101	46.182	-18.862	15.125	1.00	13.16	N
ATOM	504	CA	ALA	A	101	47.282	-18.377	14.283	1.00	13.29	C
ATOM	505	C	ALA	A	101	47.232	-19.126	12.972	1.00	13.86	C
ATOM	506	O	ALA	A	101	46.712	-20.256	12.916	1.00	14.31	O
ATOM	507	CB	ALA	A	101	48.593	-18.637	14.928	1.00	15.74	C
ATOM	508	N	HIS	A	102	47.797	-18.518	11.931	1.00	13.73	N
ATOM	509	CA	HIS	A	102	47.859	-19.116	10.613	1.00	13.63	C
ATOM	510	C	HIS	A	102	49.035	-18.494	9.893	1.00	15.66	C
ATOM	511	O	HIS	A	102	49.248	-17.298	9.980	1.00	14.74	O
ATOM	512	CB	HIS	A	102	46.626	-18.852	9.823	1.00	13.68	C
ATOM	513	CG	HIS	A	102	46.573	-19.614	8.567	1.00	12.94	C
ATOM	514	CD2	HIS	A	102	46.800	-19.227	7.296	1.00	14.59	C
ATOM	515	ND1	HIS	A	102	46.300	-20.966	8.529	1.00	14.21	N
ATOM	516	CE1	HIS	A	102	46.359	-21.370	7.261	1.00	19.80	C
ATOM	517	NE2	HIS	A	102	46.658	-20.328	6.494	1.00	15.44	N
TER	518		HIS	A	102						
HETATM	519	CAE	VH2	A1358		52.618	-10.319	26.230	0.70	23.09	C
HETATM	520	CAF	VH2	A1358		47.945	-2.955	22.253	0.70	18.66	C
HETATM	521	CAG	VH2	A1358		47.872	-2.928	23.653	0.70	22.46	C
HETATM	522	CAH	VH2	A1358		47.781	-7.835	22.111	0.70	17.75	C
HETATM	523	CAI	VH2	A1358		52.536	-13.776	24.913	0.70	20.08	C
HETATM	524	CAJ	VH2	A1358		50.491	-13.284	26.084	0.70	16.89	C
HETATM	525	CAK	VH2	A1358		47.698	-7.874	23.500	0.70	17.95	C
HETATM	526	CAL	VH2	A1358		52.974	-12.473	25.200	0.70	22.77	C
HETATM	527	CAM	VH2	A1358		50.924	-12.008	26.365	0.70	20.99	C
HETATM	528	CAN	VH2	A1358		47.960	-4.161	21.546	0.70	19.34	C
HETATM	529	CAO	VH2	A1358		47.852	-6.598	21.461	0.70	17.56	C
HETATM	530	CAP	VH2	A1358		47.821	-4.185	24.294	0.70	21.24	C
HETATM	531	CAT	VH2	A1358		51.255	-14.121	25.349	0.70	16.80	C
HETATM	532	CAU	VH2	A1358		52.175	-11.599	25.924	0.70	21.04	C
HETATM	533	CAV	VH2	A1358		50.119	-7.344	26.544	0.70	23.56	C
HETATM	534	CAW	VH2	A1358		47.700	-6.690	24.236	0.70	19.02	C
HETATM	535	CAX	VH2	A1358		52.266	-7.931	26.743	0.70	24.92	C
HETATM	536	CAY	VH2	A1358		51.775	-9.186	26.357	0.70	21.47	C
HETATM	537	CAZ	VH2	A1358		47.844	-5.403	22.182	0.70	20.41	C
HETATM	538	NAQ	VH2	A1358		51.363	-6.960	26.839	0.70	23.45	N
HETATM	539	NAR	VH2	A1358		49.130	-6.524	26.569	0.70	22.33	N
HETATM	540	OAA	VH2	A1358		53.464	-7.707	26.969	0.70	29.62	O
HETATM	541	OAB	VH2	A1358		46.674	-5.909	26.457	0.70	19.73	O

[illegible]

ATOM N	1	N	VAL A	50	51.811	-1.374	21.143	1.00	20.76
ATOM C	2	CA	VAL A	50	50.432	-1.147	20.674	1.00	20.24
ATOM C	3	C	VAL A	50	49.678	-0.325	21.713	1.00	18.69
ATOM O	4	O	VAL A	50	49.726	-0.600	22.919	1.00	19.01
ATOM C	5	CB	VAL A	50	49.728	-2.448	20.382	1.00	20.96
ATOM C	6	CG1	VAL A	50	48.321	-2.176	19.953	1.00	22.50
ATOM C	7	CG2	VAL A	50	50.532	-3.226	19.334	1.00	22.84
TER	8		VAL A	50					
ATOM N	9	N	ILE A	52	45.916	1.062	22.708	1.00	13.85
ATOM C	10	CA	ILE A	52	44.488	0.810	22.458	1.00	14.57
ATOM C	11	C	ILE A	52	43.727	1.908	23.167	1.00	14.95
ATOM O	12	O	ILE A	52	43.754	2.063	24.387	1.00	16.13
ATOM C	13	CB	ILE A	52	43.975	-0.577	22.925	1.00	14.98
ATOM C	14	CG1	ILE A	52	44.776	-1.688	22.261	1.00	17.11
ATOM C	15	CG2	ILE A	52	42.422	-0.699	22.754	1.00	15.44
ATOM C	16	CD1	ILE A	52	44.506	-3.083	22.836	1.00	17.71
TER	17		ILE A	52					
ATOM N	18	N	ILE A	62	45.757	2.300	26.939	1.00	31.08
ATOM C	19	CA	ILE A	62	46.603	1.236	27.445	1.00	31.10
ATOM C	20	C	ILE A	62	47.671	0.930	26.412	1.00	30.97
ATOM O	21	O	ILE A	62	47.340	0.762	25.235	1.00	25.68
ATOM C	22	CB	ILE A	62	45.754	-0.016	27.813	1.00	31.05
ATOM C	23	CG1	ILE A	62	44.793	0.321	28.953	1.00	33.78
ATOM C	24	CG2	ILE A	62	46.679	-1.161	28.284	1.00	33.97
ATOM C	25	CD1	ILE A	62	43.652	-0.701	29.191	1.00	34.61
TER	26		ILE A	62					
ATOM N	27	N	ARG A	64	50.869	-1.718	25.391	1.00	34.17
ATOM C	28	CA	ARG A	64	51.452	-3.053	25.655	1.00	33.42
ATOM C	29	C	ARG A	64	52.610	-3.202	24.669	1.00	33.67
ATOM O	30	O	ARG A	64	52.527	-2.775	23.496	1.00	30.68
ATOM C	31	CB	ARG A	64	50.419	-4.224	25.515	1.00	33.63

ATOM C	32	CG	ARG A	64	50.937	-5.685	25.990	1.00	33.57
ATOM C	33	CD	ARG A	64	50.005	-6.909	25.633	1.00	35.82
ATOM N	34	NE	ARG A	64	50.322	-8.166	26.351	1.00	35.13
ATOM C	35	CZ	ARG A	64	51.374	-8.969	26.098	1.00	37.14
ATOM N	36	NH1	ARG A	64	52.256	-8.689	25.132	1.00	36.95
ATOM N	37	NH2	ARG A	64	51.574	-10.070	26.833	1.00	36.56
TER	38		ARG A	64					
ATOM N	39	N	ILE A	66	54.432	-5.099	22.040	1.00	33.08
ATOM C	40	CA	ILE A	66	54.027	-6.092	21.105	1.00	34.00
ATOM C	41	C	ILE A	66	54.801	-5.745	19.869	1.00	34.22
ATOM O	42	O	ILE A	66	54.800	-4.571	19.435	1.00	36.68
ATOM C	43	CB	ILE A	66	52.509	-6.011	20.748	1.00	34.45
ATOM C	44	CG1	ILE A	66	51.632	-6.421	21.934	1.00	35.53
ATOM C	45	CG2	ILE A	66	52.187	-6.911	19.591	1.00	36.05
ATOM C	46	CD1	ILE A	66	50.161	-6.595	21.606	1.00	37.92
ATOM N	47	N	SER A	67	55.370	-6.767	19.277	1.00	33.26
ATOM C	48	CA	SER A	67	55.987	-6.732	17.969	1.00	33.12
ATOM C	49	C	SER A	67	54.995	-7.293	16.975	1.00	30.86
ATOM O	50	O	SER A	67	54.613	-8.465	17.055	1.00	32.48
ATOM C	51	CB	SER A	67	57.236	-7.623	17.992	1.00	34.25
ATOM O	52	OG	SER A	67	56.960	-8.794	18.759	0.50	34.68
TER	53		SER A	67					
ATOM N	54	N	ILE A	80	41.990	-8.770	19.838	1.00	10.10
ATOM C	55	CA	ILE A	80	42.879	-8.093	18.881	1.00	9.24
ATOM C	56	C	ILE A	80	43.544	-9.132	17.976	1.00	10.02
ATOM O	57	O	ILE A	80	43.897	-10.227	18.457	1.00	10.54
ATOM C	58	CB	ILE A	80	43.886	-7.224	19.610	1.00	8.37
ATOM C	59	CG1	ILE A	80	44.663	-6.318	18.710	1.00	10.35
ATOM C	60	CG2	ILE A	80	44.875	-8.072	20.569	1.00	12.57
ATOM C	61	CD1	ILE A	80	45.447	-5.229	19.513	1.00	8.59
TER	62		ILE A	80					

ATOM N	63	N	LEU A	82	46.850	-9.668	15.527	1.00	11.14
ATOM C	64	CA	LEU A	82	48.197	-9.168	15.350	1.00	12.53
ATOM C	65	C	LEU A	82	48.890	-9.967	14.287	1.00	13.97
ATOM O	66	O	LEU A	82	48.804	-11.194	14.226	1.00	14.12
ATOM C	67	CB	LEU A	82	48.950	-9.317	16.680	1.00	14.32
ATOM C	68	CG	LEU A	82	48.415	-8.573	17.888	1.00	16.99
ATOM C	69	CD1	LEU A	82	49.197	-9.000	19.129	1.00	16.28
ATOM C	70	CD2	LEU A	82	48.515	-7.035	17.632	1.00	16.08
TER	71		LEU A	82					
ATOM N	72	N	GLN A	89	48.005	-13.948	14.742	1.00	10.61
ATOM C	73	CA	GLN A	89	47.501	-14.381	15.999	1.00	11.17
ATOM C	74	C	GLN A	89	46.298	-13.545	16.366	1.00	11.64
ATOM O	75	O	GLN A	89	46.208	-12.356	16.018	1.00	13.08
ATOM C	76	CB	GLN A	89	48.564	-14.249	17.085	1.00	13.19
ATOM C	77	CG	GLN A	89	49.810	-15.094	16.808	1.00	15.83
ATOM C	78	CD	GLN A	89	50.749	-15.031	18.014	1.00	24.48
ATOM N	79	NE2	GLN A	89	51.475	-13.930	18.143	1.00	26.63
ATOM O	80	OE1	GLN A	89	50.773	-15.930	18.825	1.00	29.59
TER	81		GLN A	89					
ATOM N	82	N	PHE A	91	44.187	-12.609	19.979	1.00	11.33
ATOM C	83	CA	PHE A	91	44.182	-12.653	21.436	1.00	11.82
ATOM C	84	C	PHE A	91	42.946	-12.106	22.080	1.00	14.39
ATOM O	85	O	PHE A	91	42.402	-11.092	21.653	1.00	13.16
ATOM C	86	CB	PHE A	91	45.289	-11.822	21.975	1.00	13.35
ATOM C	87	CG	PHE A	91	46.575	-12.395	21.752	1.00	16.16
ATOM C	88	CD1	PHE A	91	47.245	-12.140	20.591	1.00	18.73
ATOM C	89	CD2	PHE A	91	47.145	-13.253	22.725	1.00	18.04
ATOM C	90	CE1	PHE A	91	48.549	-12.688	20.354	1.00	20.46
ATOM C	91	CE2	PHE A	91	48.403	-13.843	22.472	1.00	23.63
ATOM C	92	CZ	PHE A	91	49.084	-13.540	21.290	1.00	23.16
TER	93		PHE A	91					

END

FIGURE 16

ATOM	1	N	VAL	A	50	52.043	-0.739	20.384	1.00	17.98	N
ATOM	2	CA	VAL	A	50	50.643	-0.700	19.870	1.00	18.61	C
ATOM	3	C	VAL	A	50	49.779	-0.043	20.942	1.00	16.71	C
ATOM	4	O	VAL	A	50	49.853	-0.381	22.159	1.00	17.03	O
ATOM	5	CB	VAL	A	50	50.162	-2.029	19.496	1.00	17.99	C
ATOM	6	CG1	VAL	A	50	48.747	-1.957	18.924	1.00	21.60	C
ATOM	7	CG2	VAL	A	50	51.106	-2.668	18.480	1.00	18.77	C
TER	8		VAL	A	50						
ATOM	9	N	ILE	A	52	45.899	0.970	21.999	1.00	12.92	N
ATOM	10	CA	ILE	A	52	44.425	0.763	21.834	1.00	13.58	C
ATOM	11	C	ILE	A	52	43.643	1.851	22.592	1.00	15.99	C
ATOM	12	O	ILE	A	52	43.754	1.998	23.830	1.00	18.98	O
ATOM	13	CB	ILE	A	52	43.980	-0.641	22.280	1.00	14.38	C
ATOM	14	CG1	ILE	A	52	44.772	-1.706	21.505	1.00	15.39	C
ATOM	15	CG2	ILE	A	52	42.507	-0.792	22.033	1.00	12.30	C
ATOM	16	CD1	ILE	A	52	44.673	-3.120	22.122	1.00	18.71	C
TER	17		ILE	A	52						
ATOM	18	N	ILE	A	62	45.537	2.202	26.121	1.00	28.15	N
ATOM	19	CA	ILE	A	62	46.441	1.153	26.585	1.00	26.83	C
ATOM	20	C	ILE	A	62	47.557	0.980	25.600	1.00	24.36	C
ATOM	21	O	ILE	A	62	47.317	0.689	24.433	1.00	20.85	O
ATOM	22	CB	ILE	A	62	45.708	-0.155	26.761	1.00	27.96	C
ATOM	23	CG1	ILE	A	62	44.544	0.027	27.752	1.00	32.63	C
ATOM	24	CG2	ILE	A	62	46.679	-1.239	27.268	1.00	27.57	C
ATOM	25	CD1	ILE	A	62	44.939	0.558	29.155	1.00	32.97	C
TER	26		ILE	A	62						
ATOM	27	N	ARG	A	64	51.321	-0.854	24.702	1.00	20.93	N
ATOM	28	CA	ARG	A	64	52.191	-2.002	24.998	1.00	20.86	C
ATOM	29	C	ARG	A	64	53.230	-2.243	23.950	1.00	21.22	C
ATOM	30	O	ARG	A	64	53.050	-1.912	22.759	1.00	19.81	O
ATOM	31	CB	ARG	A	64	51.354	-3.264	25.234	1.00	20.33	C
ATOM	32	CG	ARG	A	64	50.461	-3.276	26.497	1.00	25.97	C
ATOM	33	CD	ARG	A	64	51.264	-3.250	27.862	1.00	29.48	C
ATOM	34	NE	ARG	A	64	52.194	-4.386	27.966	1.00	30.21	N
ATOM	35	CZ	ARG	A	64	53.380	-4.386	28.577	1.00	33.57	C
ATOM	36	NH1	ARG	A	64	53.850	-3.269	29.137	1.00	34.03	N
ATOM	37	NH2	ARG	A	64	54.139	-5.496	28.558	1.00	30.11	N
TER	38		ARG	A	64						
ATOM	39	N	ILE	A	80	41.983	-8.788	19.124	1.00	9.85	N
ATOM	40	CA	ILE	A	80	42.840	-8.038	18.134	1.00	11.16	C
ATOM	41	C	ILE	A	80	43.490	-9.025	17.175	1.00	11.45	C
ATOM	42	O	ILE	A	80	43.846	-10.146	17.603	1.00	11.66	O
ATOM	43	CB	ILE	A	80	43.878	-7.156	18.850	1.00	11.02	C
ATOM	44	CG1	ILE	A	80	44.529	-6.227	17.839	1.00	13.79	C
ATOM	45	CG2	ILE	A	80	44.932	-7.985	19.614	1.00	12.94	C
ATOM	46	CD1	ILE	A	80	45.279	-5.076	18.572	1.00	13.72	C
TER	47		ILE	A	80						
ATOM	48	N	PHE	A	91	44.172	-12.625	18.947	1.00	10.83	N
ATOM	49	CA	PHE	A	91	44.415	-12.658	20.379	1.00	11.04	C
ATOM	50	C	PHE	A	91	43.230	-12.114	21.149	1.00	13.67	C
ATOM	51	O	PHE	A	91	42.637	-11.108	20.792	1.00	13.42	O
ATOM	52	CB	PHE	A	91	45.757	-11.866	20.616	1.00	11.64	C
ATOM	53	CG	PHE	A	91	46.291	-11.936	21.985	1.00	14.30	C
ATOM	54	CD1	PHE	A	91	47.113	-12.970	22.335	1.00	14.61	C
ATOM	55	CD2	PHE	A	91	45.942	-10.967	22.913	1.00	15.12	C
ATOM	56	CE1	PHE	A	91	47.622	-13.056	23.600	1.00	14.77	C
ATOM	57	CE2	PHE	A	91	46.430	-10.991	24.242	1.00	14.15	C
ATOM	58	CZ	PHE	A	91	47.341	-12.109	24.562	1.00	14.61	C

END

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2012/000858

A. CLASSIFICATION OF SUBJECT MATTER

A61K 31/00 (2006.01) A61P 31/00 (2006.01) A61P 25/00 (2006.01) C40B 30/02 (2006.01) C40B 50/02 (2006.01) G06F 19/18 (2011.01) G01N 33/15 (2006.01) C07C 13/547 (2006.01) C07C 13/573 (2006.01) C07C 13/66 (2006.01) C07D 221/14 (2006.01) C07D 221/16 (2006.01) C07D 277/20 (2006.01) C07D 277/36 (2006.01) C07D 277/38 (2006.01) C07D 277/34 (2006.01)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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

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

CA: Structure search of Formula I, Formula V; keywords - therapy, clathrin, protein, pitstop and related terms

WPI, EPODOC Medline: keywords - clathrin, inhibitor, terminal domain, protein, pitstop, epilepsy, Alzheimer's disease, Parkinson's disease, lewy body, dementia, amyloid, Dent's disease, clathrin mediated endocytosis, CME, pain, encephalopathy, psychosis, bipolar disorder, schizophrenia, neuronal excitation, seizure, migraine, mucopolipidosis, neuro degenerative disease, neuropsychiatric, synaptic signal transduction and related terms

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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

☒ Further documents are listed in the continuation of Box C☒ See patent family annex

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

14 September 2012

Date of mailing of the international search report

17 September 2012

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INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/AU2012/000858
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2005/0142214 A1 (BROWN) 30 June 2005 see whole document, in particular -- abstract, Fig 1, [0009], Table 1, [0014], [0015], [0016]	14-36, 46-50, 58, 69-75, 99, 102-104
X	WO 2008/086008 A1 (XANTHUS PHARMACEUTICALS, INC.) 17 July 2008 see whole document, in particular -- abstract, page 1 paragraph 3 – page 2 paragraph 2, page 2 paragraph 3, page 17 paragraph 6 – page 18 paragraph 2, page 13 paragraph 2	14-36, 46-50, 58, 69-75, 99, 102-104
X	WO 2007/128538 A1 (UNIBIOSCREEN S.A.) 15 November 2007 see whole document, in particular -- abstract, page 4 lines 1-21	14-36, 46-50, 58, 69-75, 99, 102-104
X	TUMIATTI V. et al.: "Design, Synthesis, and Biological Evaluation of Substituted Naphthalene Imides and Diimides as Anticancer Agent" Journal of Medicinal Chemistry, 2009, vol. 52, no. 23, pp. 7873-7877 see whole document, in particular -- abstract, Figure 1, Scheme 2, Table 1, page 7874 left column paragraph 2, page 7875 left column paragraph 2	14-36, 46-50, 58, 69-75, 99, 102-104
X	JP 2003-300957 A (TAISHO PHARMACEUTICAL CO. LTD.) 21 October 2003 see whole document, in particular -- abstract, [0002], [0005], [0006], Table 1, Table 2	14-36, 79-92, 99, 102-104
X	WO 1994/029287 A1 (BOEHRINGER MANNHEIM GMBH) 22 December 1994 see whole document, in particular -- abstract, pages 1-3 bridging paragraph, page 4 paragraph 2	14-36, 79-92, 99, 102-104
X	WO 2004/028535 A1 (PINTEX PHARMACEUTICALS, INC.) 08 April 2004 see whole document, in particular -- abstract, page 9 line 17 – page 10 line 13, page 11 line 26 – page 12 line 6	14-36, 79-92, 99, 102-104
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A	MORGAN J. R. et al.: "A Conserved Clathrin Assembly Motif Essential for Synaptic Vesicle Endocytosis" Journal of Neuroscience, 2000, vol. 20, no. 23, pp. 8667-8676 see whole document, in particular -- abstract	1-37
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A	RAHU H. et al.: "Kaposi's Sarcoma-Associated Herpesvirus Utilizes an Actin Polymerization-Dependent Macropinocytic Pathway to Enter Human Dermal Microvascular Endothelial and Human Umbilical Vein Endothelial Cells" Journal of Virology, 2009, vol. 83, no. 10, pp. 4895-4911 see whole document, in particular -- abstract	1-37
	GAD H. et al.: "Dissociation Between Ca ²⁺ -Triggered Synaptic Vesicle Exocytosis and Clathrin-Mediated Endocytosis at a Central Synapse" Neuron, 1998, vol. 21, no. 3, pp. 607-616	

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: **38-45**
because they relate to subject matter not required to be searched by this Authority, namely:
See supplemental Box
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplemental Box for Details

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☒ No protest accompanied the payment of additional search fees.

Supplemental Box

Continuation of Box II

Rule 67 lists the subject matter which under Article 34(4)(a)(i) an international preliminary examination [or a written opinion, via the application of Rule 43bis.1(b)] is not required to be carried out. At item (v) Rule 67 specifies mere presentations of information as such matter.

Continuation of: **Box III**

This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

This Authority has found that there are different inventions based on the following features that separate the claims into distinct groups:

- Invention 1: Claims 1-37 (in part), 46-78 (full) and 99-104 (in part) are directed to compounds of Formula I, pharmaceutical compositions and methods of use thereof
- Invention 2: Claims 1-37 (in part), 79-98 (full) and 99-104 (in part) are directed to compounds of Formula V, pharmaceutical compositions and methods of use thereof
- Invention 3: Claims 1-37 (in part) and 38-45 (full) are directed to clathrin inhibitors, other than the compounds of Formula I or Formula V, which bind to at least one of amino acids Ile 52, Ile 62, Ile 80, Phe 91 or Ile 93 of the terminal domain of clathrin and their use in methods for inhibiting clathrin, methods for prophylaxis or treatment of a disease or condition, complexes of clathrin inhibitor and the terminal domain of clathrin or a fragment thereof, methods of screening and methods of providing such clathrin inhibitors

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.

When there is no special technical feature common to all the claimed inventions there is no unity of invention.

In the above groups of claims, the identified features may have the potential to make a contribution over the prior art but are not common to all the claimed inventions and therefore cannot provide the required technical relationship. Therefore there is no special technical feature common to all the claimed inventions and the requirements for unity of invention are consequently not satisfied *a priori*.

INTERNATIONAL SEARCH REPORT		International application No.			
Information on patent family members		PCT/AU2012/000858			
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.					
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Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

Form PCT/ISA/210 (Family Annex)(July 2009)

INTERNATIONAL SEARCH REPORT		International application No.	
Information on patent family members		PCT/AU2012/000858	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
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		WO 2004028535 A1	08 Apr 2004
End of Annex			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.			

Information on patent family members

PCT/AU2012/000858

Patent Document/s Cited in Search Report

Patent Family Member/s**Publication Date****Publication Date**