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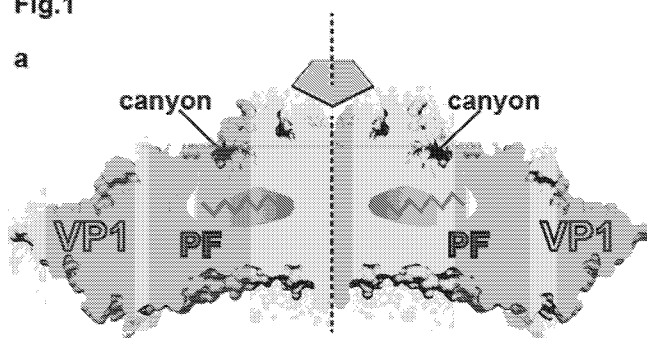
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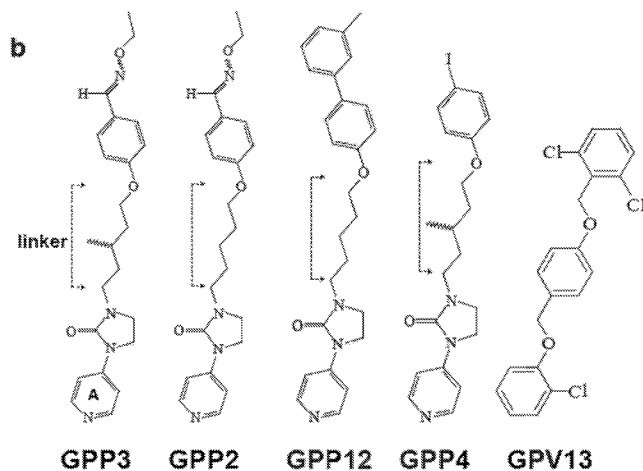
(54) Title: VIRAL INHIBITORS

Fig.1

a



b



[Continued on next page]



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VIRAL INHIBITORS

The present invention relates to inhibitors of viral infection and in particular to compounds which inhibit picornaviral infection.

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The *Picornaviridae* are a large family of pathogens with major impacts on human and animal health. The largest genera of picornavirus are the enteroviruses, and of these, apart from human rhinoviruses responsible for the majority of cases of the common cold, enterovirus 71 (EV71) is perhaps the greatest threat to public health. EV71 has been identified as responsible for periodic disease outbreaks throughout the world and in recent years there have been regular major epidemics in South Asia. These are associated with outbreaks of mild childhood exanthema, herpangina, and hand, foot and mouth disease (HFMD), however, especially in the Asia-Pacific region, fatal neurological and cardiovascular disorders can ensue (MCMINN, P. C. *Neurol J Southeast Asia* **8** 57-63 (2003); incorporated herein by reference).

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It has proved notoriously difficult to find useful therapies for picornaviral infections such as the common cold. At present replacing the hydrophobic pocket factors, expelled from many picornaviruses as they uncoat the genome (Wang, X. *et al. Nature structural & molecular biology* **19**, 424-429, doi:10.1038/nsmb.2255 (2012); incorporated herein by reference), by more robust binders is thought to be the most promising point for therapeutic intervention (Rotbart, H. A. *Antiviral research* **53**, 83-98 (2002); and Tsang, S. K. *et al. Chemistry & biology* **8**, 33-45 (2001); both of which are incorporated herein by reference).

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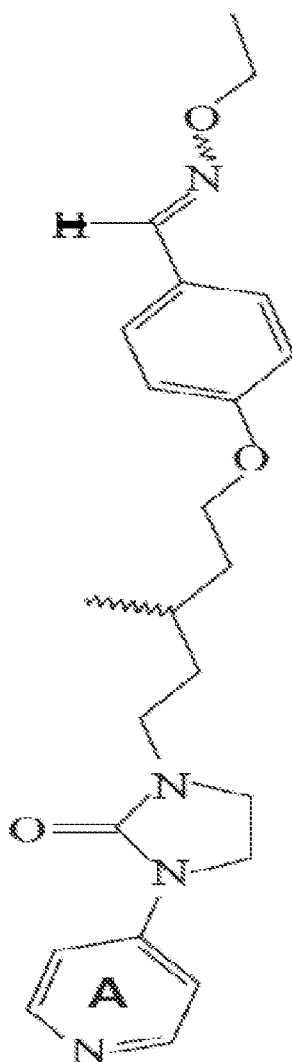
Pleconaril and BTA798 are two compounds identified as potentially able to replace the natural pocket factor (Rotbart, H. A. *Antiviral research* **53**, 83-98 (2002); and Tsang, S. K. *et al. Chemistry & biology* **8**, 33-45 (2001); both of which are incorporated herein by reference), and inhibit viral uncoating by entropic stabilization of the capsid (Phelps, D. K. & Post, C. B. *Journal of molecular biology* **254**, 544-551 (1995); and Tsang, S. K., Danthi, P., Chow, M. & Hogle, J. M. *Journal of molecular biology* **296**, 335-340, doi:10.1006/jmbi.1999.3483 (2000); both of which are incorporated herein by reference). These two have completed phase II clinical trials (Susanne C. Feil *et al. ACS Med. Chem. Lett.*, 2012, 3 (4), pp 303-307). However, there are, as yet, no approved therapies for picornavirus infections.

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There is accordingly a need for additional compounds which will inhibit picornaviral infection. There is a need for compounds which will be useful in treating picornaviral infection.

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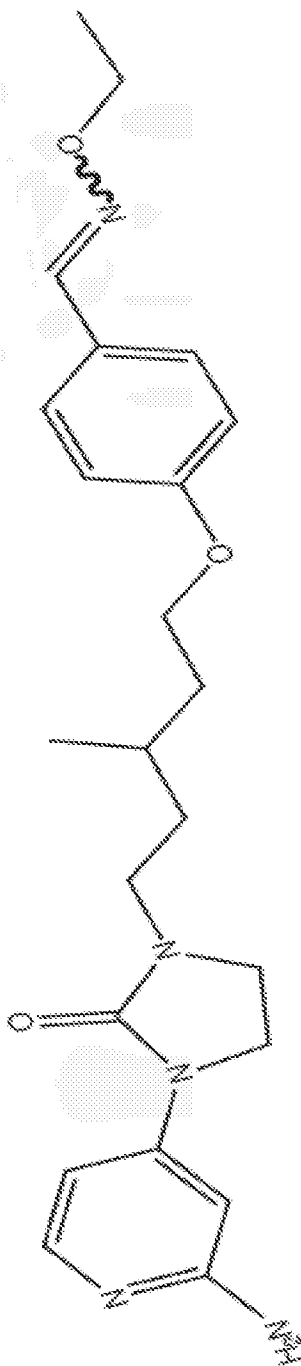
The present invention addresses the above needs by providing a compound of the formula (I):



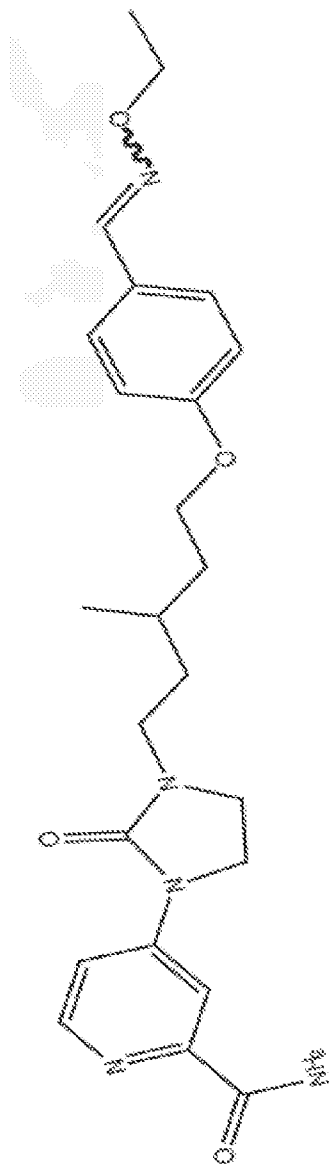
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wherein position 2 of the pyridine ring (A) is replaced with an amine or an amide.

Position 2 of the pyridine ring (A) may be replaced with a primary amine. Thus, in an embodiment, the present invention provides a compound of the formula (II):



Alternatively, position 2 of the pyridine ring (A) may be replaced with an amide. Thus, in such an embodiment, the present invention provides a compound of the formula (III):



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In the context of positions in a pyridine ring, the term “replaced” is synonymous with the term “substituted”, as exemplified by formulae (II) and (III).

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As used herein, references to “the compound of the invention” should be understood as including a compound according to the formula (I) and/or formula (II) and/or formula (III) above. The compound of the invention may also include compounds according to the formula (I) above, wherein the pyridine ring (A) is replaced with alternatives including furan, isoxazole, pyrrole or amine-thiazole.

The compound of the invention has surprisingly been found to exhibit a very high potency of inhibition against picornaviruses. In particular, a compound (formula II) of the invention may exhibit a potency of an order of magnitude greater than currently-known inhibitors. Without wishing to be bound by any theory, it is thought that the compound of the invention acts at the level of the whole virus, by targeting conformational transitions required for infection.

Accordingly, the present invention also provides a method for treating or preventing picornaviral infection. The method may comprise administering the compound of the invention to a subject. The present invention further provides the compound of the invention for use in treatment or prevention of picornaviral infection. In a related aspect, the present invention provides the use of the compound of the invention, in the preparation of a medicament for the prevention or treatment of picornaviral infection.

The picornaviral infection may be an enteroviral infection, which may be enterovirus 71 (EV71).

As used herein, the term "treatment" or "treating" embraces therapeutic or preventative/prophylactic measures, and includes post-infection therapy and amelioration.

As used herein, the term "preventing" includes preventing the initiation of infection and/or reducing the severity or intensity of infection.

The compound of the invention may be administered to a subject (typically a mammalian subject such as a human) already having a picornaviral infection, a condition or symptoms associated with a picornaviral infection, to treat or prevent the infection. In one embodiment, the subject is suspected of having come in contact with a picornavirus, or has had known contact with a picornavirus, but is not yet showing symptoms of exposure.

When administered to a subject that already has a picornaviral infection, or is showing symptoms associated with a picornaviral infection, the compound of the invention can cure, delay, reduce the severity of, or ameliorate one or more symptoms, and/or prolong the survival of a subject beyond that expected in the absence of such treatment.

The treatments and preventative therapies of the present invention are applicable to a variety of different subjects of different ages. In the context of humans, the therapies are applicable to children (e.g. infants, children under 5 years old, older children or teenagers) and adults. In the context of other animal subjects (e.g. mammals such as primates), the therapies are applicable to immature subjects and mature/adult subjects.

Enteroviruses are ubiquitous viruses that commonly infect humans, as well as a wide range of other animal species for example rhesus macaque (Oberste M.S. et al, *J. Virol.* January 2013 vol. 87 no. 1 572-580; incorporated herein by reference). Accordingly, veterinary uses are encompassed within the scope of the present invention. The present invention therefore provides the veterinary treatment or prevention of a picornaviral infection, that may be an enteroviral infection.

In a further embodiment, the invention provides a pharmaceutical composition comprising the compound of the invention and a pharmaceutically acceptable carrier. Non-limiting examples of pharmaceutically acceptable carriers include water, saline, and phosphate-buffered saline.

In addition to a pharmaceutically acceptable carrier, the pharmaceutical composition of the invention may further comprise one or more of a salt, excipient, diluent, immunoregulatory agent and/or antimicrobial compound. Pharmaceutically acceptable salts include acid addition salts formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or with organic acids such as acetic, oxalic, tartaric, maleic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

Administration of the compound or pharmaceutical composition of the invention may be by conventional routes, e.g. oral, intravenous, subcutaneous, intraperitoneal, or mucosal routes. The administration may be by parenteral injection, for example, a subcutaneous or intramuscular injection.

Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations or formulations suitable for distribution as aerosols. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1%-2%.

The compounds of the invention have low toxicity. Thus, ADME-tox properties are provided, calculated with QikProp V3.6 (www.schrodinger.com) for both compound II and compound III described above:

	ALD (compound III)	NLD (compound II)		
Predicted brain/blood partition coefficient (-3.0 - 1.2)	-2.416	-1.891		
Predicted apparent Caco-2 cell ^a permeability in nm/sec (<25 poor, >500 great)	173	460		
Predicted apparent MDCK cell ^b permeability in nm/sec (<25 poor, >500 great)	74	214		
Predicted skin permeability, log K_p (K _p in cm/h)	-2.897	-1.986		
Lipinski Rule of 5 violations (maximum is 5)	0	0		
Jorgensen Rule of 3 Violations (maximum is 3)	1	1		
% Human Oral Absorption (<25 % is poor)	89	100		
Predicted qualitative human oral absorption (> 80% is high)	Low	Low		
HERG K ⁺ Channel Blockage, logIC50 (concern below -5)	-6.939	-7.127		
5 of 1712 molecules most similar to:				
	ALD (compound III)		NLD (compound II)	
	Molecule	Similarity(%)	Molecule	Similarity(%)
	Valsartan	82.70	Valsartan	83.86
	Cisapride	80.25	Eprosartan	81.80
	Glisentide	80.19	Glisentide	80.61
	Suxibuzone	79.37	Losartan	80.07
	Rosuvastatin	78.80	Cisapride	79.74

Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders.

LIST OF FIGURES

Figure 1. The inhibitor binding site and selected structures (a) The organisation of the EV71 inhibitor-binding pocket, lying below the canyon floor, occupied by a natural pocket factor (PF). An icosahedral 5-fold axis is marked. VP1 subunits are labeled. **(b)** A selection of 3-(4-Pyridyl)-2-imidazolidinone derivatives structures, ranked according to their EC₅₀ value. The EC₅₀ for GPP3 is 10 nM, 100 nM for GPP2, 1.3 mM for GPP12, 1.6 mM for GPP4 and 40 mM for GPV13. The following chemical moieties are labeled in GPP3: A, pyridine ring; B, imidazole moiety; C, phenoxy group.

Figure 2. Single round real space averaged |Fo-Fc| omit maps of four different 3-(4-Pyridyl)-2-imidazolidinone derivatives bound to EV71. VP1 residues within 3Å from the ligand are shown in sticks; the side chain of Leu-24 of VP3 is labeled. The ligands are shown as sticks. **(a)** EV71-GPP3, **(b)** EV71-GPP2, **(c)** EV71-GPP12, **(d)** EV71-GPP4. Inset in **(a)** shows a close-up view of the methyl group on GPP3 molecule, the view is rotated by 45° about the y axis from that in the main illustration.

Figure 3. GPP3 bound to VP1 pocket and thermal stability effect of GPP3, GPP3 and GPV013. (a) VP1 is shown in cartoon representation. The side chains of hydrophilic residues at the entrance of the pocket and the hydrophobic residues surrounding the methyl moiety of GPP3 ligand are shown in stick representation. The residues in contact with the methyl of the linker group, are shown, the distances are: 4.49Å for F131, 4.75 Å for A133, 4.19 Å for M253. **(b)** The first derivatives of the fluorescence curves, measured in the PaSTRy assay are shown for the control virus incubated with SYTO9 and EV71 virions incubated with 200ug/ml GPV13, 200ug/ml GPP3 and 200ug/ml GPP2 with 72 hours incubation at room temperature, and to which SYTO9 dye has been added. **(c)** PaSTRy results as for **(b)**, using SYTO9. EV71 virions were incubated with 20ug/ml GPV13 for 72 hours at room temperature. EV71 virions were incubated with 20ug/ml GPP2 or GPP3 with 24 hour incubation at room temperature respectively. **(d)** The first derivatives of the fluorescence curves for the PaSTRy assay, using SYPRO RED. **(e)** The first derivatives of the fluorescence curves for the PaSTRy assay, using SYPRO RED are shown for the control virus and EV71 virions incubated with 200mg/ml GPP2 and 200mg/ml GPP3 with 72 hours incubation at room temperature respectively.

Figure 4. VP1 pocket and docking of the new ligands. (a) A GRID map showing the interaction energies between the probe and the explored region within the VP1 binding pocket. **(b)** Molecular docking of NLD; and **(c)** ALD, in the VP1 pocket. Both the ligands are shown as sticks. NLD hydrogen bonds with main chain nitrogen of Q202. ALD establish hydrogen bond interactions with the side chain of D112. The side chain of Ile 113 is hidden.

Figure 5. Newly designed capsid binders bound to the VP1 pocket of EV71. (a) Correlation plot of binding affinities of 3-(4-Pyridyl)-2-imidazolidinone derivatives predicted by quantum mechanics ligand docking calculations and experimental pIC_{50} values. The dots on the graph labelled "NLD" and "ALD" show the calculated pIC_{50} for the new ligands. **(b)** and **(c)** show the crystal structures of the drugs bound to the virus. Single round real space averaged $|Fo-Fc|$ omit maps of **(b)** ALD ligand in EV71-ALD complex; and **(c)** NLD ligand in EV71-NLD complex. **(d)** and **(e)** show the first derivatives of the fluorescence curves for the PaSTRy assay. **(d)** compares the control virus incubated with SYTO9 dye and EV71 virions incubated with 200mg/ml NLD, ALD, GPP12 and GPP4 with 24 hours incubation at room temperature. **(e)** compares the control virus incubated with SYPRO RED with EV71 virions incubated with 200ug/ml NLD, 200ug/ml ALD, 200mg/ml GPP12 and 200ug/ml, GPP4 with 24 hours incubation at room temperature (legends are the same as for **(d)** above). **(f)** EV71 samples were titrated via $TCID_{50}$ in the presence of a range of concentrations of ALD, NLD and GPP3. Non-linear regression was used to determine the IC_{50} value. The IC_{50} is the point at which the $TCID_{50}$ value is reduced by 50%. For clarity the curves are represented on a logarithmic scale.

Figure 6. Structure-based drug design.

Molecules of similar size (319.68 Å and 331.08 Å).

Figure 7. Large-scale chemical synthesis and quality control.

Chemical structures are provided in (a) with analytical results shown in (b)-(e).

Figure 8. Survival curve – medium dosage (10 mg/kg)

Plot of % survival in mice over time, following EV71 infection.

Figure 9. Survival curve – high dosage (25 mg/kg)

Plot of % survival in mice over time, following EV71 infection.

Figure 10. Oral suspension delivered 3 days after infection

5 Plot of % survival in mice over time, following EV71 infection.

Figure 11. Oral suspension delivered 4 days after infection

Plot of % survival in mice over time, following EV71 infection.

Figure 12. Oral suspension delivered 5 days after infection

10 Plot of % survival in mice over time, following EV71 infection.

Figure 13. Oral suspension delivered 6 days after infection

Plot of % survival in mice over time, following EV71 infection.

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Figure 14. Experiment of pharmaceutical preparation.

Representation of LC/MS and HPLC data.

EXAMPLES

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The invention will be further clarified by the following examples, which are intended to be purely exemplary of the invention and are in no way limiting.

Methods and materials

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Synthesis of compound ALD

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Methyl 4-[3-(5-[4-[(ethoxyimino)methyl]phenoxy]-3-methylpentyl)-2-oxoimidazolidin-1-yl]pyridine-2-carboxylate (45 mg, 0.10 mmol, 1.00 equiv), methanol (5 mL), NH₃·H₂O (10 mL) and NH₄Cl (1.7 mg, 0.33 equiv) were placed in a 100-mL 3-necked round-bottom flask. The resulting solution was stirred overnight at 40°C, and then concentrated under vacuum. The residue was diluted with 15 mL of water. The resulting solution was extracted with 3x10 mL of ethyl acetate and the organic layers combined. The mixture was dried over anhydrous sodium sulfate and concentrated under vacuum. The residue

was washed with 2x5 mL of ether/hexane (1:1). This yielded 30 mg of ALD as a white solid.

Synthesis of compound NLD

- 5 A solution of tert-butyl N-[4-[3-(5-[4-[(ethoxyimino)methyl]phenoxy]-3-methylpentyl)-2-oxoimidazolidin-1-yl]pyridin-2-yl]carbamate (200 mg, 0.38 mmol, 1.00 equiv) in TFA/CH₂Cl₂ (1:1) (20 mL) was placed in a 50-mL round-bottom flask. The resulting solution was stirred for 6 h at room temperature and then concentrated under vacuum. The crude product was purified by Flash-Prep-HPLC. This yielded 30 mg of NLD as a
10 light yellow solid.

Virus purification and crystallisation

- Cells were cultured and virus stocks prepared as described previously (Wang, X. *et al. Nature structural & molecular biology* **19**, 424-429, doi:10.1038/nsmb.2255 (2012);
15 incorporated herein by reference). Purified live virus was concentrated to 1.5mg/ml in PBS buffer (pH 7.4). 20% of Crystal Screen1 (Hampton Research) condition 13 (30% (v/v) PEG400, 0.2 M tri-sodium citrate, 0.1 M Tris-HCl (pH 8.5)) was added to the virus solution and then equilibrated against 1.2M ammonium citrate dibasic, 0.1M sodium acetate trihydrate pH 4.6. Crystallization used nanoliter vapor diffusion in Greiner
20 CrystalQuick X plates (Walter, T. S. *et al. J Appl Crystallogr* **36**, 308-314, doi:10.1107/S0021889803001997 (2003); and Walter, T. S. *et al. Acta Crystallogr D Biol Crystallogr* **61**, 651-657, doi:10.1107/S0907444905007808 (2005); both of which are incorporated herein by reference). Cubic crystals emerged in two weeks. GPP2, GPP3, GPP4 and GPP12 were dissolved in 100% DMSO with concentrations of 19 mg/ml, 18.5
25 mg/ml, 24mg/ml and 68 mg/ml respectively. GPP3 and GPP2 stock solutions were mixed with Crystal Screen 1 (Hampton Research) condition 13 in the ratio 1:2. These solutions were further diluted to give a solution containing ~2mg/ml ligand, ~7% PEG₄₀₀, 44 mM tri-sodium citrate and 22 mM Tris-HCl (pH8.5). GPP4 and GPP2 stock solutions were diluted 55 times in water supplemented with 18% of Crystal Screen 1 (Hampton
30 Research). About 0.5 ml of this solution was added to the 0.2ml crystallization drops one to two weeks prior to data collection (one week was sufficient to allow binding to the virus).

For ALD and NLD the protocol was modified. ALD and NLD were dissolved in 100 % DMSO with concentrations of 258.3 mg/ml and 142 mg/ml. These stock solutions were diluted 100 times in 100% DMSO and then further diluted 55 times in water supplemented with 18% of Crystal Screen 1 (Hampton Research). One day of soaking was sufficient to allow full replacement of the pocket factor.

PaSTRY assay

Thermofluor experiments were performed with an MX3005p RT-PCR instrument (Agilent). SYTO9 and SYPROred (both Invitrogen) were used as fluorescent probes to detect the presence of RNA and the exposed hydrophobic regions of proteins, respectively. 50 µl reactions were set up in a thin-walled PCR plate (Agilent), containing 0.5–1.0 µg of EV71, 5 µM SYTO9 and 3X SYPROred in PBS (pH 7.4) and the temperature ramped from 25 °C to 99 °C, with fluorescence recorded in triplicate at 1 °C intervals. In order to replace the natural pocket factor with EV71 inhibitors completely, different concentrations of inhibitors (20 µg/ml and 200 µg/ml) were used with different incubation times (72 or 24 hours) at room temperature, since the natural pocket factor is expected to have a slow off-rate and the assay was performed at equilibrium. 5% DMSO was used throughout. The melting temperature, T_m , was taken as the minimum of the negative first derivative of the denaturation curve.

Structure determination.

Data were collected at room temperature (293K) from crystals in crystallization plates as described in Wang, X. *et al. Nature structural & molecular biology* **19**, 424-429, doi:10.1038/nsmb.2255 (2012); and Axford, D. *et al. Acta crystallographica. Section D, Biological crystallography* **68**, 592-600, doi:10.1107/S0907444912006749 (2012) (both of which are incorporated herein by reference), on beamlines I24 and I03 at Diamond light source. Diffraction images, each of 0.05°-0.1° rotation were recorded on a Pilatus 6M detector using an unattenuated beam of 0.05x0.05 mm² at I24 or 0.10x0.06 mm² at I03, with exposure times of 0.1s per image. Due to the fast onset of radiation damage in the microcrystals, data collection was limited to 3-10 frames per crystal. Data processing was performed using the HKL-2000 package (Otwinowski, Z. & Minor, W. *Method Enzymol* **276**, 307-326, doi:10.1016/S0076-6879(97)76066-X (1997); incorporated herein by reference). Reflections with fractional partialities of >0.7 or > 0.5 were scaled to full intensity and incorporated into the data set as fully recorded reflections (program POST: D.I.S. and Jonathan M. Diprose, unpublished program).

Averaged signal to noise was calculated with the ioversigma.py program (http://strucbio.biologie.unikonstanz.de/ccp4wiki/index.php/Calculate_average_I/).

Intensities were converted to structure factor amplitudes with TRUNCATE (K.S., F. G. S. a. W. *Acta Cryst. A*, 517 (1978); incorporated herein by reference). All crystals belonged to space group I23 with 4 pentamers in the asymmetric unit. The EV71 model PDBID: 3VBF was subjected to positional and B-factor refinement using strict NCS in CNS.1.3 (Brunger, A. T., Adams, P. D. & Rice, L. M. *Current opinion in structural biology* **8**, 606-611 (1998); incorporated herein by reference). The NCS operators were updated by rigid-body refinement of individual protomers in REFMAC5 (Nicholls, R. A., Long, F. & Murshudov, G. N. *Acta crystallographica. Section D, Biological crystallography* **68**, 404-417, doi:10.1107/S090744491105606X (2012); incorporated herein by reference) and recalculated NCS matrices used as constraints with CNS.1.3. Density modification was performed with CNS.1.3 and Parrot (Cowtan, K. *Acta crystallographica. Section D, Biological crystallography* **66**, 470-478, doi:10.1107/S090744490903947X (2010); incorporated herein by reference). Ligand coordinates were generated with PRODRG (Schüttelkopf, A. W. & van Aalten, D. M. *Acta crystallographica. Section D, Biological crystallography* **60**, 1355-1363, doi:10.1107/S0907444904011679 (2004); incorporated herein by reference), restraint dictionaries were generated by GRADE (<http://grade.globalphasing.org>). Model building was performed with COOT (Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. *Acta crystallographica. Section D, Biological crystallography* **66**, 486-501, doi:10.1107/S0907444910007493 (2010); incorporated herein by reference). Water molecules were modeled into 3.5 σ peaks of an Fo-Fc map. Models were validated using Molprobit (Chen, V. B. *et al. Acta crystallographica. Section D, Biological crystallography* **66**, 12-21, doi:10.1107/S0907444909042073 (2010); incorporated herein by reference). 93-97% of the residues were in favoured regions of the Ramachandron plot and less the 1% were outliers in all structures. Figures were prepared with PyMOL (Schrödinger, LLC).

Molecular docking and binding energy calculation

GRID calculations (Goodford PJ. *J Med Chem.* 1985 Jul;28(7):849-57.) were performed with default parameters using as probes an amine and protonated primary amine. In GOLD docking calculations, the docking site was based on the GPP2 position in the crystal structure. Docking was performed using the default settings for high-efficiency docking, using the GoldScore scoring function. The poses with highest scores were kept.

The topology file and parameters file for each ligand was generated by the ANTECHAMBER program distributed with AMBER TOOLS (University of California, San Francisco, 2010). Protein coordinates were subjected to minimization in AMBER11 (University of California, San Francisco, 2010). The energy-minimized structures were then used as an initial reference for molecular dynamics simulations. Due to the known rigidity of the structure after docking, positional restraints were applied to all C α atoms. The simulation was carried out at 310K, with constant volume periodic boundary and an integration step of 2fs. Long-range electrostatics were included using the PME (Particle Mesh Ewald) method. Prior to simulation the structures were fully TIP3 solvated, using a 10Å box of explicit water, and Cl⁻ ions were used to neutralize the surface charge. Hydrogen bonds were constrained using SHAKE (Ryckaert, J. P. et al. *J Comput Phys* 23, 327-341 (1977)). Each solvated assembly was simulated for 1ns, with a snapshot taken every 10ps. The ΔG of binding was calculated from snapshots using the MMPSA.py (Miller B.R. et al. *J Chem Theory Comput* 8, 3314-3321 (2012)) program as implemented in AMBER11 package (University of California, San Francisco, 2010). All calculations were performed on a computer cluster consisting 672 cores, made up of two types of nodes, AMD nodes with 2.67 GB/core and intel nodes with 8GB/core. Each MD simulation was run in parallel distributed over 12 cores. It took on average 42h to complete a single MD run (47 being required for a full benchmark set). Docking with Rosetta ligand docking (Davis, I. W. et al. *Journal of molecular biology* 385, 381-392 (2009)) used the position of C5 of GPP2 to define the target position of the ligand. Experiments were performed with the following modification to the default protocol. To search for rotations that maximize the attractive and repulsive score the ligands were randomly rotated 1000 times.

The final method tested was that embodied in the Schrödinger software. Docking of into the rigid binding pocket of EV71 was performed with Glide SP (Glide5.5 standard precision, Friesner, R. A. et al. *Journal of medicinal chemistry* 47, 1739-1749 (2004)) procedure. Small molecule coordinates were generated by PRODRG (Schüttelkopf, A. W. & van Aalten, D. M. *Acta crystallographica. Section D, Biological crystallography* **60**, 1355-1363, doi:10.1107/S0907444904011679 (2004); incorporated herein by reference) and energy minimized using Ligprep in Schrödinger suite at pH 7.0 and using the OPLS_2005 force field (Banks, J. L. et al. *Journal of computational chemistry* **26**, 1752-1780, doi:10.1002/jcc.20292 (2005); incorporated herein by reference). The standard conversion procedure with full hydrogen optimization was applied to prepare the protein

with the Protein Preparation work-flow. The VP1 binding pocket in the crystal structure in complex with GPP2 ligand was taken as the receptor structure. These processed coordinates were used for the subsequent grid generation and ligand docking procedures. The docking box was centered on the centroid of the GPP2 molecule with an exhaustive search being performed on a box, generally of $8 \times 8 \times 8 \text{ \AA}^3$ around this point (the larger volume over which the grid potentials were computed extended beyond the likely positions of any ligand atoms). Default values were used for all other parameters. As positional constraints, the hydrogen bond between the imidazole moiety of the GPP2 molecule and the carbonyl group of VP1 Ile113 were used as well as hydrophobic constraints corresponding to the region identified as a hydrophobic trap. To improve the accuracy of the binding pose the QMPLD (Quantum Mechanics Polarized Ligand Docking; CHO AE, GUALLAR V, BERNE BJ, FRIESNER R. Importance of Accurate Charges in Molecular Docking: Quantum Mechanical/Molecular Mechanical (QM/MM) Approach. *Journal of computational chemistry* 2005;26(9):915-931; incorporated herein by reference) protocol was used as implemented in the Schrödinger suite. The most reliable binding pose for each small molecule was selected according to the preservation of key interactions observed in the crystal structure and on the van der Waals and electrostatic interactions.

Viral Titration

EV71 genotype B2 strain MS742387 was used for all *in vitro* experiments and Vero cells for viral propagation. TCID₅₀ was employed to measure viral titre. Viral samples were serially diluted (10^{-2} to 10^{-9}) and added to Vero cells grown in 96 well plates. Each dilution was replicated 10 times along with two control wells that contained no virus. The concentration of each drug was kept constant in each plate. Plates were incubated for seven days at 37°C, the plates were then stained with crystal violet and the cytopathic effect (CPE) was evaluated. TCID₅₀ values were calculated using the Reed-Muench method (Reed, L. J. M., H. *The American Journal of Hygiene* **27**, 493-497 (1938); incorporated herein by reference). Each experiment was repeated three times.

Example 1: Analysis of 3-(4-pyridyl)-2-imidazolidinones as potential inhibitors of picornaviral activity

Background

Picornaviruses are small positive-stranded RNA viruses with non-enveloped icosahedral capsids. The capsid comprises 60 copies of proteins VP1-4. Proteins VP1-3 each adopt a β -barrel configuration and are arranged with icosahedral symmetry such that VP1 surrounds the 5-fold axes and VP2 and VP3 alternate about the 2 and 3 fold axes, whilst VP4 is internal (Rossmann, M. G. *et al. Nature* **317**, 145-153 (1985); incorporated herein by reference). Depressions encircling the five-fold axes in enteroviruses, referred to as ‘canyons’, have been shown to be, frequently, the sites of receptor attachment (Fig. 1a).

Uncoating, whereby the capsid opens to release the viral genome into the host cell cytosol in order to replicate, is key to picornavirus infection. Like most enteroviruses, EV71 harbors within its capsid 60 copies of a hydrophobic “pocket factor”, a natural lipid (sphingosine), buried in a hydrophobic pocket, which lies at the base of the canyon, in the capsid protein VP1 (Fig. 1a). Expulsion of this molecule following binding of the virus to its receptor triggers a cascade of structural rearrangements, which result in the capsid opening to facilitate genome release (Ren, J. *et al. Nature communications* **4**, 1929, doi:10.1038/ncomms2889 (2013); and Wang, X. *et al. Nature structural & molecular biology* **19**, 424-429, doi:10.1038/nsmb.2255 (2012); both of which are incorporated herein by reference). There are several low molecular weight hydrophobic compounds which may be able to replace the natural pocket factor (Rotbart, H. A. *Antiviral research* **53**, 83-98 (2002); and Tsang, S. K. *et al. Chemistry & biology* **8**, 33-45 (2001); both of which are incorporated herein by reference) and inhibit viral uncoating by entropic stabilization of the capsid (Phelps, D. K. & Post, C. B. *Journal of molecular biology* **254**, 544-551 (1995); and Tsang, S. K., Danthi, P., Chow, M. & Hogle, J. M. *Journal of molecular biology* **296**, 335-340, doi:10.1006/jmbi.1999.3483 (2000); both of which are incorporated herein by reference).

Here, a novel class of imidazolidinones has been synthesized using the skeletons of Pleconaril (Pevear, D. C., Tull, T. M., Seipel, M. E. & Groarke, J. M. *Antimicrobial agents and chemotherapy* **43**, 2109-2115 (1999); incorporated herein by reference) and related molecules, having anti-EV71 activity and exhibiting an IC₅₀ in the range of 0.001-25 μ M (Shia, K. S. *et al. Journal of medicinal chemistry* **45**, 1644-1655 (2002); and Yu, M. X.,

Slater, M. R. & Ackermann, H. W. *Arch Virol* **151**, 663-679, doi:10.1007/s00705-005-0667-x (2006); both of which are incorporated herein by reference). The recent crystal structures of native and inactivated EV71 particles (Wang, X. *et al. Nature structural & molecular biology* **19**, 424-429, doi:10.1038/nsmb.2255 (2012); and Plevka, P., Perera, R., Cardoso, J., Kuhn, R. J. & Rossmann, M. G. *Science* **336**, 1274, doi:10.1126/science.1218713 (2012); Plevka P, *et al. Proc Natl Acad Sci U S A*. 2013 Apr 2;110(14):5463-7; all of which are incorporated herein by reference) provides the opportunity for the rational design of improved EV71 inhibitors.

The structures, at room temperature, of EV71 in complex with four 3-(-4-pyridyl)-2-imidazolidinone derivatives (GPP2, GPP3, GPP4 and GPP12) were determined (Figs. 1 & 2). Data were collected *in situ* at the Diamond Light Source (Axford, D. *et al. Acta crystallographica. Section D, Biological crystallography* **68**, 592-600, doi:10.1107/S0907444912006749 (2012); incorporated herein by reference), providing structures at between 2.65 and 2.8 Å resolutions (methods as described above). The compounds replace sphingosine in the VP1 pocket, with some very small shifts (0.1 Å - 0.4 Å) in Ca positions as expected from their shape similarity with sphingosine. The solvent accessible area, calculated by Areaimol (Winn, M. D. *et al. Acta Crystallogr D* **67**, 235-242, doi:10.1107/S0907444910045749 (2011); incorporated herein by reference), is 8Å² for GPP3, 11Å² for GPP2, 12Å² for GPP4, 9Å² for GPP12 and for the natural pocket factor 9 Å². Without wishing to be bound by any theory, the present inventors propose that all of these molecules are essentially fully buried, with perhaps GPP3 slightly deeper in the pocket. All compounds bind with the pyridine ring close to the entrance of the pocket, the imidazole moiety hydrogen-bonding residue I113, as seen with sphingosine, and the phenoxy-ring sandwiched between two phenylalanines (F135, F155) (Fig. 2). The introduction of an additional methyl group at the linker region of GPP3 results in an order of magnitude tighter binding compared to GPP2 (Fig. 1, Fig. 3a).

Evaluation of in silico docking methods

To attempt to establish a reliable protocol for the prediction of binding affinities for novel pocket binders, a database was assembled of published inhibition data for 49 EV71 inhibitors (Ke *et al.* (2006), Shia *et al.* (2002) and Chang *et al.* (2002); each of which is incorporated herein by reference). Correlation plots were generated between the published IC₅₀ values (Rotbart, H. A. *Antiviral research* **53**, 83-98 (2002); and Yu, M. X.,

Slater, M. R. & Ackermann, H. W. *Arch Virol* **151**, 663-679, doi:10.1007/s00705-005-0667-x (2006); both of which are incorporated herein by reference) and the energy of interaction computed from their docking pose in the VP1 pocket for several current methods: GOLD (Jones, G., Willett, P., Glen, R. C., Leach, A. R. & Taylor, R. *Journal of molecular biology* **267**, 727-748, doi:10.1006/jmbi.1996.0897 (1997); incorporated herein by reference), Rosetta Ligand Dock (Davis, I. W. & Baker, D. *Journal of molecular biology* **385**, 381-392, doi:10.1016/j.jmb.2008.11.010 (2009); incorporated herein by reference), Glide5.5 (Friesner, R. A. *et al. Journal of medicinal chemistry* **47**, 1739-1749, doi:10.1021/jm0306430 (2004); incorporated herein by reference) and Quantum Mechanics Polarised Ligand Docking (QMPLD) (; CHO AE, GUALLAR V, BERNE BJ, FRIESNER R. Importance of Accurate Charges in Molecular Docking: Quantum Mechanical/Molecular Mechanical (QM/MM) Approach. *Journal of computational chemistry* 2005;26(9):915-931); incorporated herein by reference) implemented in the Schrödinger suite (<http://www.schrodinger.com>). Protocols are set out above.

For GOLD, the docked ligand poses were subjected to further molecular dynamics runs to calculate the free energy of binding ($\Delta G_{\text{binding}}$) using MMPBSA.py algorithms implemented in AMBER 11, nevertheless the overall correlation between the IC₅₀ and $\Delta G_{\text{binding}}$ (0.26) was found to be weak. Similar agreement was obtained with Rosetta Ligand Dock, which allows ligand flexibility by minimizing ligand torsion angles. Energy values generated by Glide 5.5 scored somewhat better but the correlation with measured values was still below 0.67. However the QMPLD computed energy data provided a compelling correlation of 0.81 (Fig 5a).

It was reviewed how well the methods predicted the poses observed experimentally. Once again, whilst the other methods performed rather poorly the quantum mechanical optimized procedure was broadly reliable. In particular when applied to GPP2 and GPP3, the predicted docking poses had a root-mean-square deviation (RMSD) of less than 2 Å from the observed crystal structures.

In vitro inhibitor characterisation

Using inhibitors GPP3 and GPP2, thermostability was examined as a potential measure of compound potency using a plate-based high-throughput thermofluor assay, PaSTRy (Walter, T. S. *et al. Journal of virological methods* **185**, 166-170, doi:10.1016/j.jviromet.2012.06.014 (2012); incorporated herein by reference), developed

to assess viral stability and examine the dynamics of uncoating (Ren, J. *et al. Nature communications* **4**, 1929, doi:10.1038/ncomms2889 (2013); and Wang, X. *et al. Nature structural & molecular biology* **19**, 424-429, doi:10.1038/nsmb.2255 (2012); both of which are incorporated herein by reference). GPV13 (1'-[1,4-phenylenebis(methyleneoxy)]bis(2-chloro-6-chlorobenzene)), an inhibitor similar to the SCH47820 compound known for its strong antiviral properties against poliovirus type 2, several echoviruses and Coxsackieviruses, but only weakly effective against EV71 (De Palma, A. M., Vliegen, I., De Clercq, E. & Neyts, J. *Medicinal research reviews* **28**, 823-884, doi:10.1002/med.20125 (2008); incorporated herein by reference), provided a negative control.

The purified virus releases its RNA genome (T_R) at $\sim 58^\circ\text{C}$, however after incubation with 200ug/ml GPV13, GPP3 and GPP2 for 72 hours incubation at room temperature T_R is raised to $60-61^\circ\text{C}$ (Fig.3b). Even at a concentration of 20mg/ml and a 24-hour incubation, GPP3 and GPP2 showed increased particle stability but a similar concentration of GPV13 had little effect even after 72 h (Fig.3c). These results are consistent with the EC_{50} s for GPP3, GPP2 and GPV13 of 10 nM, 100 nM and 40 mM respectively (the only measured IC_{50} , for GPP2, was 1nM) (Shia, K. S. *et al. Journal of medicinal chemistry* **45**, 1644-1655 (2002); incorporated herein by reference). Interestingly the protein melting temperatures, T_m s, for untreated virus occurred in two distinct steps with values of $\sim 58^\circ\text{C}$ and $\sim 65^\circ\text{C}$. Taken together with T_R , this indicates a two-stage transition in protein conformation, with the lower temperature transition corresponding to the release of RNA from expanded particles (Fig. 3d) and the higher temperature the protein melting. In contrast only the higher transition was found after incubation with 200ug/ml GPP3 or GPP2 and the T_m peaks were sharper (Fig.3e).

Summary

This Example demonstrates the utility of an extremely rapid plate-based fluorescence assay for inhibitor binding which replicates the rank order of previously reported *in vitro* assays. In addition by measuring RNA accessibility alongside protein unfolding it was determined that potent inhibitors elevate the capsid conformational transition associated with genome release to the point at which the capsid proteins melt. Several *in silico* methods were compared in order to provide a robust basis for structure-based design for docking and binding affinity calculations. Although most were of limited value, the QMPLD method provided, with guidance from the observed crystal structures, extremely

reliable docking results and excellent predictions of binding strength (correlation coefficient 0.81 against a database of 49 prior results). The remarkable power of this method is presumably partly due to the fact it uses quantum mechanics to take into account the ligand polarization of the protein environment during the docking process.

5

Example 2: Design and analysis of improved EV71 inhibitors

Design

Having determined the crystal structures of EV71 in complex with four ligands, GPP3, GPP2, GPP4 and GPP12, it was found that the molecules have a broad range of affinities, which are explained by the structures of the complexes. GPP4, the shortest of the four, only partially occupies the EV71 binding pocket and has the poorest EC₅₀. Without wishing to be bound by any theory, the present inventors propose that this finding suggests that there is an optimal drug size, which correlates with the efficiency of binding, so that molecules of the right length better fill the pocket and are therefore better inhibitors. Inhibitors that satisfy this requirement should offer an aromatic moiety at the correct point to occupy a hydrophobic trap formed by F135 and F155. Indeed in crystal structures of picornavirus-inhibitor complexes a pair of hydrophobic residues is found at positions equivalent to those occupied by these aromatic residues in EV71. Thus rhinovirus 14 in complex with Pleconavir (Zhang, Y. *et al. Journal of virology* **78**, 11061-11069, doi:10.1128/JVI.78.20.11061-11069.2004 (2004); incorporated herein by reference) and poliovirus 2 in complex with a Schering-Plough compound (Lentz, K. N. *et al. Structure* **5**, 961-978 (1997); incorporated herein by reference) show the antiviral agent located between the structurally equivalent residues Y128 and Y152, and F134 and Y159 respectively. The presence of this hydrophobic trap constrains the extent of penetration of such inhibitors and hence the length of the molecule. Thus increasing the length of the inhibitor causes a mis-alignment of the phenoxy group with respect to F135 and F155, decreasing the binding energy and undermining the inhibitory effect. Conversely, in GPP4, where the molecule is shorter (bearing just an iodine atom at position 6 on the phenoxy group), the trap locks the molecule, preventing it from completely filling the cavity (Fig. 2d). GPP3 additionally largely fills a hydrophobic pocket with a methyl group in the linker region giving it the lowest EC₅₀ value of 0.01 mM. The methyl group is surrounded by a set of hydrophobic residues (F131, A133, M253), which leave some space which might possibly accommodate a slightly bigger substituent (Fig.3a), however it is thought (Chang, C. S. *et al. Journal of medicinal chemistry* **48**,

3522-3535, doi:10.1021/jm050033v (2005); incorporated herein by reference) that phenyl, dimethyl, ethyl and propyl groups are not appropriate for filling the EV71 pocket, since they decrease the affinity. Similarly in rhinovirus bulkier substituents are unfavourable (Diana, G. D. *et al. Journal of medicinal chemistry* **33**, 1306-1311 (1990);
5 incorporated herein by reference), so it seems likely that a methyl group is close to optimal. It was noted that the binding pocket of EV71 is more exposed to the solvent compared to other picornaviruses (Wang, X. *et al. Nature structural & molecular biology* **19**, 424-429, doi:10.1038/nsmb.2255 (2012); and Plevka, P., Perera, R., Cardoso, J., Kuhn, R. J. & Rossmann, M. G. *Science* **336**, 1274, doi:10.1126/science.1218713
10 (2012); both of which are incorporated herein by reference). Based on inspection of the pocket entrance at the bottom of the canyon, it was postulated that introducing a functional group such as an amine or amide on the pyridine ring (Fig. 3a) might simultaneously increase the solubility of the compound and enhance its affinity for the virion, by allowing the formation of hydrogen bonds with polar residues (for instance
15 Q202 or D112) (Fig. 3a).

To test this hypothesis, the pocket surface was scanned *in silico* with a collection of probes using GRID (Goodford, P. J. *Journal of medicinal chemistry* **28**, 849-857 (1985); incorporated herein by reference), to identify hotspots for ligand binding. Using an amine
20 with a lone pair as a probe identified a hotspot around the residue D112 with an overall energy of interaction of -15 kcal mol⁻¹. This increased to -17 kcal mol⁻¹ when the probe was an amine cation. The region around Q202 is also a hotspot for binding, with interaction energy -20 kcal mol⁻¹ (Fig. 4a). These results support the hypothesis that
25 introducing an amino group on the pyridine ring would significantly increase the overall binding energy due to the formation of a hydrogen bond with residues on the canyon floor. On this basis modifications of the GPP3 molecule were generated *in silico*, exploring the effect of multiple substituents on the pyridine ring and replacing the pyridine with alternative moieties of different size, such as furan, isoxazole, pyrrole, and amine-thiazole.

30 Putting these results together with an *in silico* mapping of the pocket entrance for additional polar interactions, the above structural data were used to design more potent compounds, specifically, optimized versions of the GPP3 molecule. The QMPLD generated docking poses for the two of these molecules, named ALD and NLD (Fig. 4 b,
35 c), confirmed additional putative hydrogen bonds with residues lining the pocket cavity.

Moreover the hydrophilic substituents responsible for these additional interactions offered the additional benefit of increasing the solubility (Lipinski, C. A., Lombardo, F., Dominy, B. W. & Feeney, P. J. *Adv Drug Deliver Rev* **23**, 3-25, doi:Doi 10.1016/S0169-409x(96)00423-1 (1997); incorporated herein by reference). The binding energy of these molecules estimated using the QMPLD method, suggested that ALD and NLD were likely to bind far more tightly to EV71 than any previously reported compounds, potentially translating into a pico-molar IC₅₀ for the tightest binder, NLD (Fig. 4c).

NLD and ALD (Fig. 4b, 4c), were synthesized as described above. NLD (molecular weight 448 Da) is a variant of GPP3 where position 2 of the pyridine ring has been replaced with a primary amine (Fig.4b). The docking poses show this molecule engaged with its amino group forming a hydrogen bond to the carbonyl group of Q202, increasing the number of virus-ligand interactions. To achieve this, docking flips the pyridine ring by 180° compared to its orientation in the GPP3-EV71 complex. The second molecule, ALD (molecular weight 476 Da), has position 2 of the pyridine ring replaced with an amide (Fig.4c). The docking pose suggests that this substituent will form hydrogen bonds with the side chain of D112.

LogP, the logarithm of the n-octanol/water partition coefficient, is a useful measure of solubility (lower logP = increased solubility). LogP for GPP3 is computed to be 3.8, whereas for ALD and NLD this decreases to 3.0 and 3.6, respectively. The predicted IC₅₀ values were 2.56 pM and 0.84 pM for ALD and NLD respectively (Fig. 5a).

Analysis

ALD and NLD were soaked into EV71 crystals. As expected the compounds were more soluble than the GPP series and soaking with the protocol used for these led to rapid degradation of the crystals. The soaking procedure was therefore modified to reduce the effective concentration of the compounds 550-fold, allowing data to be collected and room temperature structures determined at 2.75Å resolution using *in situ* X-ray analysis. Both ligands preserve the key interactions described above (Fig. 5 b, c). Both ligands maintain the key useful interactions described above (Fig. 5a, b). The presence of the amide group on the pyridine moiety allows ALD to establish hydrogen bonds with the side chain of D112, exactly as predicted by *in silico* docking (Fig. 5b). However for NLD, the crystal structure shows that the amine group on the pyridine moiety, rather than interacting with the peptide carbonyl moiety of Q202 as predicted by *in silico* docking, is

rotated by almost 180° to interact instead with the side chain of D112, in a similar way to that observed for ALD (Fig. 5b). With the exception of this reorientation, the experimental results agree with the predicted docking poses (r.m.s. deviation <2 Å). To investigate the NLD docking, LigPrep (<http://www.schrodinger.com>) was used, which suggested that at pH 7 the pyridine nitrogen could be protonated. Redocking NLD with a protonated pyridine produced an essentially correct pose of similar energy (r.m.s. deviation of 0.5 Å in comparison to experimental result).

It was accordingly determined that ALD and NLD readily replace the natural pocket factor in EV71. PaSTRy analysis confirmed that NLD and ALD are potent capsid stabilizers, enhancing stabilization at lower concentrations than known tight binders such as GPP3 (Fig. 5 d, e). Quantification for such tight binders is difficult, since the concentration of binding sites (60 times the virus concentration of 0.2 µM), and of competing pocket factor (at least equal to the concentration of binding sites) are far above the binding constant for NLD and ALD. The concentration needed to produce thermal stabilization may therefore significantly underestimate the IC₅₀ (Kenakin, T. *Pharmacologic Analysis of Drug-Receptor Interaction*. (1993); incorporated herein by reference). Therefore, the inhibitory activities of ALD and NLD were also compared to those of GPP3 and GPP4 by *in vitro* TCID₅₀ assay in Vero cells. Ten-fold serial dilutions of virus were used in the presence of different concentrations of the compounds. Control wells were exposed to the equivalent concentration of the compound solvent (DMSO) to ensure that this had no cytopathic effect on uninfected cells or on virus titre. NLD was shown to be the most effective inhibitor with an IC₅₀ of ~0.025 nM and was able to inhibit the viral titre to below 5% at concentrations over 0.05 nM. ALD has an IC₅₀ of 8.54 nM (although the TCID₅₀ results suggested a more complex biphasic effect). GPP4 did not display inhibitory effects at concentrations up to 1000 nM (Fig. 5 f).

IC₅₀ values from cell-based assays accordingly underline the extraordinary potency of NLD, protecting cells from EV71 infection at a concentration of 25 pM (Fig. 5 f).

In summary, the invention provides an experimental and computational pipeline for the design of picornavirus inhibitors, applied to the complete virus capsid. Each element of this pipeline is both highly efficient (X-ray data collection, *in silico* analysis, and thermal stability validation each taking only a few hours) and reliable, with *in silico* analysis proving to be a powerful predictor of potency *in vitro*.

Using these components a single round of design allowed the provision, starting from a nM prior compound with limited solubility, a next generation, more soluble, pM inhibitor, with many drug-like properties. The present inventors propose that this approach can markedly facilitate the design of more efficient inhibitors targeted at a range of other picornaviruses, including rhinoviruses, poliovirus and Coxsackieviruses.

Example 3: *In vivo* analysis of candidate drug toxicity

NLD was dissolved in 0.5%DMSO, 99.5% saline, and administered via the intraperitoneal route. Mice were not infected with EV71:

	Dosage	Concentration	Animal tested	Animal survival
Solvent- control	—	—	10	10
Low dosage	1mg/kg	50ug/ml	10	10
Medium dosage	5mg/kg	250ug/ml	10	10
High dosage	25mg/kg	1250ug/ml	10	10

Experiments into long-term toxicity reveal low toxicity (>1000mg/kg) in adult mice.

Example 4: *In vivo* analysis of candidate drug effectiveness

NLD was dissolved in 0.5%DMSO, 99.5% saline. Mice were administered drug via the intraperitoneal route two hours after infection with EV71:

	Dosage	Concentration	Animal tested	Animal survival
Blank control	10LD ₅₀ /0.1ml	/	10	0
Solvent control	—	—	10	0
Low dosage	1mg/kg	50ug/ml	10	1
Medium dosage	5mg/kg	250ug/ml	10	9
High dosage	25mg/kg	1250ug/ml	10	10

NLD was tested by a new oral suspension formulation, and administered several days after EV71 infection.

	Time after EV71 infection (hours)									
	74	98	122	146	170	194	218	242	266	290
Medium dosage 1	√	√	√	√	√	√	√			
Medium dosage 2		√	√	√	√	√	√	√		
Medium dosage 3			√	√	√	√	√	√	√	
Medium dosage 4				√	√	√	√	√	√	√
High dosage 1	√	√	√	√	√	√	√			
High dosage 2		√	√	√	√	√	√	√		
High dosage 3			√	√	√	√	√	√	√	
High dosage 4				√	√	√	√	√	√	√

5

Survival results following medium dosage (10 mg/kg) are provided in Figure 8. Survival results following high dosage (10 mg/kg) are provided in Figure 9.

Figure 10 shows the survival results when oral suspension was administered 3 days after infection. Figures 11-13 show the survival results when oral suspension was administered 4, 5, or 6 days after infection. These data demonstrate that mice administered with NLD significantly outperform controls, even when administered 6 days after infection.

15 LC/MS and HPLC methods for determination of NLD oral suspension in mice and human plasma have been performed to study drug metabolism and pharmacokinetics.

CLAIMS

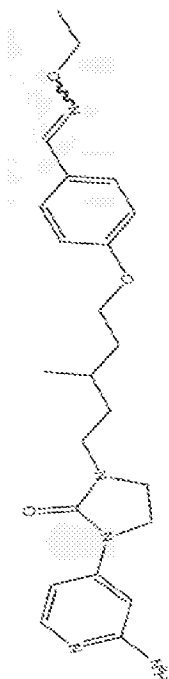
1. A compound of the formula (I):



5

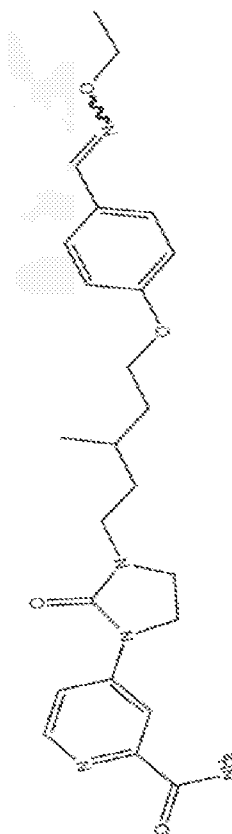
wherein position 2 of the pyridine ring (A) is replaced with an amine or an amide.

2. The compound of claim 1, having the formula (II):



10

3. The compound of claim 1, having the formula (III):



4. A method for treating or preventing picornaviral infection, the method comprising administering the compound of any one of claims 1 to 3 to a subject.
5. The compound of any one of claims 1 to 3 for use in treatment or prevention of picornaviral infection.
6. The use of the compound of any one of claims 1 to 3, in the preparation of a medicament for the prevention or treatment of picornaviral infection.
7. The method of claim 4, or the compound of claim 5, or the use of claim 6, wherein the picornaviral infection is an enteroviral infection.
8. The method, compound or use of claim 7, wherein the enteroviral infection is enterovirus 71 (EV71).

9. The method of claim 4, or the compound of claim 5, or the use of claim 6, wherein the picornaviral infection is an infection by a rhinovirus, poliovirus or Coxsackievirus.
- 5 10. A pharmaceutical composition comprising the compound of any one of claims 1 to 3 and a pharmaceutically acceptable carrier.
- 10 11. The compound of any one of claims 1 to 3, or the pharmaceutical composition of claim 10, formulated for intravenous, subcutaneous, intraperitoneal, parenteral, oral, intramuscular or mucosal delivery.
12. The compound or pharmaceutical composition of claim 11, formulated for oral delivery.
- 15 13. The compound or pharmaceutical composition of claim 11, formulated for intraperitoneal delivery.
14. The compound of any one of claims 1 to 3, or the pharmaceutical composition of claim 10, formulated for veterinary use.

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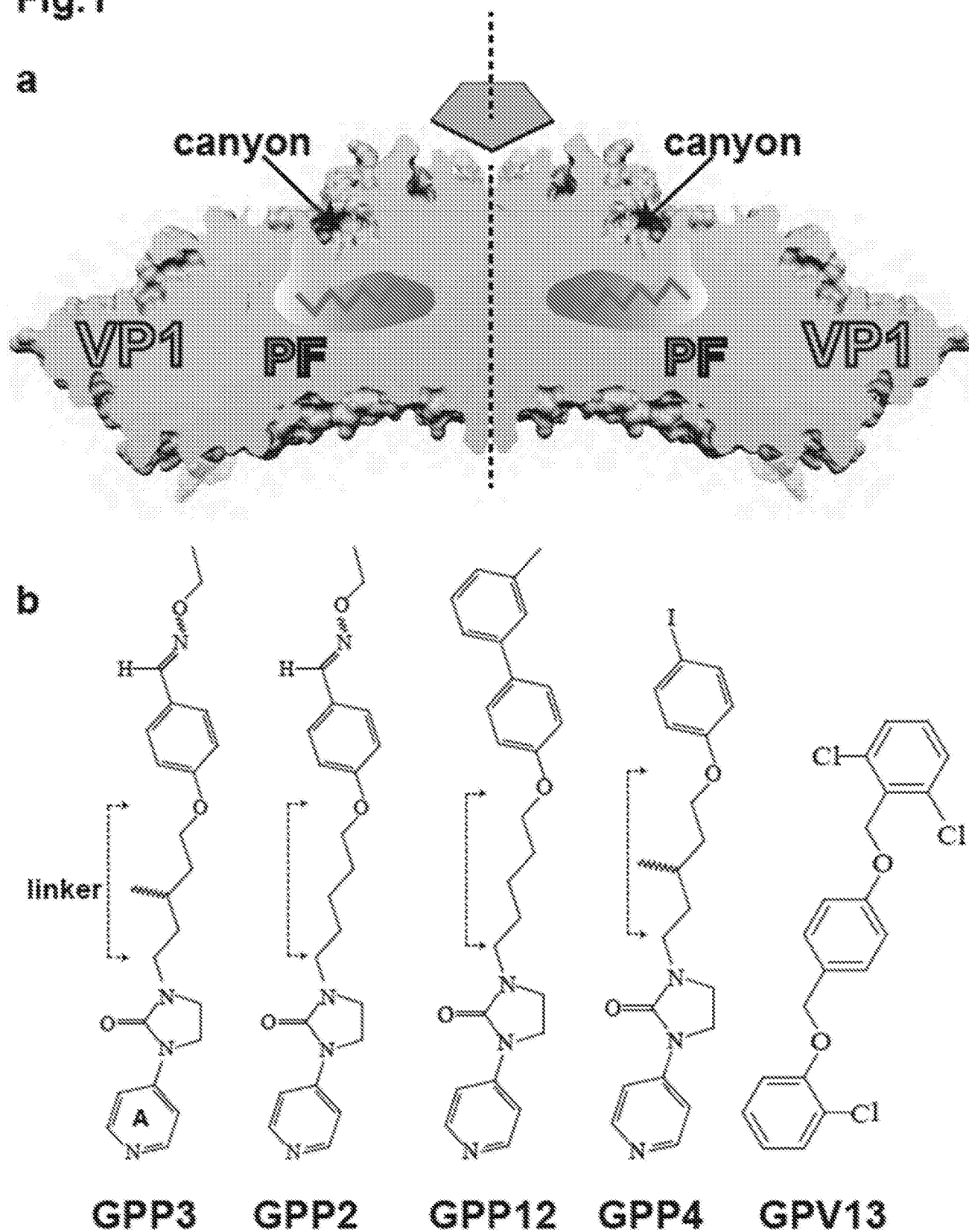
Fig.1

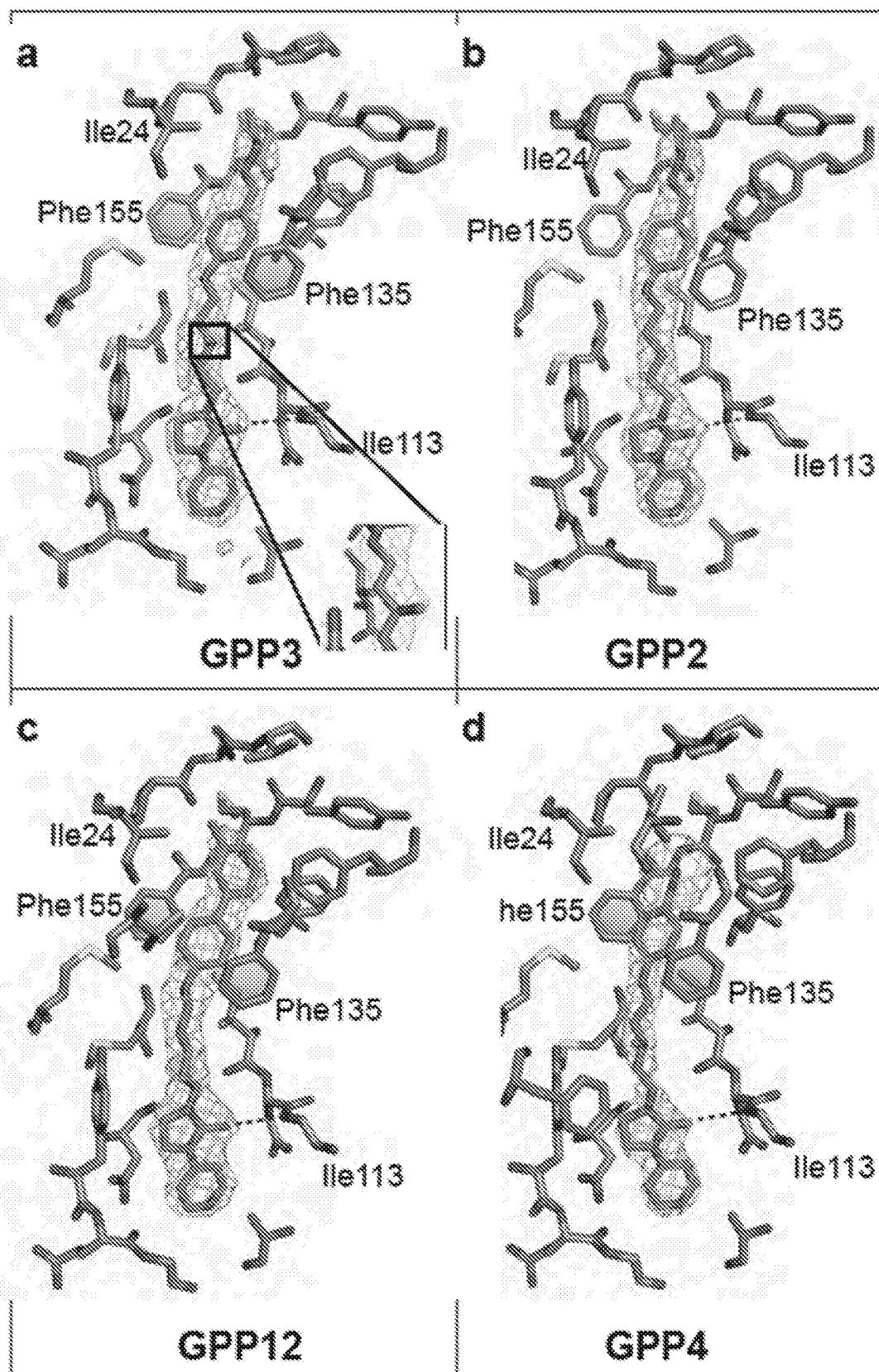
Fig.2

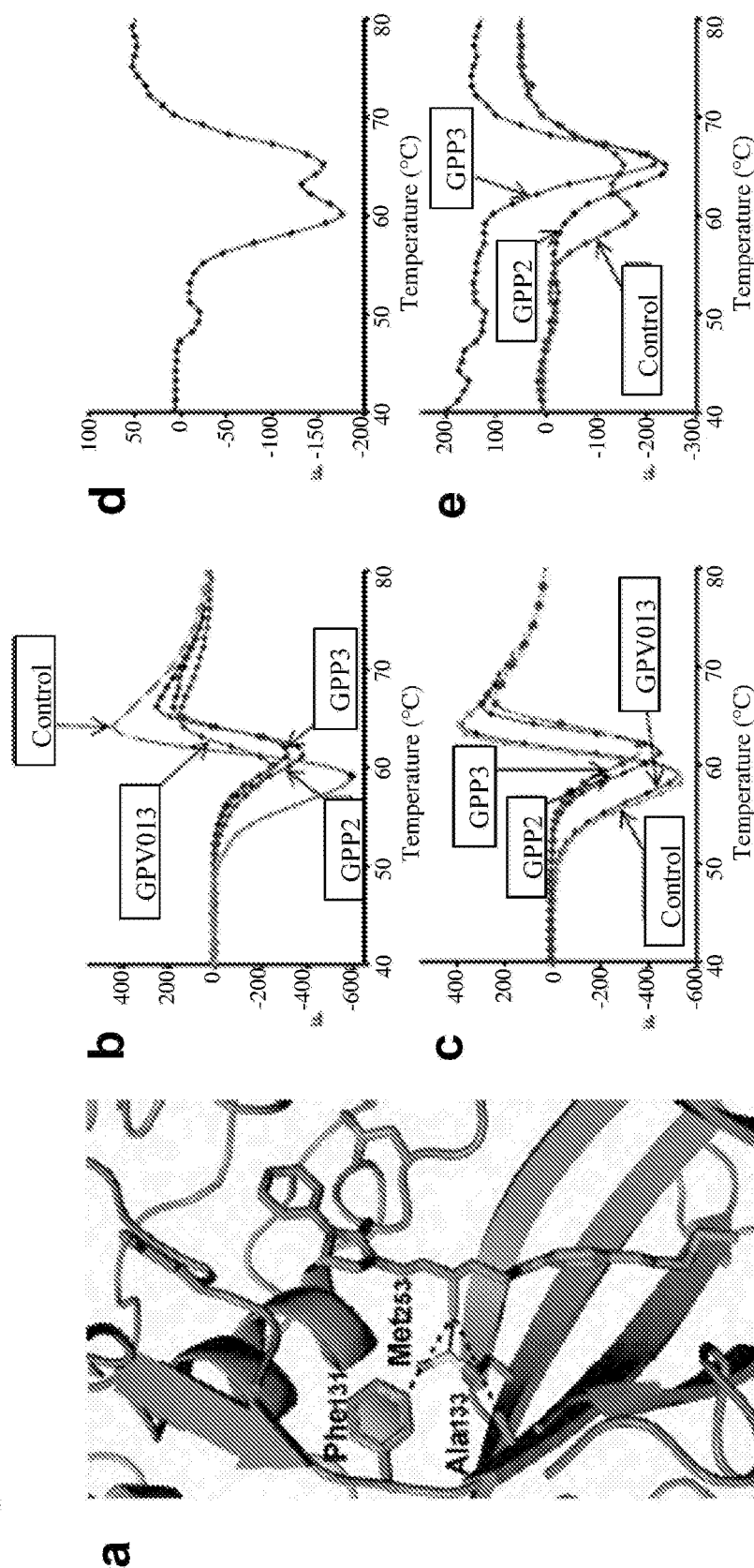
Fig. 3

Fig. 4

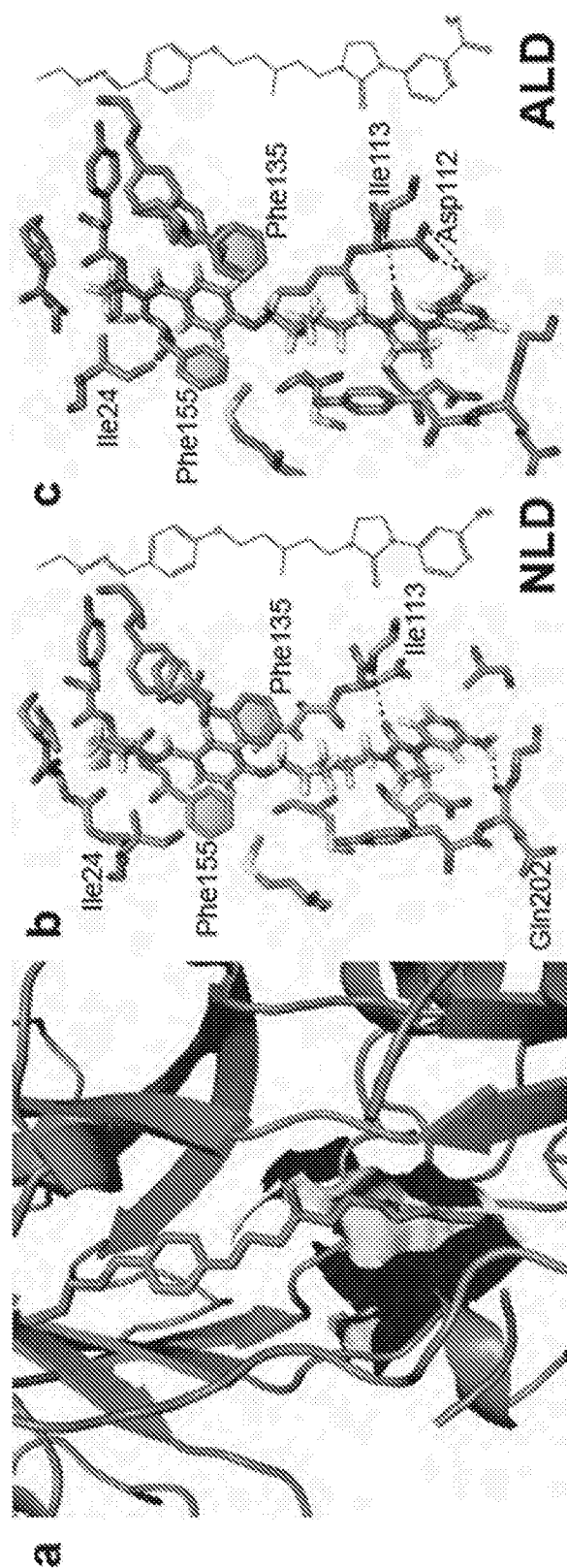


Fig. 5

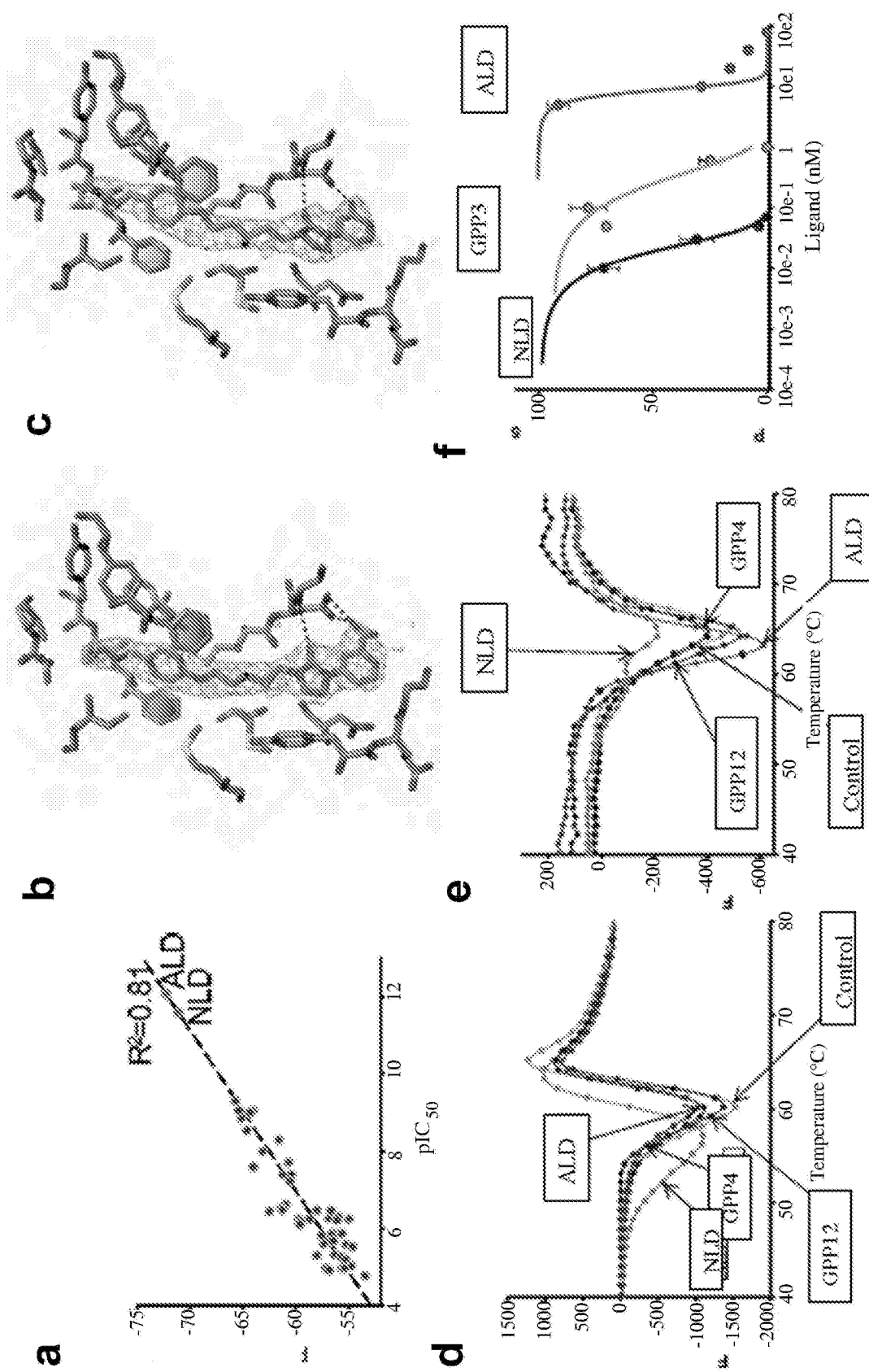


Fig. 6

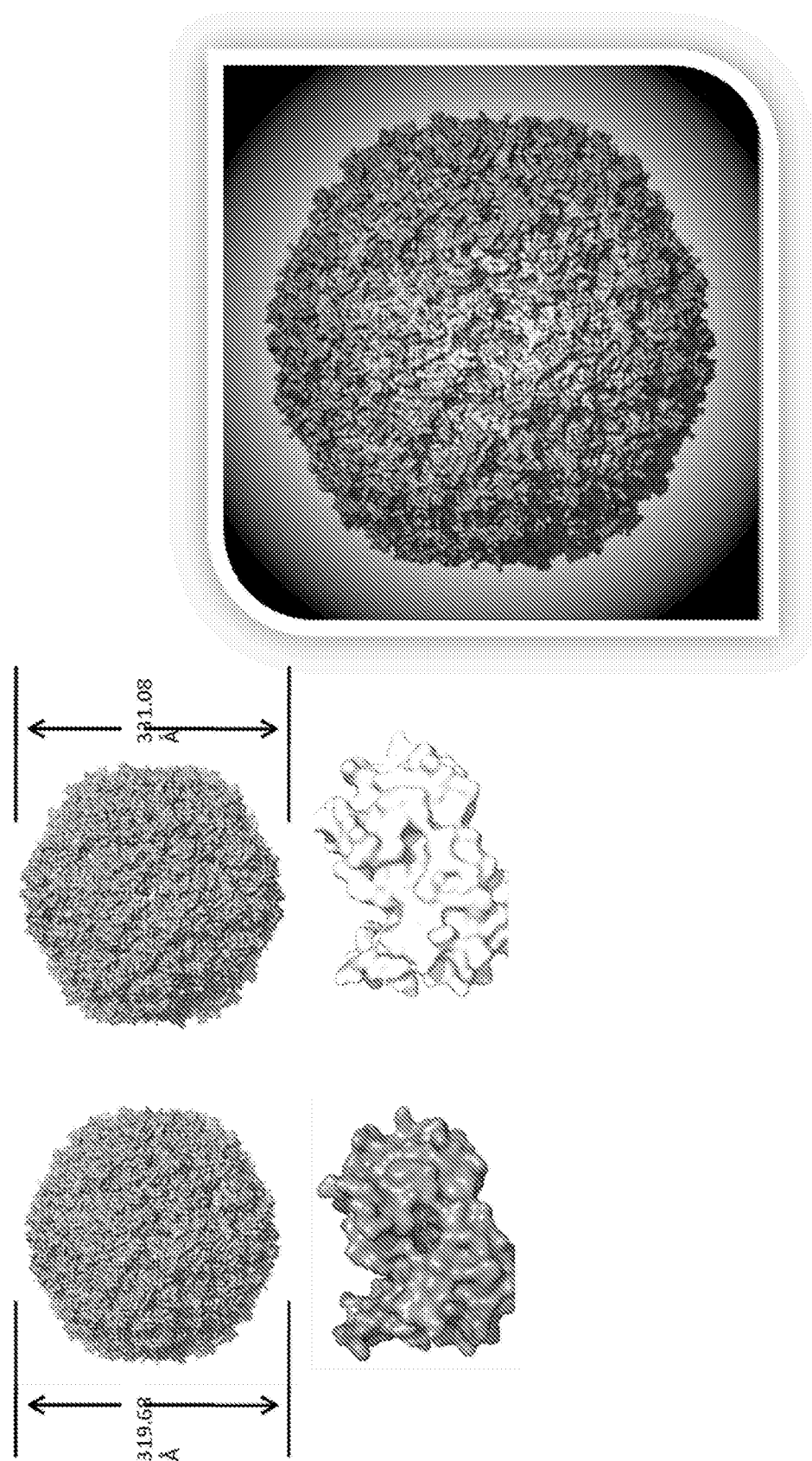


Fig. 7 (a)

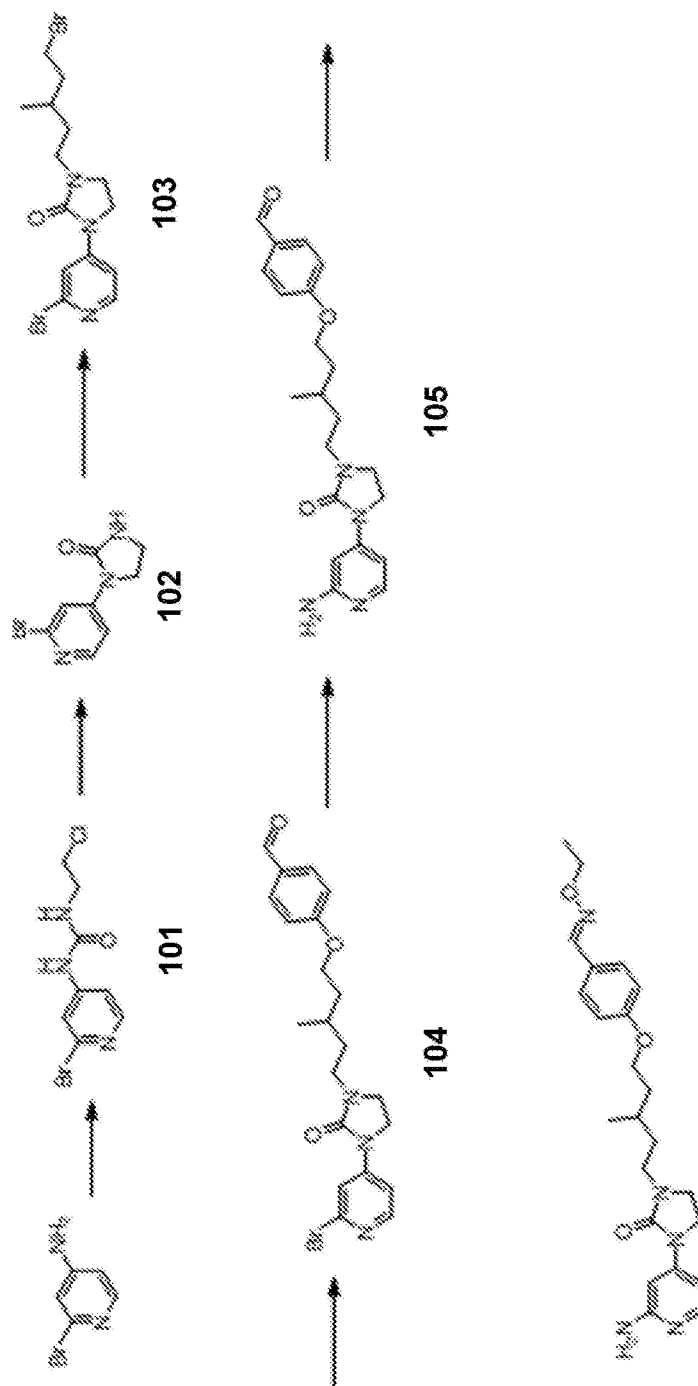


Fig. 7(b)

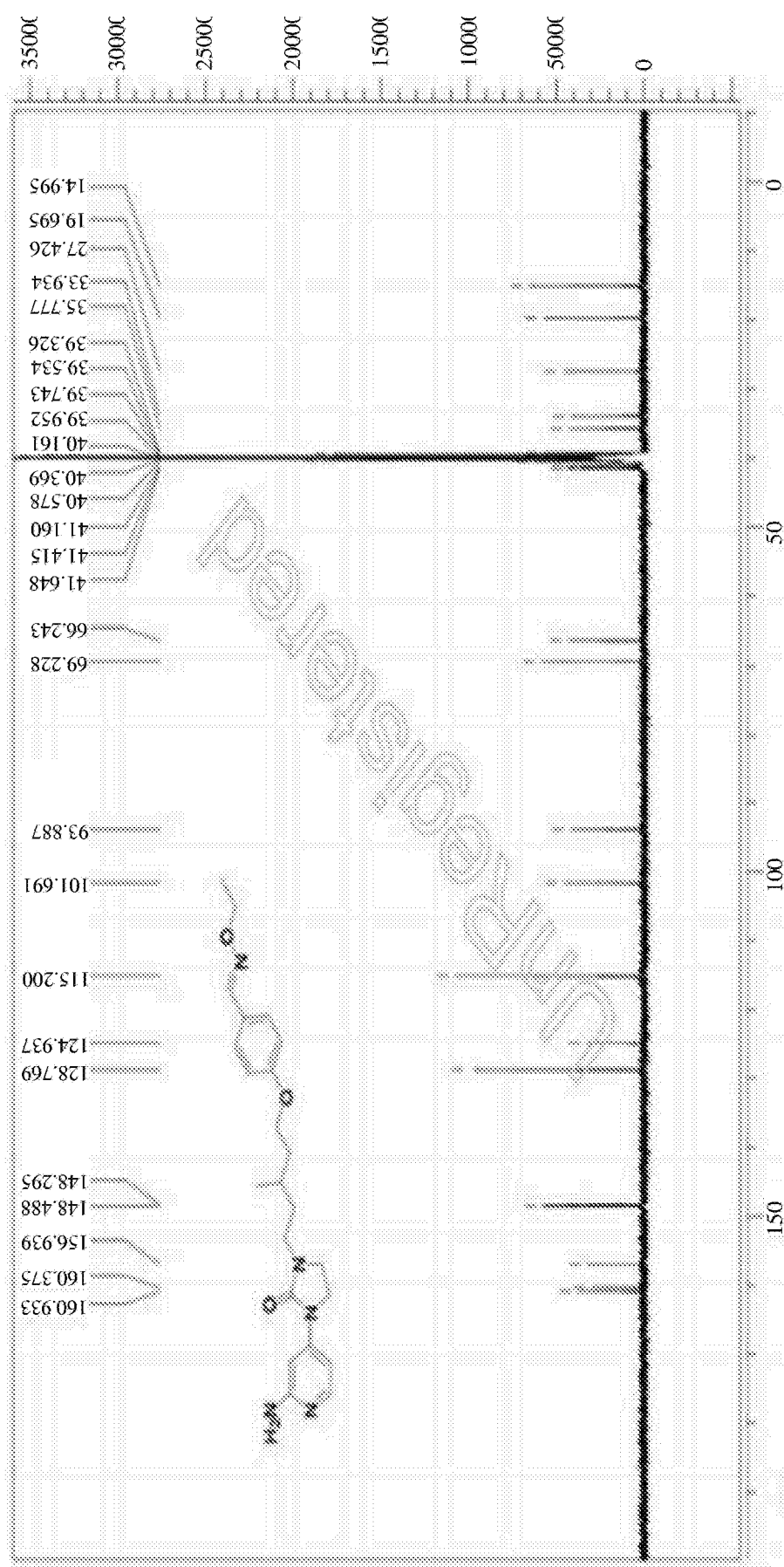


Fig. 7(c)

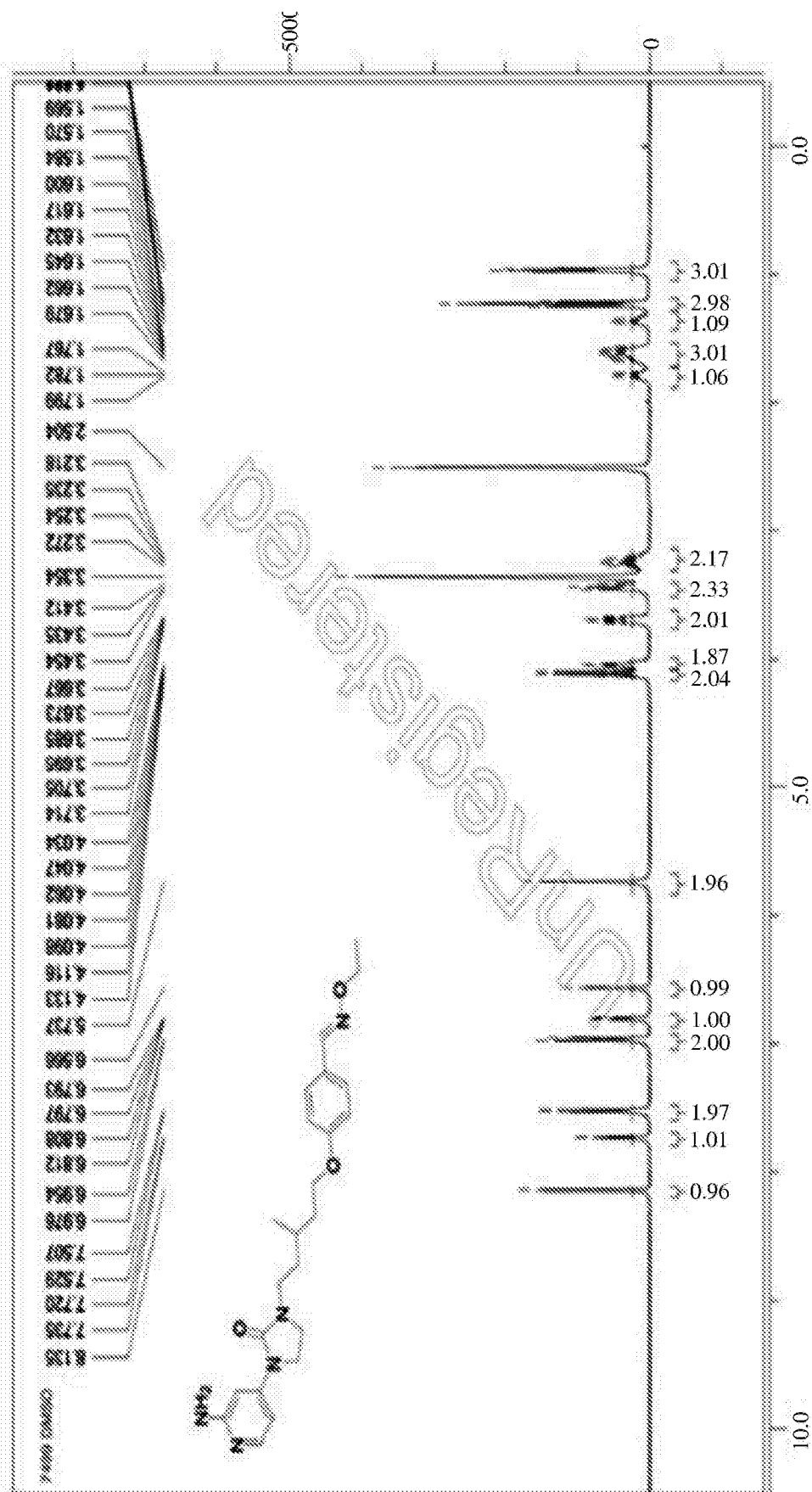


Fig. 7(d)

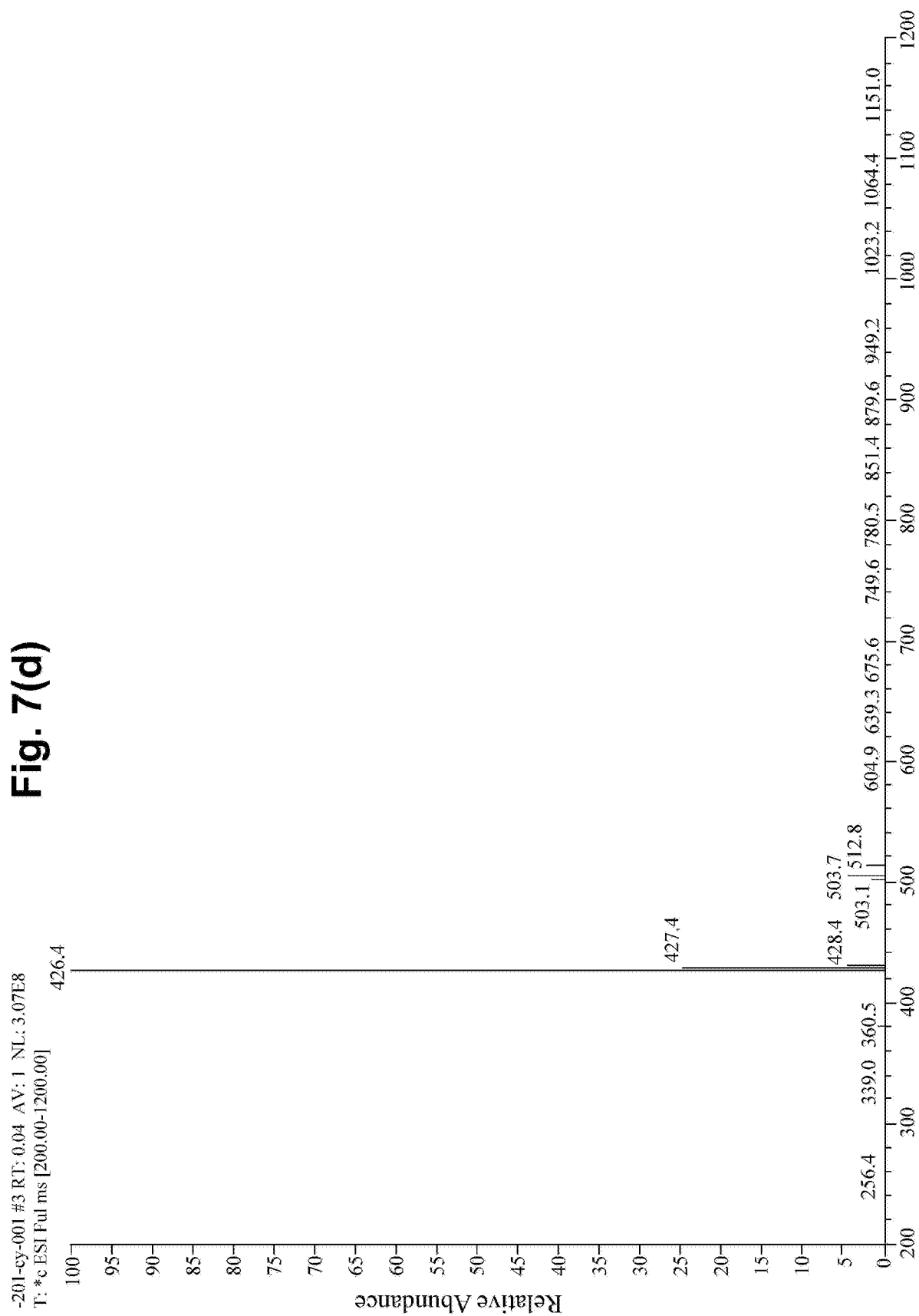


Fig. 7 (e)

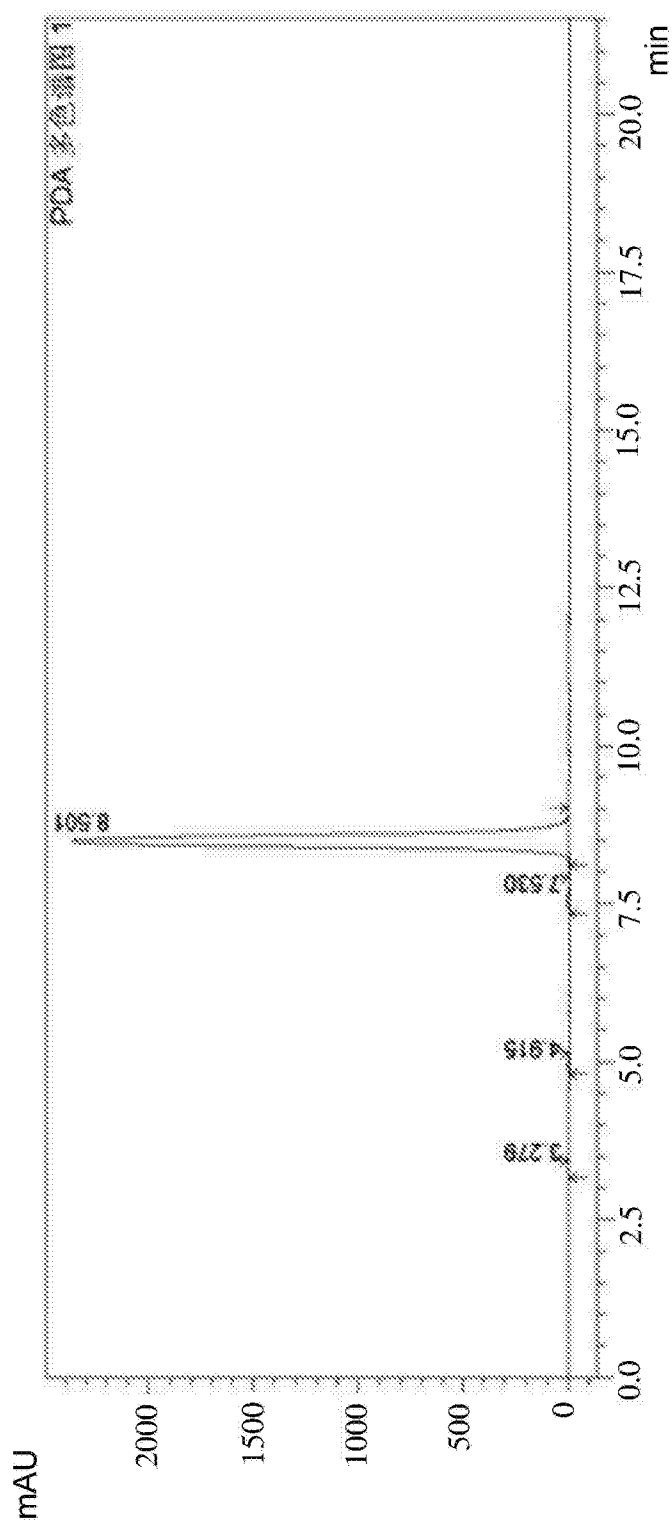
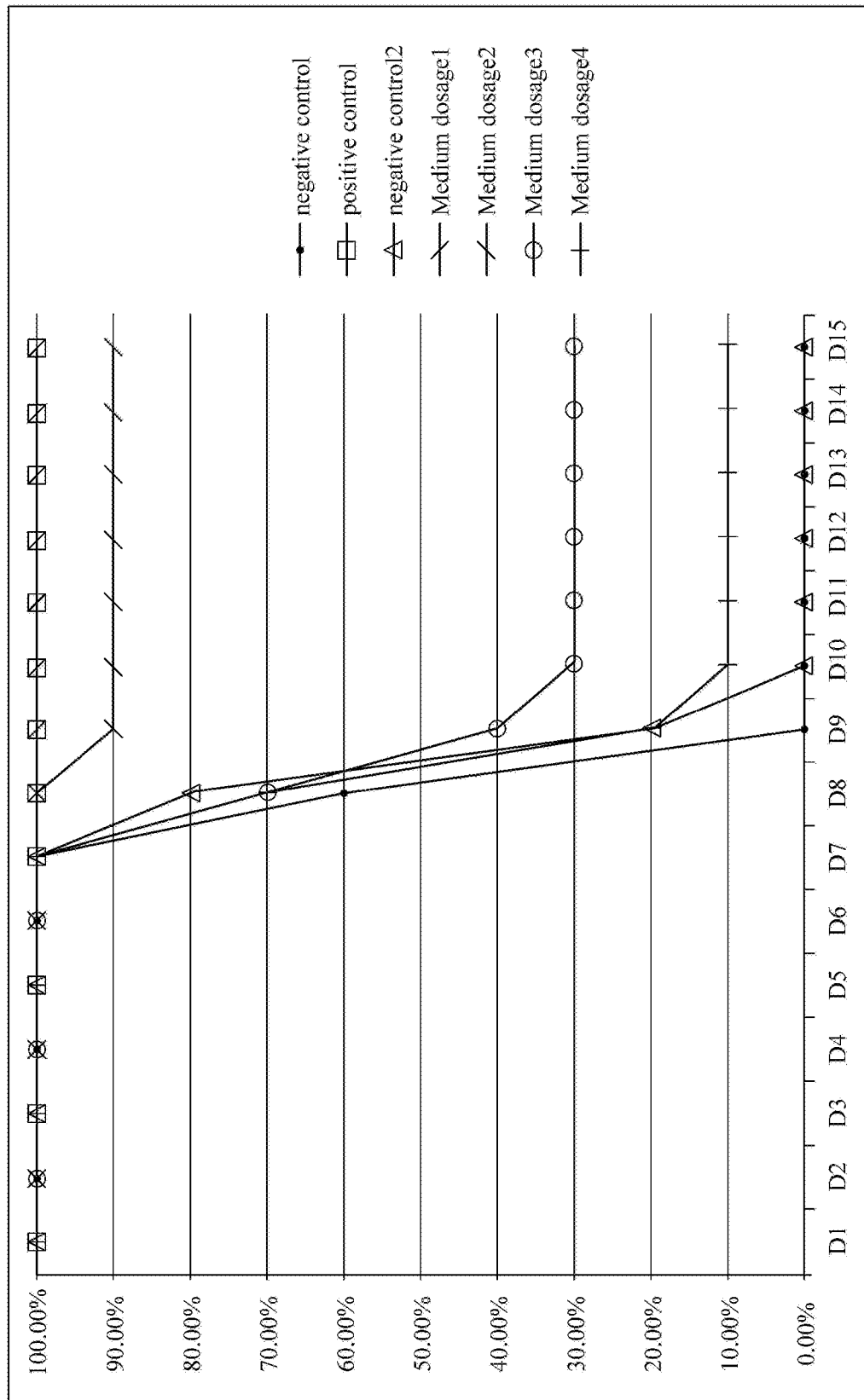


Fig. 8



Medium dosage (10mg/kg) survival curve

Fig. 9

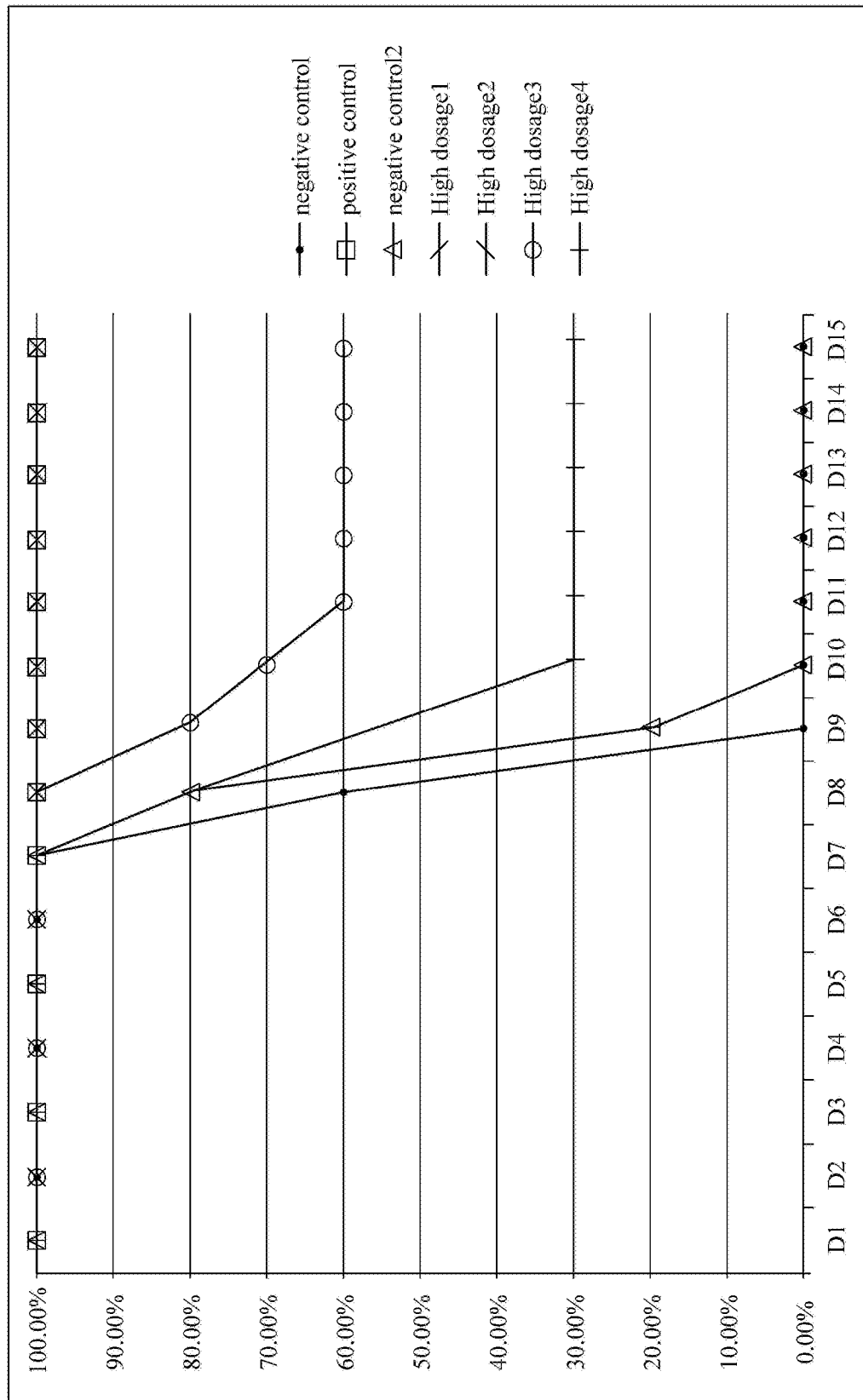
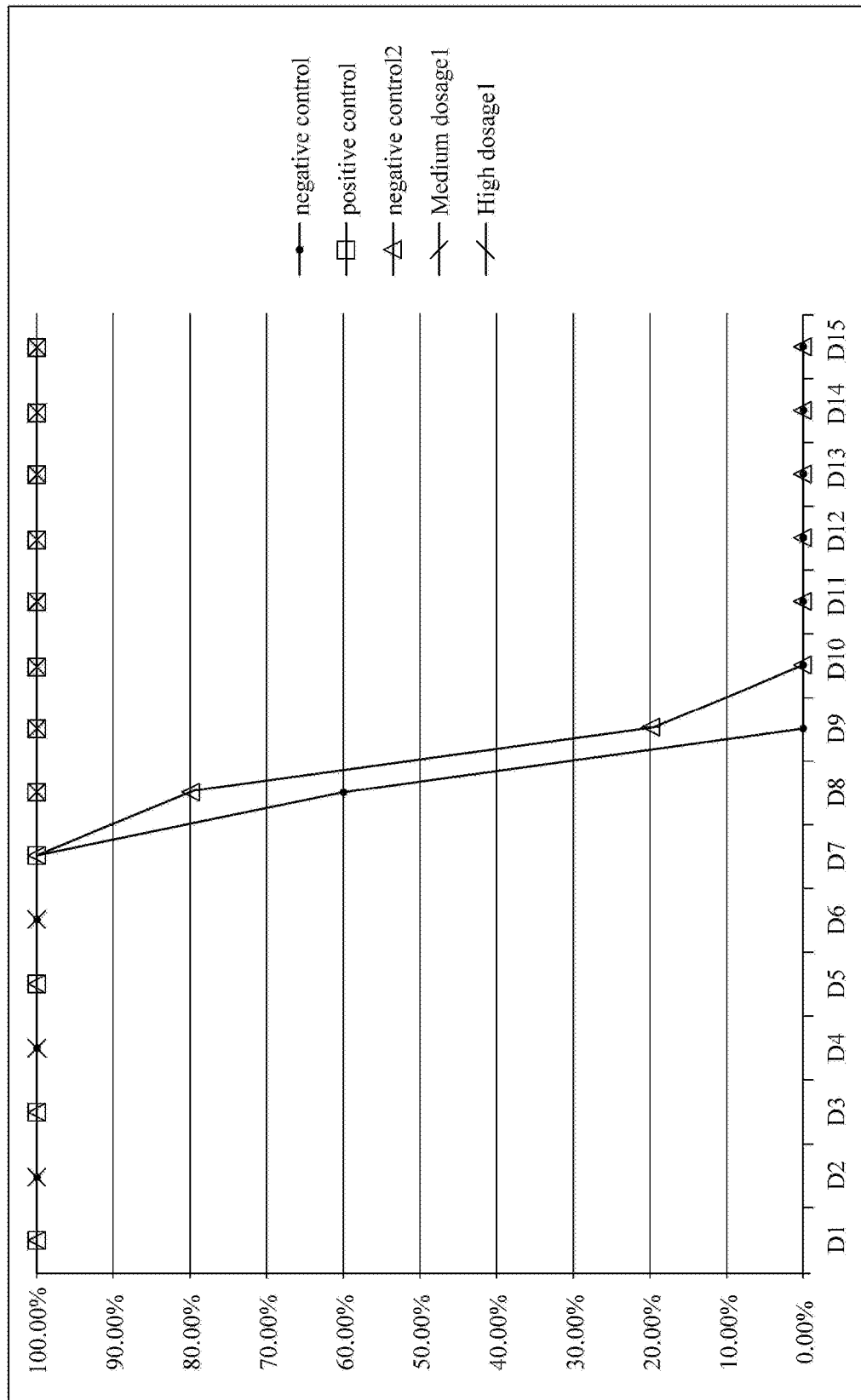
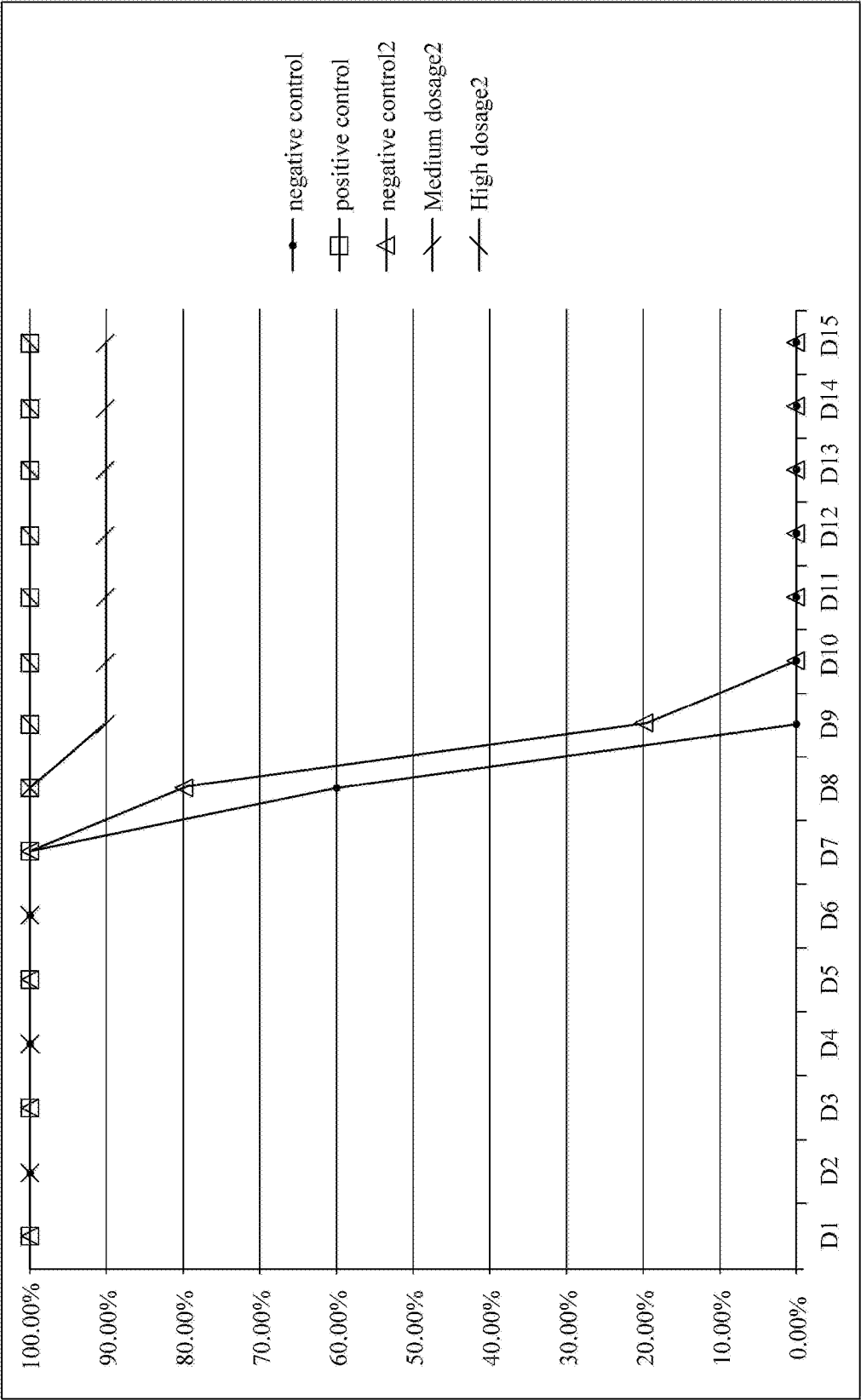


Fig. 10



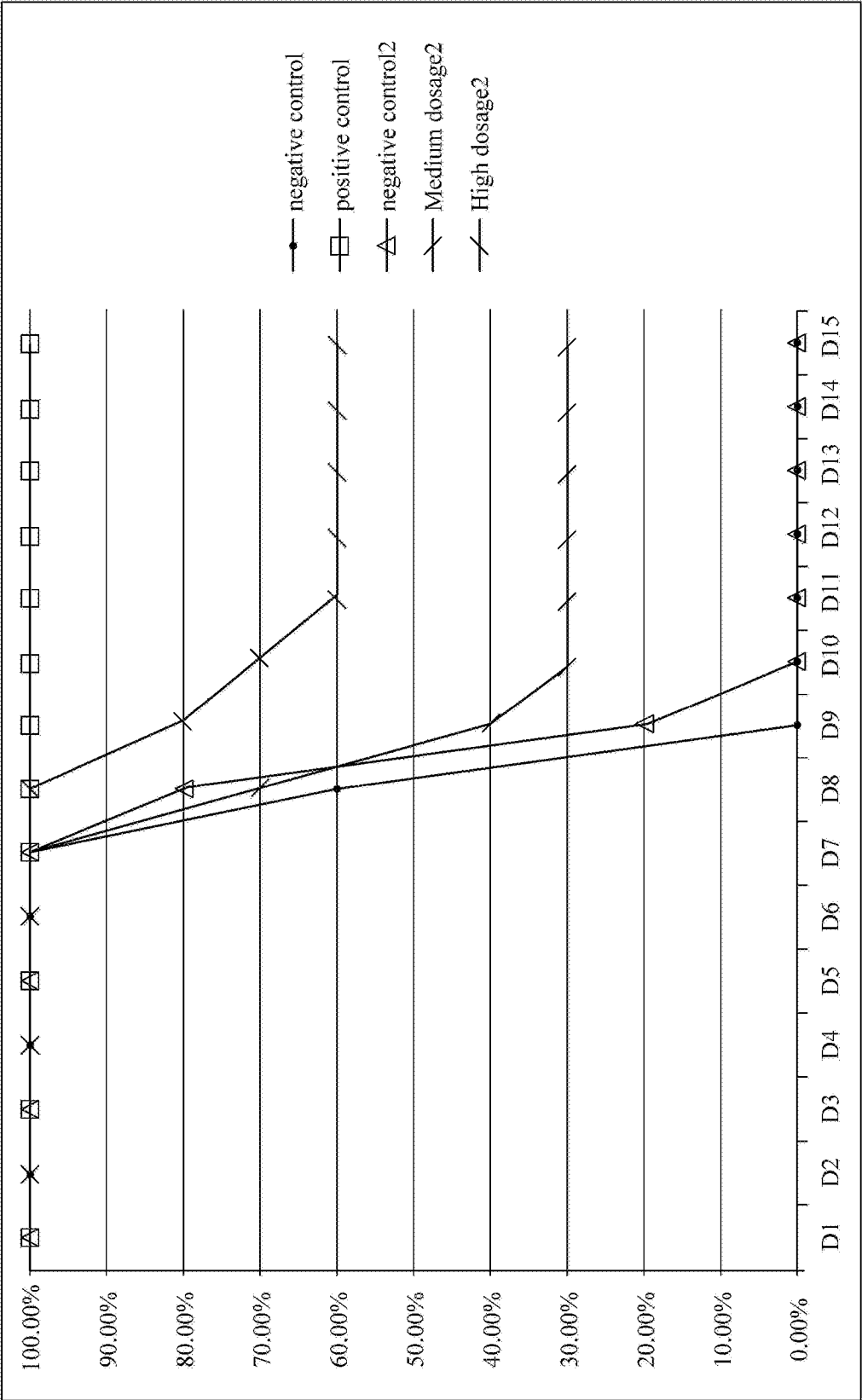
Oral suspension administered 3 days after infection

Fig. 11



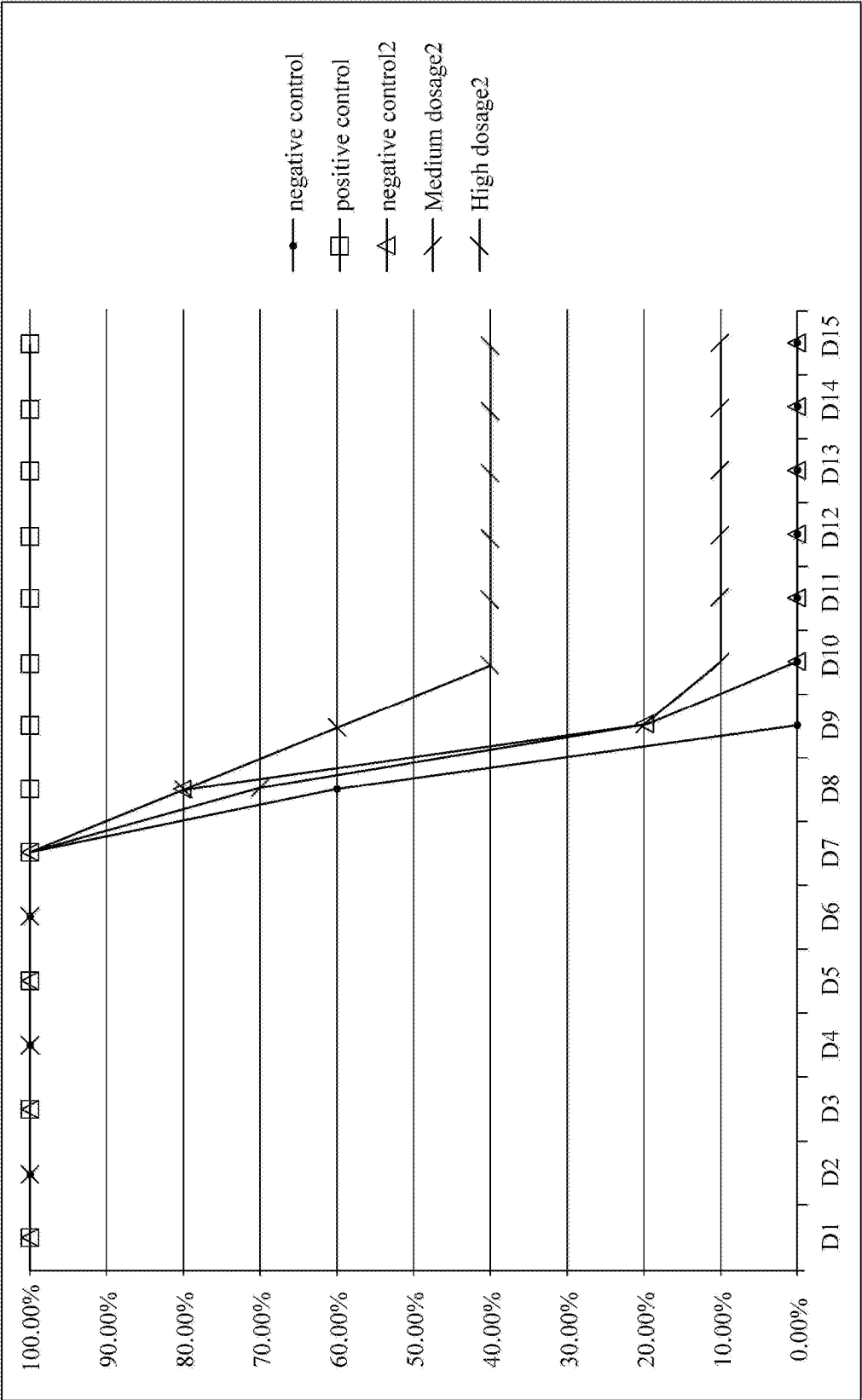
Oral suspension administered 4 days after infection

Fig. 12

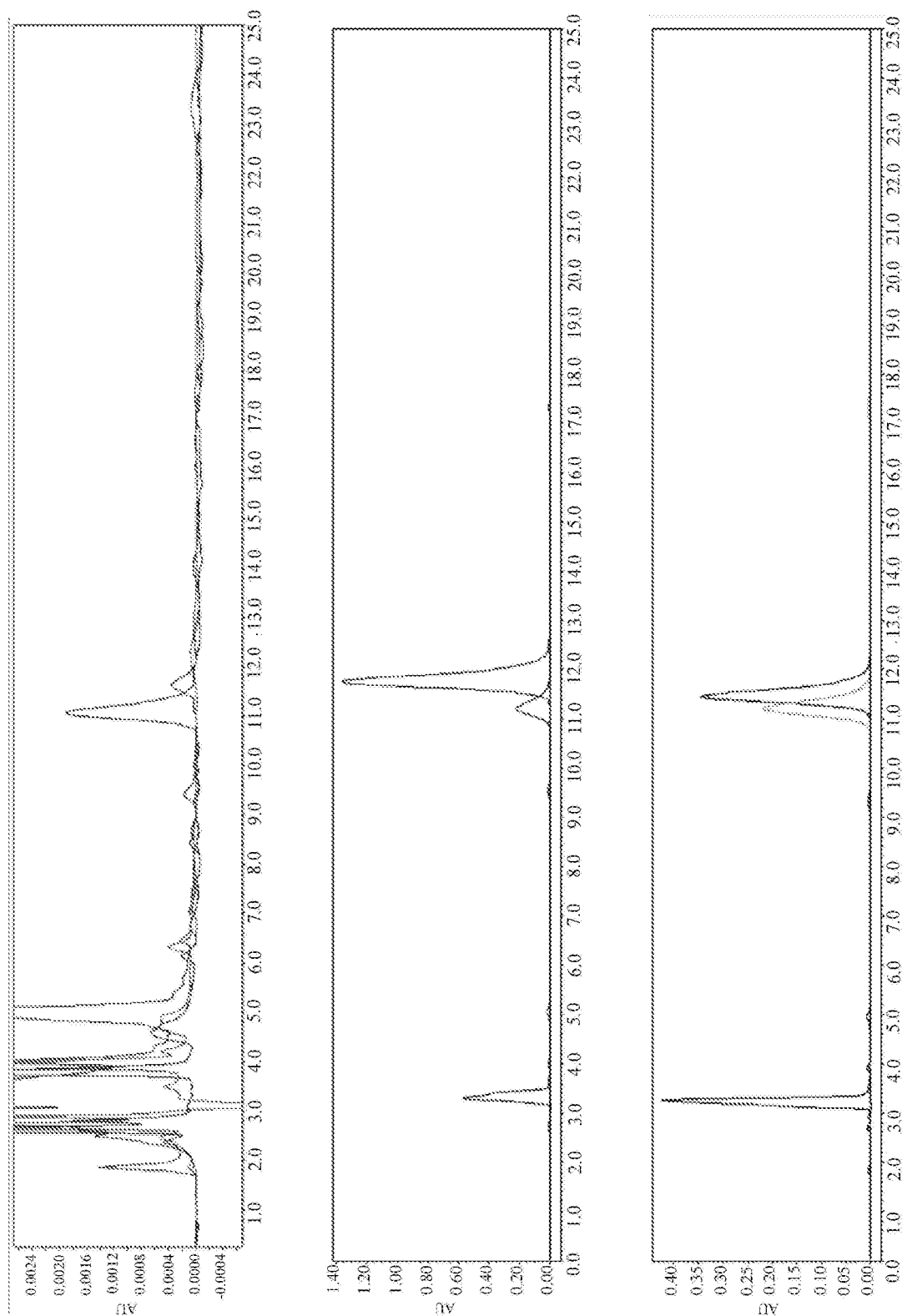


Oral suspension administered 5 days after infection

Fig. 13



Oral suspension administered 6 days after infection

Fig. 14

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2015/050351

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07D401/04 A61K31/55 A61P31/14
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07D A61K A61P

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)

EPO-Internal, CHEM ABS Data, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2005/267164 A1 (CHERN JYH-HAUR [TW] ET AL) 1 December 2005 (2005-12-01) page 1, paragraph [0004]; compounds 5-8 -----	1-14



Further documents are listed in the continuation of Box C.



See patent family annex.

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"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

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"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

"&" document member of the same patent family

Date of the actual completion of the international search

3 March 2015

Date of mailing of the international search report

17/03/2015

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Authorized officer

Schmid, Arnold

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2015/050351

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2005267164	A1	01-12-2005	NONE
