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# SuperPain—a resource on pain-relieving compounds targeting ion channels

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## ABSTRACT

**Pain is more than an unpleasant sensory experience associated with actual or potential tissue damage: it is the most common reason for physician consultation and often dramatically affects quality of life. The management of pain is often difficult and new targets are required for more effective and specific treatment. SuperPain (http://bioinformatics.charite.de/superpain/) is freely available database for pain-stimulating and pain-relieving compounds, which bind or potentially bind to ion channels that are involved in the transmission of pain signals to the central nervous system, such as TRPV1, TRPM8, TRPA1, TREK1, TRESK, hERG, ASIC, P2X and voltage-gated sodium channels. The database consists of ~8700 ligands, which are characterized by experimentally measured binding affinities. Additionally, 100 000 putative ligands are included. Moreover, the database provides 3D structures of receptors and predicted ligand-binding poses. These binding poses and a structural classification scheme provide hints for the design of new analgesic compounds. A user-friendly graphical interface allows similarity searching, visualization of ligands docked into the receptor, etc.**

## INTRODUCTION

Ion channels are proteins forming a pore that allows the flow of ions across membranes. Ion channels are voltage or ligand gated. Some of these proteins help nerve cells to transmit pain signals to the central nervous system and are therefore promising targets for the development of pain therapeutics.

Transient receptor potential channels (TRPs) are a family of 28 human cellular ion channels, varying in homology to each other but all with six transmembrane regions in common, and are nonselectively permeable to cations. There are seven subfamilies that can be divided into two groups. Group 1 includes TRPC, TRPV, TRPA, TRPM, TRPN, and group 2 comprises TRPP and TRPML (1). Compounds perceived as hot stimulate the vanilloid receptor (TRPV). At the same time, this receptor plays a crucial role in pain mediation and is therefore an interesting drug target. Known pungent chemicals with high receptor affinity such as capsaicin or resiniferatoxin were lead structures in drug development toward desensitization (2). Recent research focuses on novel analgesic mechanisms like positive allosteric modulation (3) and broad-spectrum TRP antagonists (4).

The mechanism of cold-induced analgesia was unclear until the discovery of TRPM8. This receptor is also known as the cold or menthol receptor (5). It is activated by cold temperatures and cooling agents, such as menthol or icilin, allowing the entry of $Na^+$ and $Ca^{2+}$ to the cell (6). Two modulating mechanisms are generally discussed. Whereas antagonists physically block the receptor for cold and menthol, agonists activate TRPM8 and generate a cooling sensation. Selective ligands could be used as a new generation of analgesic drugs in neuropathic pain (7,8).

TRPA1 plays a key role in chemical sensing in the inflammatory pain pathway. Many small molecules, including ingredients like wasabi, horseradish, garlic and mustard oil, can activate the channel. Recently, it has been shown that desensitizing TRPA1 could help in the treatment of neuropathic pain (9,10).

The human Ether-à-go-go Related Gene (hERG) channel or KCNH2 is a voltage-gated potassium channel. It has been the focus of pharmaceutical research for years because the inhibition of hERG potassium channels by drugs can lead to cardiac arrhythmia (11). In 2010, Stary and colleagues published a homology model (12) and a group from Canada investigated structural mechanisms of state-dependent

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drug binding (13). The different binding sites for ions and compounds are defined and the International Conference of Harmonization set up guidelines for drug development. Therefore, new screening methods for the prediction of drug liability to the hERG channel have been developed (14).

Voltage-gated sodium channels are activated through action potential firing. They represent the target for local anesthetic agents. It is challenging to find selective inhibitors of sodium channels in the pain pathway. Genotyping of families suffering from congenital indifference to pain identified mutations in gene coding for Nav1.7 channels (15). A lot of research is performed to find specific blockers to the treatment of pain and epilepsy (16).

There are some complementary resources on ion channels. Multiple analysis for voltage-gated potassium channels from different species is compiled in the voltage-gated potassium channel database VKCDB (17). Protein–protein interactions (PPIs) are the focus of the TRIP database (18): Shin and colleagues manually curated 653 PPIs for mammalian TRP channels. MoleOnline 2.0 (19) is a web server that provides interactive channel analysis to identify active sites.

The International Union of Basic and Clinical Pharmacology (IUPHAR) database provides information on human and rodent receptors (20).

There is a need for a specific resource for pain-relieving compounds targeting ion channels and their 3D homology models so researchers can identify targets and putative ligands.

## MATERIALS AND METHODS

Here, we shortly describe our methods used. If you are interested in further details, please read the 'About' page on our Web site.

### Compounds

To identify pain-related receptors and ligands, scientific literature was screened via text mining and subsequent manual evaluation. Therefore, we first downloaded Medline/PubMed data from the NCBI FTP site in xml-format. Using the search engine library Apache Lucene (http://lucene.apache.org) and a tool kit for processing text with computational linguistics (http://alias-i.com/lingpipe), the data was indexed. The search engine, written in Java, dynamically queries the indexed data and results in a structured query language (SQL) file containing the textmining hits. An example for a query for the literature search is as follows: [lidocaine (TI) AND ic50 (TI)] OR [lidocaine (abstract) AND ic50 (abstract)]. Found hits were put in a preliminary database and are displayed with keywords highlighted in different colors. In the manual evaluation process, confirmed experimentally determined affinities were moved to the final database. In some cases, the full text of the article was checked. Further information regarding the molecules was retrieved from the PubChem database (21). PubChem is a freely available database, which is provided by the National Center for Biotechnology Information (NCBI). It contains detailed information on ~30 million compounds. All those compounds and additional information were put in a MySQL database. Additional affinities were found via a search in BindingDB (22). BindingDB is a database on experimentally determined protein–ligand interactions. It provides ~782 000 affinities for 6500 protein targets.

### Compound clustering

The experimentally determined ligands had to be clustered regarding their similarity to each other by means of a K-means algorithm. As in the K-means algorithm, the number of clusters has to be defined the algorithm was slightly modified. To ensure that the most similar compounds are members of one cluster, a neighbor-joining algorithm was used. The R package with heatmap.2 was used to display the compound similarities in a heatmap.

### *In silico* screening

Putative ligands were indentified through a similarity search. Therefore, the structural fingerprints of each ligand were calculated and used for a similarity search within the PubChem Compound database. The similarity between experimentally determined ligands and potential ligands was calculated with the Tanimoto coefficient. Depending on the chemical topological properties it gives values between zero (no similarity) and one (identical). Compounds with a coefficient $>0.90$ were classified as putative ligands and included in the database.

### Receptor structure

Currently, no radiographic crystallographic structures are available for most of the receptors, but there is some information on the active sites of some of the molecules. To perform dockings a homology model for TRPV1 had to be created. The template structure for the homology modeling of TRPV1 and the models of P2X and ASIC were retrieved from the Protein Data Bank (PDB). The PDB is a freely available database on 3D structures of proteins und nucleic acids (23,24). Models are downloadable and provide coordinates of each atom within the molecule. A homology model of the hERG channel was retrieved from a research group in Germany (12).

### Docking

To obtain an understanding of binding mechanisms, the homology model of TRPV1 and the models of P2X, ASIC and hERG were imported into Accelrys Discovery Studio (25). About 6000 ligands were docked into the binding sites with the integrated Docking Tool LibDock. LibDock is a high-throughput docking algorithm. Based on polar and apolar interaction sites up to 1000 ligand conformations were positioned in the binding site. The five best-ranked poses regarding energetic conditions are displayed on the Web site. 'JSmol' was used to implement a molecular viewer, which is JavaScript based.

### Web site

The Web site is based on PHP (http://www.php.net/); web access is enabled through Apache HTTP Server (http://httpd.apache.org/). We recommend a recent version of Mozilla Firefox or Google Chrome; alternative browsers like Microsoft Internet Explorer and Apple Safari were tested with some configurations. JavaScript must be enabled, as it is required for all features of the site.

## RESULTS

### Compound database

A total of 100 000 putative channel modulators were included of which 12 000 are purchasable and 8700 ligands have measured binding affinities.

### Compound clustering

A structural clustering of the 8700 compounds with an internal similarity (Tanimoto) above 0.7 was performed and resulted in clusters with member sizes up to 52 compounds. For a better visualization, the member size was limited to 30. The neighbor-joining algorithm resulted in 684 clusters. The similarities are displayed in interactive heatmaps.

### Receptor structure

The homology model of TRPV1 was based on the known structure of the Kv1.2 potassium channel, which exhibits significant similarity and a high resolution (2.4 •). The structure was downloaded in PDB format (PDB-ID: 2R9R). Homology modeling was carried out in accordance with Fernandez-Ballester et al. (26) using the SWISS-MODEL server alignment mode. PyMOL was used to create a tetramer and manual refinement was carried out with Accelrys Discovery Studio.

### Docking

The docking of 6000 TRPV1, P2X, ASIC and hERG ligands in >1000 different binding poses revealed five binding poses for each ligand. Figure 1 shows capsaicin in the binding site of TRPV1 in an active interactive view. These binding poses were chosen regarding energetic conditions. The results are embedded into the database. Each ligand can be displayed in different manners, as well as the receptor itself (ball and stick, spacefill, etc.). Holding the left mouse button while moving the mouse leads to a rotation. A right click brings up a drop-down menu for zooming, measurements, color adjustments, etc. Safari or Tablet users can use their common gestures to navigate through the docking results. Each binding pose can be exported as a graphic (PNG) or file (MOL).

### Usage of the database

There are different ways to use the database and browse through the data. Figure 2 summarizes the main functionalities of the Web site.

'Receptor' holds information on pain-related ion channels. The homology model of TRPV1 and docking results can be found there.

There are two search boxes in the 'Compounds' section. Specific compounds can be found through a property search by typing in the name, PubChem ID, IUPAC or SMILES. It is also possible to search for a specific target such as TRPV1 or hERG or to select certain features like purchasability, IC50, EC50, molweight, rotatable bonds, etc. On the results page, information on compounds including vendors is listed in a table. This table can be used as a starting point for a similarity search or to

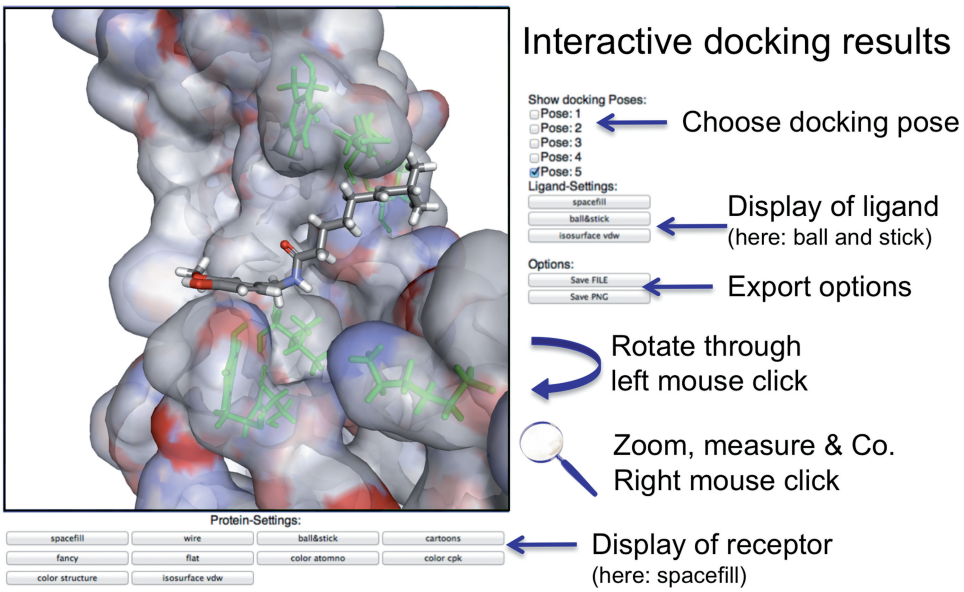


**Figure 1.** Capsaicin in TRPV1. Capsaicin was put into the homology model of TRPV1 using a high-throughput docking algorithm. Each docked ligand is shown in the interactive view.

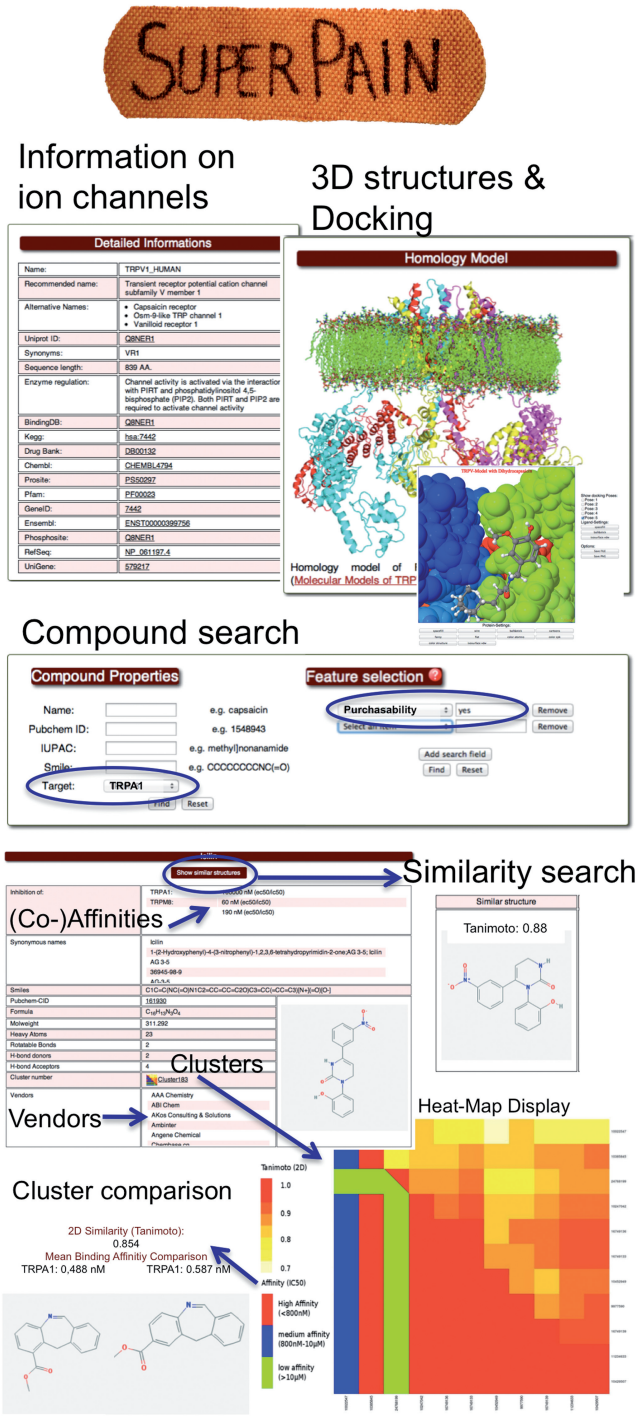


**Figure 2.** Main functionalities of 'SuperPain'. The database provides information on pain-related ion channels, as well as 3D structures and interactive docking results. The 'Compound' search enables users to find ligands regarding properties or features (name, SMILES, target, $IC_{50}$, molweight, purchasability, etc.). The 'Results' page allows to perform similarity searches or to view docking results or to see heatmaps with similar structures to compare Tanimoto coefficient or affinities.

compare structures directly by viewing the compound clusters. In the 'Cluster' section all clusters are listed with one compound of the cluster. A search box enables quick access to a distinct cluster. Clicking on a compound also enables browsing through different clusters. The heatmaps are diagonally divided to compare similarity (Tanimoto) and affinity (IC50). Mouseover shows the structure of the compared compounds. Clicking leads to a comparison page with information about (co-) affinities, as well as chemical information and vendors.

## DISCUSSION

SuperPain aims at providing a comprehensive resource on ligands for pain-related ion channels.

Acid sensing ion channels (ASICs) belong to the degenerin-epithelial sodium channel superfamily and are voltage independent. They are activated by extracellular acidosis and are involved in different processes, such as taste, mechanosensation and nociception (27). Recently, it has been shown that mambalgin, a three-finger peptide from the venom of the black mamba, suppresses pain in mice without toxicity and fewer side effects than morphine (28). These findings show that ASICs are promising targets in the treatment of pain, especially to avoid addiction problems (29).

In traditional Chinese medicine, tetramethylprazine, sodium ferrulate and puerarin are used in the therapy of pain. It has been found that these compounds target some P2X receptors. These ligand-gated ion channels open in response to binding of adenosine 5′-triphosphaste (ATP). Advances in radiographic crystallography allow *in silico* ligand docking and P2X receptors are becoming therapeutically important drug targets (30).

Further potassium channels of interest are the TWIK-related spinal cord potassium channel (TRESK) and TREK-1. TRESK is a two-pore domain potassium channel that is mainly expressed in dorsal root and trigeminal ganglia (31). This channel is involved in acute and chronic pain and plays an important role in migraine pathogenesis, which makes it a promising target (32). TREK-1 is an interesting target because of its co-expression with TRPV1 and its involvement in polymodal pain perception (33). The present intensive research on 3D structures will improve the development of channel modulators. For example, there are different binding sites for agonists and antagonists (34). The binding sites of the ion channels are rather large, which allows binding of a variety of compounds if certain physiochemical property conditions are fulfilled. This is reflected by the great diversity of the pain-related compounds. They show a low mean similarity of 0.4 compared with other targets like PARP (0.6). It is also important to find out more about the interactions or co-inhibition of different receptors for pain. In the database, they can be found by choosing 'MultiTarget' from the 'Compound Properties' search box. For example, menthol is known to be an inhibitor of TRPM8 with a half maximal effective concentration ($EC_{50}$) of 29 000 nM. At the same time, it has also inhibitory potency on TRPA1 with an $EC_{50}$ of 28 400 nM. Cannabigerol is a compound that occurs naturally in hemp strains. In contrast to other cannabinoids, it is nonpsychoactive because it is not only a $\alpha_2$-adrenergic receptor agonist, but also a mild $CB_1$ receptor antagonist

(35). Although the compound is not well-studied, it has been found to lower the intraocular pressure (36) and there is some research on treating inflammatory bowel diseases with cannabigerol (37). 'SuperPain' stores affinities for TRPA1, TRPM8 and TRPV1 for the compound. These experimentally determined co-affinities suggest that these multi-target compounds might be promising for the development of pain therapeutics (38).

### Availability

'SuperPain' is publicly available via http://bioinformatics.charite.de/superpain and should be used under the terms of the Creative Commons Attribution-Noncommercial-Share Alike 3.0 License.

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## REFERENCES

1. Montell,C., Birnbaumer,L., Flockerzi,V., Bindels,R.J., Bruford,E.A., Caterina,M.J., Clapham,D.E., Harteneck,C., Heller,S., Julius,D. *et al.* (2002) A unified nomenclature for the superfamily of TRP cation channels. *Mol. Cell*, **9**, 229–231.
2. Schwartz,E.S., La,J.H., Scheff,N.N., Davis,B.M., Albers,K.M. and Gebhart,G.F. (2013) TRPV1 and TRPA1 antagonists prevent the transition of acute to chronic inflammation and pain in chronic pancreatitis. *J. Neurosci.*, **33**, 5603–5611.
3. Lebovitz,E.E., Keller,J.M., Kominsky,H., Kaszas,K., Maric,D. and Iadarola,M.J. (2012) Positive allosteric modulation of TRPV1 as a novel analgesic mechanism. *Mol. Pain*, **8**, 70.
4. Premkumar,L.S. and Abooj,M. (2013) TRP channels and analgesia. *Life Sci.*, **92**, 415–424.
5. Bharate,S.S. and Bharate,S.B. (2012) Modulation of thermoreceptor TRPM8 by cooling compounds. *ACS Chem. Neurosci.*, **3**, 248–267.
6. Selescu,T., Ciobanu,A.C., Dobre,C., Reid,G. and Babes,A. (2013) Camphor activates and sensitizes transient receptor potential melastatin 8 (TRPM8) to cooling and icilin. *Chem. Senses.*, **38**, 563–575.
7. Journigan,V.B. and Zaveri,N.T. (2013) TRPM8 ion channel ligands for new therapeutic applications and as probes to study menthol pharmacology. *Life Sci.*, **92**, 425–437.
8. Storey,D.J., Colvin,L.A., Mackean,M.J., Mitchell,R., Fleetwood-Walker,S.M. and Fallon,M.T. (2010) Reversal of dose-limiting carboplatin-induced peripheral neuropathy with TRPM8 activator, menthol, enables further effective chemotherapy delivery. *J. Pain Symptom. Manage.*, **39**, e2–e4.
9. Docherty,R.J., Ginsberg,L., Jadoon,S., Orrell,R.W. and Bhattacharjee,A. (2013) TRPA1 insensitivity of human sural nerve axons after exposure to lidocaine. *Pain*, **154**, 1569–1577.
10. Nativi,C., Gualdani,R., Dragoni,E., Di Cesare Mannelli,L., Sostegni,S., Norcini,M., Gabrielli,G., la Marca,G., Richichi,B., Francesconi,O. *et al.* (2013) A TRPA1 antagonist reverts oxaliplatin-induced neuropathic pain. *Sci. Rep.*, **3**, 2005.
11. Thomas,D., Karle,C.A. and Kiehn,J. (2006) The cardiac hERG/IKr potassium channel as pharmacological target: structure, function, regulation, and clinical applications. *Curr. Pharm. Des.*, **12**, 2271–2283.
12. Stary,A., Wacker,S.J., Boukharta,L., Zachariae,U., Karimi-Nejad,Y., Aqvist,J., Vriend,G. and de Groot,B.L. (2010) Toward a consensus model of the HERG potassium channel. *Chem. Med. Chem.*, **5**, 455–467.
13. Durdagi,S., Deshpande,S., Duff,H.J. and Noskov,S.Y. (2012) Modeling of open, closed, and open-inactivated states of the hERG1 channel: structural mechanisms of the state-dependent drug binding. *J. Chem. Inf. Model*, **52**, 2760–2774.
14. Rayan,A., Falah,M., Raiyn,J., Da'adoosh,B., Kadan,S., Zaid,H. and Goldblum,A. (2013) Indexing molecules for their hERG liability. *Eur. J. Med. Chem.*, **65C**, 304–314.
15. Fischer,T.Z. and Waxman,S.G. (2010) Familial pain syndromes from mutations of the NaV1.7 sodium channel. *Ann. N. Y. Acad. Sci.*, **1184**, 196–207.
16. Hilder,T.A. and Chung,S.H. (2013) Designing a C84 fullerene as a specific voltage-gated sodium channel blocker. *Nanoscale Res. Lett.*, **8**, 323.
17. Gallin,W.J. and Boutet,P.A. (2011) VKCDB: voltage-gated K+ channel database updated and upgraded. *Nucleic Acids Res.*, **39**, D362–D366.
18. Shin,Y.C., Shin,S.Y., Chun,J.N., Cho,H.S., Lim,J.M., Kim,H.G., So,I., Kwon,D. and Jeon,J.H. (2012) TRIP database 2.0: a manually curated information hub for accessing TRP channel interaction network. *PLoS One*, **7**, e47165.
19. Berka,K., Hanak,O., Sehnal,D., Banas,P., Navratilova,V., Jaiswal,D., Ionescu,C.M., Svobodova Varekova,R., Koca,J. and Otyepka,M. (2012) MOLEonline 2.0: interactive web-based analysis of biomacromolecular channels. *Nucleic Acids Res.*, **40**, W222–W227.
20. Sharman,J.L., Benson,H.E., Pawson,A.J., Lukito,V., Mpamhanga,C.P., Bombail,V., Davenport,A.P., Peters,J.A., Spedding,M. and Harmar,A.J. (2013) IUPHAR-DB: updated database content and new features. *Nucleic Acids Res.*, **41**, D1083–D1088.
21. Wang,Y., Bolton,E., Dracheva,S., Karapetyan,K., Shoemaker,B.A., Suzek,T.O., Wang,J., Xiao,J., Zhang,J. and Bryant,S.H. (2010) An overview of the PubChem BioAssay resource. *Nucleic Acids Res.*, **38**, D255–D266.
22. Liu,T., Lin,Y., Wen,X., Jorissen,R.N. and Gilson,M.K. (2007) BindingDB: a web-accessible database of experimentally determined protein-ligand binding affinities. *Nucleic Acids Res.*, **35**, D198–D201.
23. Bhat,T.N., Bourne,P., Feng,Z., Gilliland,G., Jain,S., Ravichandran,V., Schneider,B., Schneider,K., Thanki,N., Weissig,H. *et al.* (2001) The PDB data uniformity project. *Nucleic Acids Res.*, **29**, 214–218.
24. Prlic,A., Bliven,S., Rose,P.W., Bluhm,W.F., Bizon,C., Godzik,A. and Bourne,P.E. (2010) Pre-calculated protein structure alignments at the RCSB PDB website. *Bioinformatics*, **26**, 2983–2985.
25. Laskowski,R.A. and Swindells,M.B. (2011) LigPlot+: multiple ligand-protein interaction diagrams for drug discovery. *J. Chem. Inf. Model*, **51**, 2778–2786.
26. Fernandez-Ballester,G. and Ferrer-Montiel,A. (2008) Molecular modeling of the full-length human TRPV1 channel in closed and desensitized states. *J. Membr. Biol.*, **223**, 161–172.
27. Jasti,J., Furukawa,H., Gonzales,E.B. and Gouaux,E. (2007) Structure of acid-sensing ion channel 1 at 1.9 A resolution and low pH. *Nature*, **449**, 316–323.
28. Diochot,S., Baron,A., Salinas,M., Douguet,D., Scarzello,S., Dabert-Gay,A.S., Debayle,D., Friend,V., Alloui,A., Lazdunski,M. *et al.* (2012) Black mamba venom peptides target acid-sensing ion channels to abolish pain. *Nature*, **490**, 552–555.
29. Pietra,F. (2012) Putative binding sites, and pathways to them, for amidine and guanidine current inhibitors on acid-sensing ion channels (ASIC). A theoretical approach with hASIC1a homology model. *Chem. Biodivers.*, **9**, 331–351.
30. North,R.A. and Jarvis,M.F. (2013) P2X receptors as drug targets. *Mol. Pharmacol.*, **83**, 759–769.
31. Enyedi,P., Braun,G. and Czirjak,G. (2012) TRESK: the lone ranger of two-pore domain potassium channels. *Mol. Cell Endocrinol.*, **353**, 75–81.

32. Lafreniere,R.G. and Rouleau,G.A. (2011) Migraine: role of the TRESK two-pore potassium channel. *Int. J. Biochem. Cell Biol.*, **43**, 1533–1536.
33. Alloui,A., Zimmermann,K., Mamet,J., Duprat,F., Noel,J., Chemin,J., Guy,N., Blondeau,N., Voilley,N., Rubat-Coudert,C. *et al.* (2006) TREK-1, a K+ channel involved in polymodal pain perception. *Embo J.*, **25**, 2368–2376.
34. Messeguer,A., Planells-Cases,R. and Ferrer-Montiel,A. (2006) Physiology and pharmacology of the vanilloid receptor. *Curr. Neuropharmacol.*, **4**, 1–15.
35. Cascio,M.G., Gauson,L.A., Stevenson,L.A., Ross,R.A. and Pertwee,R.G. (2010) Evidence that the plant cannabinoid cannabigerol is a highly potent alpha2-adrenoceptor agonist and moderately potent 5HT1A receptor antagonist. *Br. J. Pharmacol.*, **159**, 129–141.
36. Colasanti,B.K., Craig,C.R. and Allara,R.D. (1984) Intraocular pressure, ocular toxicity and neurotoxicity after administration of cannabinol or cannabigerol. *Exp. Eye Res.*, **39**, 251–259.
37. Borrelli,F., Fasolino,I., Romano,B., Capasso,R., Maiello,F., Coppola,D., Orlando,P., Battista,G., Pagano,E., Di Marzo,V. *et al.* (2013) Beneficial effect of the non-psychotropic plant cannabinoid cannabigerol on experimental inflammatory bowel disease. *Biochem. Pharmacol.*, **85**, 1306–1316.
38. De Petrocellis,L., Vellani,V., Schiano-Moriello,A., Marini,P., Magherini,P.C., Orlando,P. and Di Marzo,V. (2008) Plant-derived cannabinoids modulate the activity of transient receptor potential channels of ankyrin type-1 and melastatin type-8. *J. Pharmacol. Exp. Ther.*, **325**, 1007–1015.