Human Pleckstrin Homology Domain Interacting Protein (PHIP)

A Target Enabling Package (TEP)

Gene ID / UniProt ID / EC	PHIP, 55023 / Q8WWQ0 / -
Target Nominator	SGC Internal Nomination
SGC Authors	Oakley B. Cox, Tobias Krojer, Octovia Monteiro, Romain Talon, Anthony Bradley, Oleg Fedorov, Brian D. Marsden, John Spencer, Frank von Delft, and Paul E. Brennan
Collaborating Authors	Patrick Collins[1], Jahangir Amin[2]
Target PI	Paul Brennan (SGC Oxford)
Therapeutic Area(s)	Cancer
Disease Relevance	PHIP has been linked with cancer
Date approved by TEP Evaluation Group	17th June 2016
Document version	Version 6
Document version date	October 2020
Citation	Oakley B. Cox, Tobias Krojer, Patrick Collins, Octovia Monteiro, Romain Talon, Anthony Bradley, Oleg Fedorov, Jahangir Amin, Brian D. Marsden, John Spencer, Frank von Delft, and Paul Brennan (2016) Human Pleckstrin Homology domain Interacting Protein (PHIP); A Target Enabling Package. 10.5281/zenodo.1219712.
Affiliations	1. Diamond Light Source, Harwell Science and Innovation Campus 2. Department of Chemistry, School of Life Sciences, University of Sussex

We respectfully request that this document is cited using the DOI reference as given above if the content is used in your work.

USEFUL LINKS

(Please note that the inclusion of links to external sites should not be taken as an endorsement of that site by the SGC in any way)

SUMMARY OF PROJECT

SGC Oxford has expressed, purified and crystallized the second bromodomain of PHIP as part of the probe programme. Fragment screening and X-ray crystallography identified binders, some of which optimised to micromolar affinity. However, molecules with probe properties were not obtained. Consequently it has been decided to put the information generated into the public domain.

SCIENTIFIC BACKGROUND

PHIP is a member of the BRWD1-3 Family (Bromodomain and WD repeat-containing proteins). PHIP (BRWD2, WDR11) contains a WD40 repeat (Kme binder) and 2 Bromodomains (Kac binder). PHIP was initially identified through interactions with the pleckstrin homology domain of Insulin Receptor Signalling proteins, has been shown to mediate transcriptional responses in pancreatic islet cells and is important for postnatal growth. Although PHIP plays a role in IGF signalling, its involvement in cancer has only recently been reported. PHIP has been identified as the gene most highly overexpressed in metastatic melanomas, compared with primary tumors (1), noted that activation of PHIP promotes melanoma metastasis, can be used to classify a subset of primary melanomas and is a prognostic biomarker for melanoma (2) and that elevated PHIP copy number was associated with significantly reduced distant metastasis–free survival and disease specific survival by Kaplan-Meier analyses and that PHIP plays an important role as a molecular marker of melanoma ulceration, metastasis and survival (3). Recently PHIP has been identified as a susceptibility locus in breast cancer (4) and as a tumour suppressor in Group 3 medulobalstoma (5).

Objectives of this TEP:

- Demonstrate that PHIP protein can be expressed, purified and crystallized as recombinant proteins.
- Identify chemical starting points for a PHIP inhibitor.

RESULTS – THE TEP

PHIP (BRWD2) contains a WD40 repeat (Kme binder) and 2 Bromodomains (Kac binder)

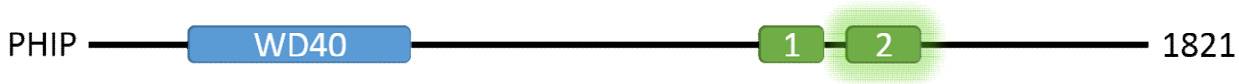


Fig. 1 PHIP

Purified proteins

Using *E. coli* as the expression host, we expressed and purified samples of the second bromodomain of PHIP

Structures

We have obtained the 7 crystal structures high resolution diffraction structures of PHIP (e.g. PDB IDs 3MB3, 5ENB, 5ENC, 5ENE, 5ENF, 5ENH, 5ENI, 5ENJ) either as an apo- structure (2.2A resolution) or as complexes with fragments (1.4-2.0 resolution). These structures have been deposited at the PDB.

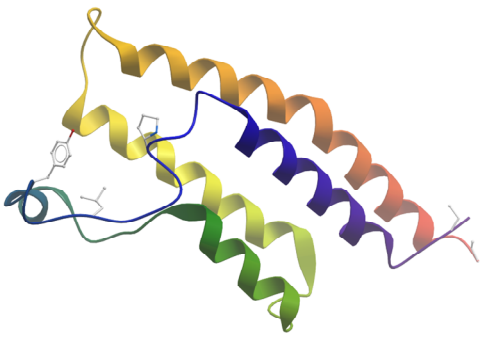


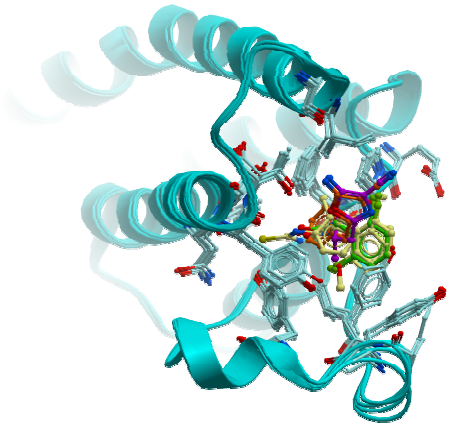
Fig. 2 Structures of the PHIP bromodomain, PDB ID: 3MB3

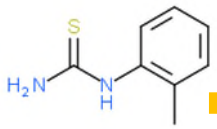
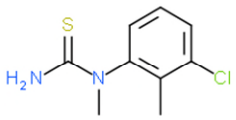
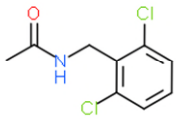
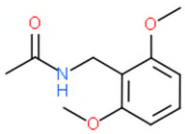
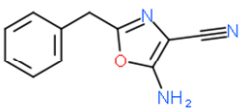
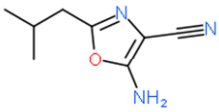
Binding assays

We have developed AlphaScreen [H4(1-20)ac4], ITC, BLI and DSF assays against the PHIP second bromodomain. Additionally we have a BRD Selectivity Panel (containing 25 out of the 61 human bromodomain proteins and a DSF Selectivity Panel containing 48 out of the 61 human bromodomain proteins)

Chemical starting points

A Fragment soaking campaign delivered 4 hits, 2 of which were optimised to compounds with µM activity.



ID		$IC_{50(\mu M)}$	Ligand Efficiency	Ligand Lipophilicity Efficiency
XTS942 (original hit)		768	0.39	1.47
FMOOA463 (follow-up)		68	0.45	1.81
FMOMB76b (original hit)		>5000	N/A	N/A
FMOOA322a (follow-up)		190	0.45	2.42
FMSOA1544		588	0.30	1.78
FMOSA1515		269	0.42	2.33

IMPORTANT: Please note that the existence of small molecules within this TEP may indicate that chemical matter can bind to the protein in a functionally relevant pocket. As such these molecules should not be used as tools for functional studies of the protein unless otherwise stated as they are not sufficiently potent or well-characterised to be used in cellular studies. The small molecule ligands are intended to be used as the basis for future chemistry optimisation to increase potency and selectivity and yield a chemical probe or lead series.

Future work

- Develop chemical probes for PHIP(2) based on TEP hits
- ULTRA-DD
- Express and screen for hits of other PHIP annotated domains (PHIP(1) and PHIP WD40)

Key SGC-Oxford contributors

- Paul Brennan
- Oleg Fedorov
- Susanne Muller-Knapp

Collaborations

Collaboration with David DiSemir, Mohammed Kashani-Sabet (Cal. Pac. Med. Center) on effect of PHIP inhibitors in triple negative melanoma and glioblastoma.

A recent global ligand docking challenge used a second set of over 50 fragment hits based on a second XChem screen (https://github.com/samplchallenges/SAMPL7/tree/PHIP2-analysis).

CONCLUSION

We have generated protein, assays, crystal structures and chemical matter that has been shown to bind to, and inhibit the actions of the PHIP second bromodomain.

FUNDING INFORMATION

The work at the SGC has been supported by the Innovative Medicines Initiative Joint Undertaking (IMI JU) under grant agreement [115766].

ADDITIONAL INFORMATION

Structure Files

PDB ID	Structure Details
3MB3	Structure of PHIP bromodomain
5ENB	PHIP bromodomain in complex with fragment
5ENC	PHIP bromodomain in complex with fragment
5ENE	PHIP bromodomain in complex with fragment
5ENF	PHIP bromodomain in complex with fragment
5ENH	PHIP bromodomain in complex with fragment
5ENI	PHIP bromodomain in complex with fragment
5ENJ	PHIP bromodomain in complex with fragment

Materials and Methods

1. Protein expression and purification
Vector: pNIC28-Bsa4
Cell line: BL21-Rosetta
Tags and additions: N-terminal, TEV protease cleavable hexahistidine tag
Final protein sequence
MHHHHHHSSGVDLGTENLYFQ*SMSYDIQAWKKQCEELLNLIFQCEDSEPFRQPVDLLEYPDYRDIIDTPMDFATVRETLEA
GNYESPMELCKDVRLIFSNSKAYTPSKRSRIYSMSLRLSAFFEEHISSVLSDYKSALRFHKRNTITKR
(underlined sequence contains vector encoded His-tag and TEV protease cleavage site*)

Cell Lysis
Lysis Buffer: 50 mM Hepes pH 7.5, 500 mM NaCl, 5% Glycerol, 10 mM Imidazole pH 7.4, 0.5 mM TCEP, protease inhibitors (Sigma, set III)
Cell pellet was dissolved in lysis buffer and broken by 2 cycles of sonication for 6 minutes (3s on, 3s off). The cell debris was removed by centrifugation for 45 minutes at 35000 x g.

Column 1: Ni-Sepharose 6 FF (5 ml volume in a gravity-flow column)
Wash Buffer: 50 mM Hepes pH 7.5, 500 mM NaCl, 5% Glycerol, 10 mM Imidazole, 0.5 mM TCEP
Elution Buffer: 50 mM Hepes pH 7.5, 250 mM NaCl, 5% Glycerol, 250 mM Imidazole, 0.5 mM TCEP
The clarified cell extract was added to 5 ml of Ni-Sepharose 6 FF pre-equilibrated with Wash buffer and passed through a glass column. The column was then washed with 150ml Wash Buffer. The protein was eluted with 30ml Elution Buffer.

Tag cleavage
3mg of TEV protease was added to the eluted protein and the digestion was performed overnight at 4 °C.

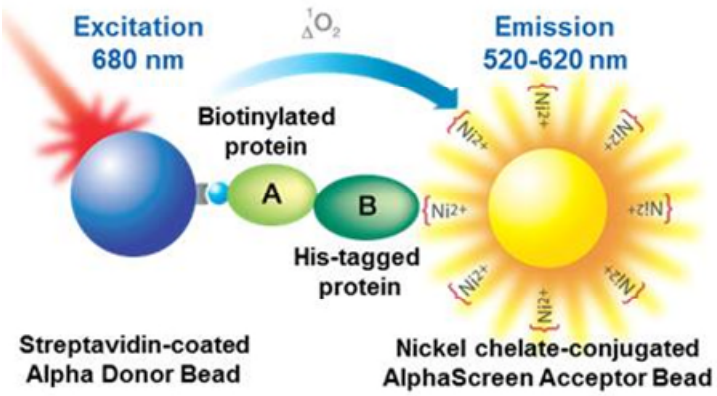
Column 2: Superdex75 HiPrep Gel Filtration
GF Buffer: 50 mM Hepes pH 7.5, 500 mM NaCl, 5% Glycerol
Successful TEV cleavage was confirmed by mass spectrometry. The protein was concentrated and injected into a Superdex75 HiPrep column (pre-equilibrated in GF Buffer) at 1.2 ml/min. Eluted fractions where analysed by SDS-PAGE and only the purest samples where pooled for re-binding on a Ni-Sepharose column.

Column 3: Ni-Sepharose 6 FF (1 ml volume in a gravity-flow column)
The column was equilibrated with gel filtration buffer, the protein was applied and only the flow through was taken forward. The protein was concentrated to 13 mg/ml using a 10 kDa MWCO concentrator, flash frozen in liquid nitrogen and stored at -80°C.

2. Protein crystallization
PHIPA(2) was crystallized by mixing 100nl of 13mg/ml protein in 20mM HEPES pH7.5, 500mM NaCl, 5% Glycerol with 100nl of reservoir solution containing 0.1M HEPES pH 7.5, 0.15M Magnesium Chloride, 34% PEG3350. Crystals appeared overnight from sitting drop plates at 4°C. PHIP(2) crystallized in space group

$P2_12_12$ with unit cell dimensions of a=60Å, b=92Å, c=24Å, corresponding to one PHIP(2) molecule in the asymmetric unit. The crystals typically diffract between 1.5 and 1.8 Å

3. PHIP AlphaScreen Assay



Introduction

This procedure is intended to measure the interaction between the second bromodomain of PHIP and a peptide that has acetylated lysine residues and can be used to assay compounds which bind to the bromodomains and inhibit this interaction. The assay uses a 6HIS tagged bromodomain protein and a biotinylated peptide in conjunction with the Ni-NTA AlphaScreen kit. The excitation of the donor bead (680 nm) causes the generation of short lived singlet oxygen which can only diffuse 200 nm in solution. When the donor and acceptor beads are in close proximity, by binding of the peptide by the PHIP, the singlet oxygen can transfer its energy to the acceptor bead resulting in a chemilluminescent signal.

A. Materials and Equipment

A.1 Equipment

- Access™ Laboratory Workstation fitted with Echo®-555 Acoustic Liquid Handler.
  (Labcyte USA)
- Multidrop® Combi Reagent Dispenser fitted with Small Tube, Plastic Tip Dispensing Cassette.
  (Thermo Scientific, USA)
- PHERAstar FS Plate reader
  (BMG Labtech, Germany)

A.2 Consumables

- ProxiPlate-384 Plus, White 384-shallow well Microplate, 6008280.
  (Perkin Elmer, USA)
- Echo® Qualified 384-Well Polypropylene Microplate, Clear, Flat Bottom, P-05525.
  (Labcyte, USA)
- Echo® Qualified 384-Well COC Microplate, Low Dead Volume, Clear, Flat Bottom, LP-0200.
  (Labcyte, USA)
- Microplate lid, low profile, sterile, 656191.
  (Greiner Bio-One, UK)

A.3 Chemicals and reagents

- AlphaScreen® Histidine (Nickel Chelate) Detection Kit, 6760619M. (Perkin Elmer, USA)

For the Assay buffer (25 mM HEPES pH7.4, 100mM NaCl, 0.1% BSA, 0.05% CHAPS)

- Gibco® Ultra-Pure HEPES (N-2-Hydroxyethylpiperazine-N-2-Ethane Sulfonic Acid), 11344-041. (Life Technologies, USA)
- CHAPS (3-[3-(Cholamidopropyl)dimethylammonio]-1-propanesulfonate), C-1019. (AG Scientific, USA)
- **BSA (Bovine Serum Albumin) lyophilized powder, ≥98%, A7030. (Sigma-Aldrich, USA)**
- **NaCl (**Sodium chloride) AnalaR NORMAPUR®, 27810.364. (VWR, USA)
- DMSO (Dimethyl sulfoxide) ReagentPlus®, ≥99.5%, D5879. **(Sigma-Aldrich, USA)**

A.4 Protein and peptide

- HIS tagged PHIP Bromodomain protein, SGC: stored in aliquots at -80°C.
- Biotinylated Histone H4 tetra-acetylated Peptide: SGRGK(ac)GGK(ac)GLGK(ac)GGAK(ac)RHRK(biotin), stored at -20°C in 10 µl aliquots at a concentration of 1 mM.

B. Methods

B.1 Dose response

B.1.1 Protein and peptide are prepared to 16 µM from which are prepared 14 1:2 serial dilutions in assay buffer plus one buffer only.

B.1.2 Add 4 µl of assay buffer to all wells of columns 1-16 of a ProxiPlate-384 Plus assay plate, using the matrix multichannel.

B.1.3 Add 4 µl of the protein dilution series to columns 1-16 of the assay plate using the matrix multichannel.

B.1.4 Add 4 µl of the peptide dilution series to rows A-P of the assay plate using the matrix multichannel.

B.1.5 Cover the plate and incubate at room temperature for 30 minutes.

B.1.6 Prepare an Alpha bead suspension at 1:300 (15 µl of each bead type in 4500 µl assay buffer) under light restricted conditions.

B.1.7 Add 8 µl of the bead suspension to columns 1-16 of the assay plate using the Multidrop combi.

B.1.8 Cover the assay plate to protect it from light and incubate at room temperature for 1 hour.

B.1.9 Taking care to protect from light, read the assay plate using the PHERAstar microplate reader.

B.2 Inhibition Assay

B.2.1 Dispense compounds into assay plates using the Echo liquid handler. Into columns 12 and 24 dispense DMSO into alternate wells these will be the positive controls, alternate wells are used to monitor the effect of DMSO in the assay.

B.2.2 Prepare a protein peptide mix that is :
375 nM protein and 50 nM peptide (225 nM and 50 nM final assay concentration)

B.2.3 Add 12 µl of the protein peptide mix to the assay plates using the Multidrop combi and incubate for 30 minutes at room temperature.

B.2.4 Prepare Alpha beads at 1:300 in assay buffer and add 8 µl to the assay plates using the Multidrop combi under light restricted conditions, cover and incubate for 1 hour at room temperature.

B.2.5 Taking care to protect from light, read the assay plate using the PHERAstar microplate reader.

B.2.6 Calculate % inhibition values as follows

$$\% I = \frac{positive\ control - signal}{positive\ control} \times 100$$

Determine IC50 values by plotting % inhibition vs compound concentration and fitting the data to a non-linear sigmoidal dose response equation (4 parameter fit).

References

1. Hagg, M., and Wennstrom, S. (2005) Activation of hypoxia-induced transcription in normoxia. *Exp Cell Res* **306**, 180-191
2. De Semir, D., Nosrati, M., Bezrookove, V., Dar, A. A., Federman, S., Bienvenu, G., Venna, S., Rangel, J., Climent, J., Meyer Tamgüney, T. M., Thummala, S., Tong, S., Leong, S. P. L., Haqq, C., Billings, P., Miller, J. R., Sagebiel, R. W., Debs, R., and Kashani-Sabet, M. (2012) Pleckstrin homology domain-interacting protein (PHIP) as a marker and mediator of melanoma metastasis. *Proceedings of the National Academy of Sciences* **109**, 7067-7072
3. Bezrookove, V., De Semir, D., Nosrati, M., Tong, S., Wu, C., Thummala, S., Dar, A. A., Leong, S. P. L., Cleaver, J. E., Sagebiel, R. W., Miller, J. R., and Kashani-Sabet, M. (2014) Prognostic Impact of PHIP Copy Number in Melanoma: Linkage to Ulceration. *The Journal of investigative dermatology* **134**, 783-790
4. Jiao, X., Aravidis, C., Marikkannu, R., Rantala, J., Picelli, S., Adamovic, T., Liu, T., Maguire, P., Kremeyer, B., Luo, L., von Holst, S., Kontham, V., Thutkawkorapin, J., Margolin, S., Du, Q., Lundin, J., Michailidou, K., Bolla, M. K., Wang, Q., Dennis, J., Lush, M., Ambrosone, C. B., Andrulis, I. L., Anton-Culver, H., Antonenkova, N. N., Arndt, V., Beckmann, M. W., Blomqvist, C., Blot, W., Boeckx, B., Bojesen, S. E., Bonanni, B., Brand, J. S., Brauch, H., Brenner, H., Broeks, A., Bruning, T., Burwinkel, B., Cai, Q., Chang-Claude, J., Couch, F. J., Cox, A., Cross, S. S., Deming-Halverson, S. L., Devilee, P., Dos-Santos-Silva, I., Dork, T., Eriksson, M., Fasching, P. A., Figueroa, J., Flesch-Janys, D., Flyger, H., Gabrielson, M., Garcia-Closas, M., Giles, G. G., Gonzalez-Neira, A., Guenel, P., Guo, Q., Gundert, M., Haiman, C. A., Hallberg, E., Hamann, U., Harrington, P., Hooning, M. J., Hopper, J. L., Huang, G., Jakubowska, A., Jones, M. E., Kerin, M. J., Kosma, V. M., Kristensen, V. N., Lambrechts, D., Le Marchand, L., Lubinski, J., Mannermaa, A., Martens, J. W. M., Meindl, A., Milne, R. L., Mulligan, A. M., Neuhausen, S. L., Nevanlinna, H., Peto, J., Pylkas, K., Radice, P., Rhenius, V., Sawyer, E. J., Schmidt, M. K., Schmutzler, R. K., Seynaeve, C., Shah, M., Simard, J., Southey, M. C., Swerdlow, A. J., Truong, T., Wendt, C., Winqvist, R., Zheng, W., Benitez, J., Dunning, A. M., Pharoah, P. D. P., Easton, D. F., Czene, K., Hall, P., and Lindblom, A. (2017) PHIP - a novel candidate breast cancer susceptibility locus on 6q14.1. *Oncotarget* **8**, 102769-102782
5. Wei, L., Murphy, B. L., Wu, G., Parker, M., Easton, J., Gilbertson, R. J., Zhang, J., and Roussel, M. F. (2017) Exome sequencing analysis of murine medulloblastoma models identifies WDR11 as a potential tumor suppressor in Group 3 tumors. *Oncotarget* **8**, 64685-64697

TEP IMPACT

Publications arising from this work:

Filippakopoulos, P., Picaud, S., Mangos, M., Keates, T., Lambert, J.P., Barsyte-Lovejoy, D., Felletar, I., Volkmer, R., Muller, S., Pawson, T., et al. (2012). Histone recognition and large-scale structural analysis of the human bromodomain family. Cell 149, 214-231.

Cox, O.B., Krojer, T., Collins, P., Monteiro, O., Talon, R., Bradley, A., Fedorov, O., Amin, J., Marsden, B.D., Spencer, J., et al. (2016). A poised fragment library enables rapid synthetic expansion yielding the first reported inhibitors of PHIP(2), an atypical bromodomain. Chem Sci 7, 2322-2330.

We respectfully request that this document is cited using the DOI value as given above if the content is used in your work.