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Makwana, M., dos Santos Souza, C. orcid.org/0000-0001-5314-6263, Pickup, B.T. orcid.org/0000-0002-2728-5486 et al. (6 more authors) (2023) Chemical tools for study of phosphohistidine: generation of selective T-phosphohistidine and Π-phosphohistidine antibodies. ChemBioChem. e202300182. ISSN 1439-4227

https://doi.org/10.1002/cbic.202300182

ChemBioChem

Research Article
doi.org/10.1002/cbic.202300182

Chemistry Europe
European Chemical Societies Publishing

www.chembiochem.org

Chemical Tools for Studying Phosphohistidine: Generation of Selective τ-Phosphohistidine and π-Phosphohistidine Antibodies**

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Nonhydrolysable stable analogues of τ-phosphohistidine (τ-pHis) and π-pHis have been designed, aided by electrostatic surface potential calculations, and subsequently synthesized. The τ-pHis and π-pHis analogues (phosphopyrazole **8** and pyridyl amino amide **13**, respectively) were used as haptens to generate pHis polyclonal antibodies. Both τ-pHis and π-pHis conjugates in the form of BSA-glutaraldehyde-τ-pHis and BSA-glutaraldehyde-π-pHis were synthesized and characterized by ^{31}P NMR spectroscopy. Commercially available τ-pHis (SC56-2) and π-pHis (SC1-1; SC50-3) monoclonal antibodies were used to show that the BSA–G-τ-pHis and BSA–G-π-pHis conjugates could be used to assess the selectivity of pHis antibodies in a competitive ELISA. Subsequently, the selectivity of the pHis antibodies generated by using phosphopyrazole **8** and pyridyl amino amide **13** as haptens was assessed by competitive ELISA against His, pSer, pThr, pTyr, τ-pHis and π-pHis. Antibodies generated by using phosphopyrazole **8** as a hapten were found to be selective for τ-pHis, and antibodies generated by using pyridyl amino amide **13** were found to be selective for π-pHis. Both τ- and π-pHis antibodies were shown to be effective in immunological experiments, including ELISA, western blot, and immunofluorescence. The τ-pHis antibody was also shown to be useful in the immunoprecipitation of proteins containing pHis.

Introduction

The phosphorylation of histidine (His) residues is a post-translational modification (PTM) that occurs by formation of a phosphorus nitrogen bond (also known as a phosphoramidate bond).[1,2] Unlike, the O-phosphorylated amino acid residues phosphoserine (pSer), phosphothreonine (pThr) and phosphotyrosine (pTyr), phosphohistidine (pHis) is relatively understudied which can arguably be attributed to its acid-labile nature and insufficient/inadequate study tools.[3] Moreover, pHis is unique, in that phosphorylation of His can occur on either one of the imidazolyl group nitrogen atoms giving rise to two isomers, namely τ-pHis and π-pHis, both of which exist in nature (Figure 1).[2] There is now increasing evidence for the involvement of pHis in many eukaryotic cellular processes[3] and it is implicated in diseases states such as cancer.[4–7] Furthermore, there is mass spectrometry (MS)[8–10] and NMR spectroscopy[11] evidence that His phosphorylation is both abundant and more widely occurring than previously thought. It is therefore important to develop a range of methods for the

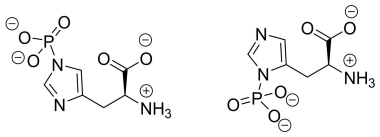

Figure 1. τ-pHis (left) and π-pHis (right) under physiological conditions.

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[**] A previous version of this manuscript has been deposited on a preprint server (https://doi.org/10.26434/chemrxiv.12993371.v1)

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbic.202300182

detection of pHis and chemical tools which will allow for pHis isomer distinction, and the study of pHis proteins *in vitro* and *in vivo*.

Antibodies have proven to be invaluable tools for studying pTyr and it is conceivable that pHis antibodies will be as effective in the study of pHis. In a 1995 review, Matthews refers to unpublished attempts to generate pHis antibodies using pHis attached to a hapten, pHis in proteins of the phosphoenol sugar transport system or phosphorylated polypHis.[12] However, none of these attempts was successful in generating pHis antibodies; the most likely explanation was the unstable nature of pHis under physiological conditions.[13] In the 1980s a pHis cross reactive pTyr antibody was generated using azobenzylphosphonate (a proposed analogue for pTyr).[14] This study was also the first to suggest that it may be possible to generate pHis antibodies using a nonhydrolysable pHis analogue, but a major challenge would be in designing pHis analogues that produced selective pHis antibodies that did not cross react with pTyr.[15]

Several stable pHis analogues have been proposed, and some have been used in pHis antibody generation studies.[3] Among these analogues, triazolyl side chains **3** and **6** have been proposed as nonhydrolysable mimics of the τ-pHis **1** and π-pHis **5** side chains respectively, and have been used in the generation of pHis antibodies (Figure 2).[16–18] However, questions remain as to whether the triazolyl side chain **3** is a suitable analogue of the τ-pHis side chain **1** both in antibody generation and as a chemical tool because there is experimental evidence which suggests that the triazolyl side chain **3** is a hybrid analogue of the τ-pHis **1** and pTyr **2** side chains.[3,16,17,19,20] In addition, it is also unclear if the triazolyl side chain **6** is an optimal analogue of the π-pHis side chain **5** because of the additional nitrogen and lone pair of electrons in the two position of the triazole aromatic ring.

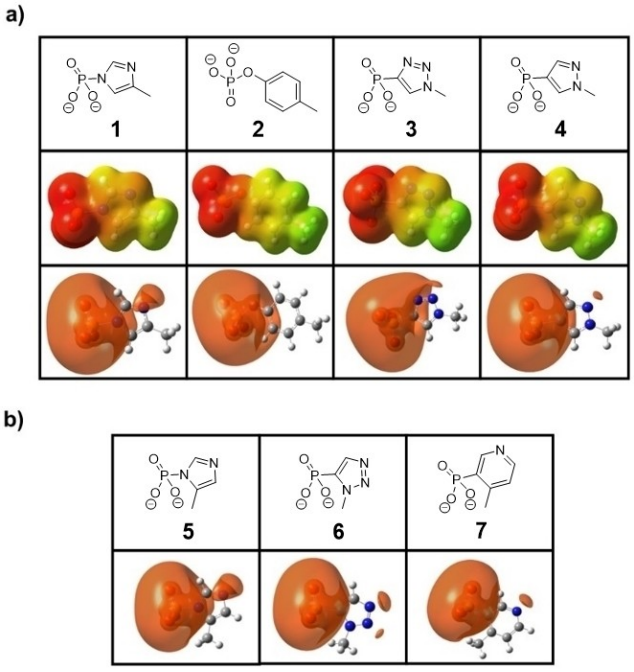

Figure 2. Density functional theory (DFT) calculations: a) Comparison of mapped ESP surfaces (middle row) and ESP surfaces (contours drawn at a potential of −0.285 au, bottom row) for model compounds (side chains) τ-pHis **1**, pTyr **2**, τ-triazolyl **3**, and pyrazolyl **4**. b) ESP surfaces (contours drawn at a potential of −0.285 au, bottom row) for model compounds π-pHis **5**, π-triazolyl **6**, and 3,4-disubstituted pyridyl **7**.

With these considerations in mind, we proposed pyrazolyl side chain **4** as a nonhydrolysable analogue of the τ-pHis side chain **1**.[21] Subsequently, we synthesized a pyrazolyl amino acid **4**, which was used to generate pHis antibodies.[22] Concurrently, in an independent report an ethylamine derivative bearing the pyrazolyl side chain **4** was also used to generate pHis antibodies.[19] The pHis antibodies generated using these analogues were both found to be selective for pHis over pTyr as assessed by dot blots and sandwich ELISA.[19,22]

Despite the significant progress made by Muir and Hunter on pHis antibody generation, the selectivity of pHis antibodies has typically been tested using dot blots[16–19,22] against proteins, peptides, amino acid conjugates or by sandwich ELISA[17,19,22] (which is a more quantitative variant of the dot blot). Recently, differential scanning fluorimetry and biolayer interferometry has been used to show the differences in binding between phosphorylated peptides and pHis Fabs.[23] The competitive ELISA, which was used to good effect to clearly show the selectivity of pTyr antibodies,[15,24] is an accurate experiment to demonstrate selectivity. The competitive ELISA used to assess pTyr antibodies[15,24] utilized pTyr conjugated to a carrier protein via a linker for pTyr antibody binding, in the presence of known quantities of competitor (e.g., phosphoamino acids[15]). Unfortunately, neither τ-pHis nor π-pHis protein conjugates have hitherto been available. Furthermore, the triazolyl side chain **6** remains the only reported stable π-pHis analogue.[21]

Here we report the generation of selective τ-pHis and π-pHis antibodies. The selectivity of these antibodies was assessed by competitive ELISA using synthesised BSA–G-τ-pHis and BSA–G-π-pHis conjugates.

Results and Discussion

Design and synthesis of τ- and π-pHis analogues

Mapped electrostatic surface potentials (ESPs) provide a visual representation of structure and charge distribution.[25] Assuming molecular recognition was based on molecular shape/structure and electrostatic interaction, such representations give an insight into what the immune system is likely responding to during an immune response. Mapped ESP calculations carried out on model compounds (side chains) for τ-pHis **1**, pTyr **2**, τ-triazolyl **3**, and pyrazolyl **4** side chains (Figure 2a, middle row) confirm that the triazolyl **3** and pyrazolyl **4** side chains are structurally similar to τ-pHis side chain **1** (Figure 2a, middle row). Similarly, previously reported mapped ESP reported by Kee *et al.*, suggested the same.[16,19] However, in the mapped ESPs of τ-pHis **1**, pTyr **2**, τ-triazolyl **3**, and pyrazolyl **4** side chains the dianion of the phosphoryl group dominates the representations and identifying subtler distinguishing features is difficult. Consequently, we considered that the electrostatic potential

field could be better understood by showing more completely the variation of ESP itself (Figure 2a, bottom row). From the ESP isocontours it was clear that for the triazolyl side chain **3**, negative regions of ESP were similar to that of the pTyr side chain **2** and do not have two separate regions of negative ESP seen with the τ-pHis side chain **1**.

Therefore, the calculations suggested the triazolyl side chain **3** was very likely a hybrid analogue of both τ-pHis and pTyr side chains which is in line with experimental data,[17] whereas the pyrazolyl side chain **4** has two separate regions of negative ESP separated by a sp^2-hybridised carbon hydrogen bond bearing a positive ESP, analogous to the τ-pHis side chain **1**. It was not surprising that preserving the electronic features of a pHis analogue was paramount. For example, we previously reported that the use of a thiophene side chain (Figure S1a in the Supporting Information) in antibody generation gave highly selective pTyr antibodies.[15] Although structurally, the thiophene side chain (Figure S1a) and the τ-pHis side chain **1** appear to be similar, the ESP isocontours of the thiophene side are more closely related to the pTyr side chain **2**. Based on ESP isocontour calculations for the thiophene side chain, the comparable ESP isocontours of Schenkel's *et al.* proposed furan side chain analogue (Figure S1b) of the τ-pHis side chain **1** would suggest it was also a likely analogue of the pTyr side chain **2**.

Comparison of model π-pHis **5** and triazolyl **6** side chain ESP surface isocontours revealed an additional negative region in the ESP present in triazolyl side chain **6** arising from the nitrogen lone pair of electrons at the two position of the triazole aromatic ring (Figure 2b). In pursuit of a closer mimic of the π-pHis side chain **5** and inspired by the possibility of using other aromatic ring sizes (see above), ESP surface isocontours of a 3,4-disubstituted pyridyl side chain **7** suggested that it might be an analogue of the π-pHis side chain **5**.

Using model side chain **7**, a nonhydrolysable pyridyl amino amide **13** was designed as a potential stable analogue of π-pHis for use in antibody generation. The pyridyl amino amide **13** was specifically chosen, because historically primary amides have worked well in the generation of peptide sequence independent phosphorylated side chain binding antibodies.[19,26,27] We had proposed pyrazolyl ethylamine **8** as a potentially useful compound for pHis antibody generation,[21] and prepared it. Pyrazolyl ethylamine **8** was independently synthesised and used in pHis antibody generation.[19] Despite the encouraging results of pyrazolyl ethylamine **8** in pHis polyclonal antibody generation, the selectivity of the pHis antibodies in detecting τ-pHis and π-pHis isomers has not been established.

Pyrazolyl ethylamine **8** was synthesised following the scheme shown in Figure S2, similar to a reported method.[19] The synthesis of pyridine amino amide **13** began with selective Negishi cross coupling of zinc reagent **10** at the four position of 3,4-dibromopyridine **9** to give pyridine **11** (Figure 3).[28] Using the Hirao cross coupling conditions reported by Bessmertnykh *et al.*,[29] palladium catalysed cross coupling of pyridine **11** and diethyl phosphite gave pyridine phosphonate **12**. Amidation of pyridine phosphonate **12** and subsequent treatment with TMSBr gave the pyridine amino amide **13** after chromatography.

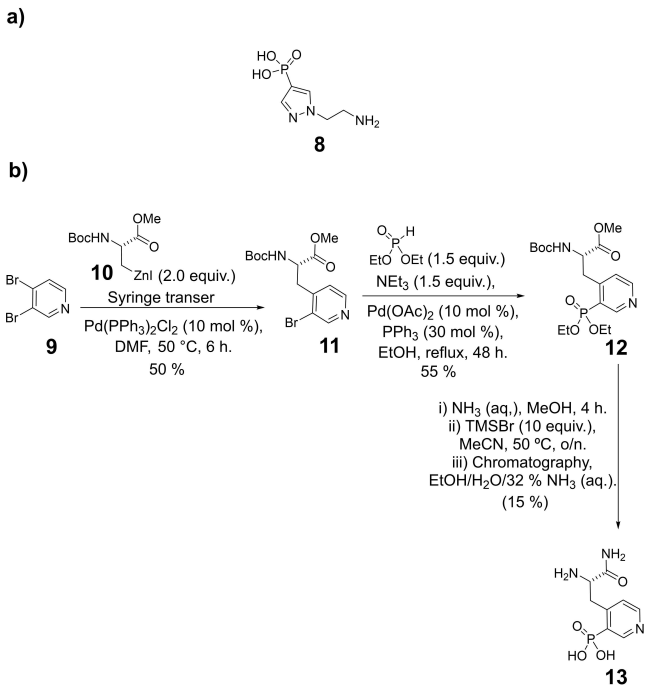

Figure 3. Proposed nonhydrolysable τ-pHis and π-pHis analogues for use as haptens. a) Pyrazolyl ethylamine analogue **8** of τ-pHis (see Figure S2 for synthesis schemes). b) Synthesis scheme of nonhydrolysable pyridyl amino amide **13** analogue of π-pHis.

Synthesis of τ-pHis and π-pHis antibody binders; BSA-G-τ-pHis and BSA-G-π-pHis

While BSA-pTyr, BSA-pSer, and BSA-pThr conjugates are commercially available BSA-pHis conjugates are not. τ-pHis[22]/pHis derivatives (phosphohistamine)[17] conjugated to the carrier protein BSA via a glutaraldehyde linker have previously been synthesized as pHis antibody binder(s), and used to assess the selectivity of pHis antibodies by dot blots or sandwich ELISA.[17,22] However, which isomeric form of pHis was conjugated to BSA or indeed whether it was a mixture of both isomers was not reported. To address this issue, samples of τ-pHis and π-pHis were first synthesized by reaction of His with potassium phosphoramidate in water.[30] Since π-pHis is known to undergo conversion to the more stable isomer (τ-pHis), the reaction was monitored by ^{31}P NMR spectroscopy to determine when the maximum amounts of τ-pHis and π-pHis respectively, had been formed (Figure S3 and S4). To isolate pure τ-pHis and π-pHis, the crude reaction mixture was purified chromatographically[30] but unlike in our previous report, pHis was not precipitated.[22] Instead, in order to suppress decomposition and bimolecular isomerisation of τ-pHis or π-pHis it was found to be important to concentrate the combined fractions of each isomer under vacuum to a specific volume under controlled conditions (pH 10–12, 25 °C).

Subsequently τ-pHis and π-pHis were each individually conjugated to BSA by using a glutaraldehyde linker[17,22] to give BSA–G-τ-pHis and BSA–G-π-pHis conjugates as pHis antibody binders (Figure 4). BSA–G-τ-pHis and BSA–G-π-pHis were both stable in 0.1 M Na_2CO_3/$NaHCO_3$, pH 10.8 buffer for at least two months stored at 4 °C or −80 °C (data not included). It is worth mentioning that both *in vitro* and *in vivo* phosphorylated NME1/NME2 and PGAM were originally used to screen commercially available τ-pHis and π-pHis monoclonal antibodies raised using triazolyl side chains **3** and **6**.[18] In vitro phosphorylated NME1/NME2 and PGAM were shown to be phosphorylated on His residues by mass spectrometry analysis but the specific isomer of pHis present in NME1/NME2 and PGAM1 proteins was not directly determined but rather inferred based on reports which have shown/suggested NME1/NME2[31] to have π-pHis and PGAM1[32] to have τ-pHis.[18] However, some previous studies have shown that NME1 may potentially also possess pSer,[33] pLys and/or τ-pHis[34] and PGAM1 has pTyr;[35] these factors mean that potential uncertainties exist with using *in vivo* phosphorylated NME1 and PGAM1 as pHis standards. Hence, it was important to establish that the samples of BSA–G-τ-pHis and BSA–G-π-pHis contained only one isomer, namely the isomer of pHis that had been used for their preparation.

While τ-pHis and π-pHis are distinguishable by ^{31}P NMR spectroscopy,[30] it is not always possible to distinguish reliably between τ-pHis and π-pHis side chains in a protein/peptide environment using τ-pHis and π-pHis ^{31}P NMR chemical shift values as references.[36] On the other hand, τ-pHis and π-pHis conjugated to BSA via a linker are essentially free from the influence of a protein/peptide environment. Indeed, the ^{31}P NMR chemical shift of BSA–G-τ-pHis (−4.86 ppm) was comparable to the chemical shift of τ-pHis (−4.78 ppm), and the chemical shift off BSA–G-π-pHis (−5.63 ppm) was comparable with the chemical shift of π-pHis (−5.61 ppm; Figure 4). Since there was no signal characteristic of π-pHis in BSA–G-τ-pHis or τ-pHis in BSA–G-π-pHis we concluded that there was no cross-contamination. To show that the BSA–G-τ-pHis and BSA–G-π-pHis ^{31}P NMR signals are distinguishable, a ^{31}P NMR analysis was also carried out on a mixture of the two conjugates (Figure 4c). The data here suggest that ^{31}P NMR spectroscopy was particularly useful for the analyses of τ-pHis and π-pHis conjugated to a protein via a linker.

An external standard was used to determine the average number of pHis phosphoryl groups per BSA protein for BSA–G-τ-pHis and BSA–G-π-pHis conjugates (Table S1). An approximate molecular weight distribution analysis of BSA–G-τ-pHis and BSA–G-π-pHis conjugates was determined by SDS PAGE and subsequent Coomassie staining (Figure S5). There was a comparable distribution of monomers (BSA–G-pHis), and higher molecular weight conjugates ([BSA–G-pHis]$_n$) in both BSA–G-τ-pHis and BSA–G-π-pHis samples.

To test the applicability of BSA–G-τ-pHis and BSA–G-π-pHis conjugates in a competitive ELISA, commercially available isomer specific τ-pHis (SC56-2) monoclonal antibody generated using a randomised combination of alanine glycine peptides containing triazolyl side chains **3**, and π-pHis (SC1-1, and SC50-3) monoclonal antibodies generated using a randomised

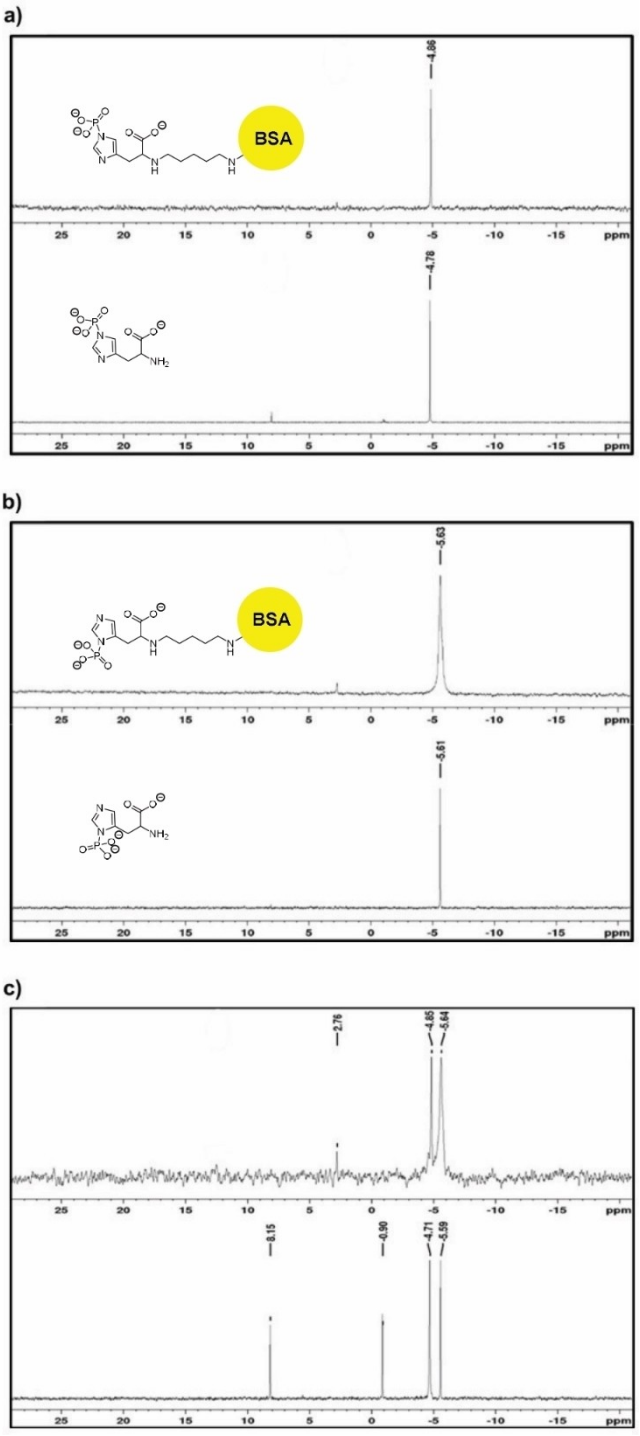

Figure 4. ^{31}P NMR spectra of τ-pHis and π-pHis antibody binder conjugates. a) BSA–G-τ-pHis (35 mg mL^{-1}) in 10% (*v/v*) deuterium oxide, 0.1 M $NaHCO_3$/Na_2CO_3 pH 10.8 buffer (top) and τ-pHis (4.9 mg mL^{-1}; bottom) in basic solution (pH ~ 12) (bottom). b) BSA–G-π-pHis (40 mg mL^{-1}) in 10% (*v/v*) deuterium oxide, 0.1 M $NaHCO_3$/Na_2CO_3 pH 10.8 buffer (top), and π-pHis pH ~ 12 (1.43 mg mL^{-1}; bottom). c) A mixture of BSA–G-π-pHis and BSA–G-τ-pHis in 10% (*v/v*) deuterium oxide, 0.1 M $NaHCO_3$/Na_2CO_3 pH 10.8 buffer (top; the minor signal at 2.75 ppm is inorganic phosphate) and a mixture of π-pHis and τ-pHis (bottom) in basic solution (pH ~ 12). The signals 8.15 ppm and −0.90 arise from an unknown impurity present in potassium phosphoramidate that was carried forward. The respective chemical shift values of τ-pHis and π-pHis do not change significantly between pH 10- 12.[11,30]

combination of alanine glycine peptides containing triazolyl side chains **6** were used (Figure 5).[18] In the report which described the generation of τ-pHis (SC56-2) and π-pHis (SC1-1, SC50-3) monoclonal antibodies, the selectivity of these antibodies were assessed by dot blot/western blots of peptides containing pTyr side chain **2**, triazolyl side chains **3** and **6**, and *in vitro* phosphorylated NME1/NME2 and PGAM1 but not ELISA.[18] Using BSA–G-τ-pHis as the τ-pHis antibody binder, the competitive ELISA profile of isomer specific τ-pHis monoclonal antibody SC56-2 against amino acid competitors His, pSer, pThr, pTyr, τ-pHis and π-pHis gave inconclusive results as none of the phosphoamino acids outcompeted the BSA–G-τ-pHis binder (Figure 5a). In fact, the low absorption (<0.1) for all amino acid's competitors would suggest the that the monoclonal antibody SC56-2 was a weak binder of τ-pHis (see below, compare absorbance of Figure 5a with b and c; and with Figure 6a, and b) and thus artefactual competition was likely observed with all amino acid competitors used. Using BSA–G-π-pHis as the π-pHis antibody binder, competitive ELISA of isomer specific π-pHis monoclonal antibodies SC1-1, and SC50-3 show that both clones are selective for π-pHis (Figure 5b and c). However, at higher amino acid concentrations, competition was observed for all phosphoamino acids, in the following order: aromatic phosphoamino acids (τ-pHis and π-pHis, pTyr), then hydroxy phosphoamino acids (pSer and pThr).

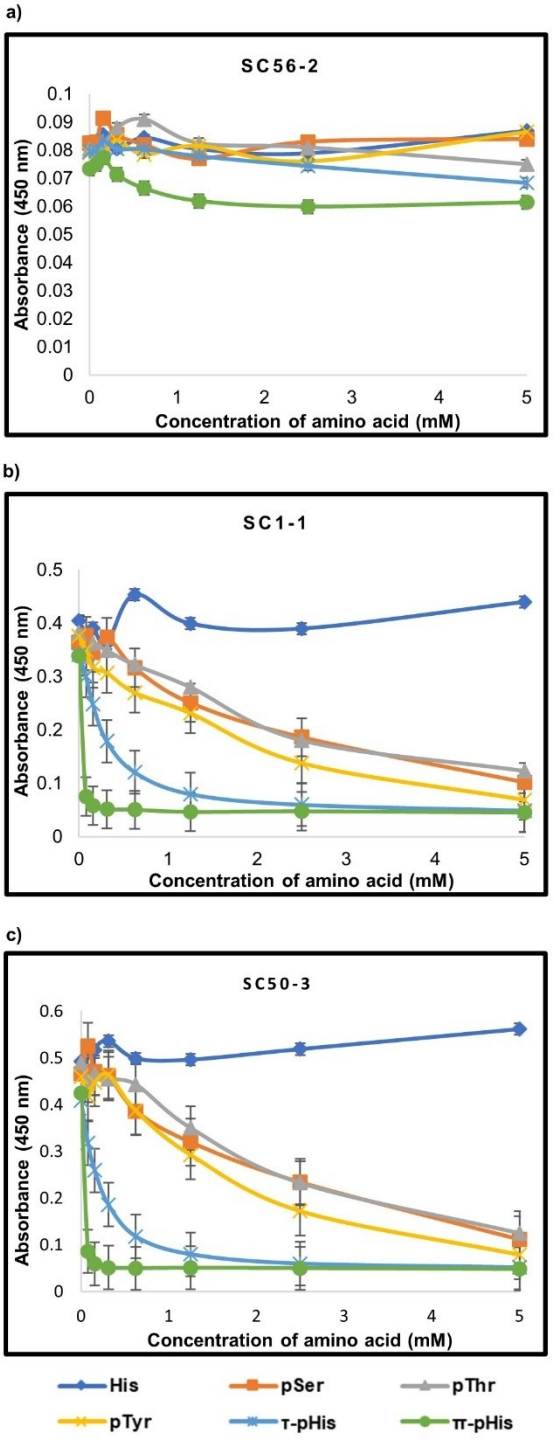

Figure 5. Competitive ELISA of commercially available pHis monoclonal antibodies with His, pSer, pThr, pTyr, τ-pHis, and π-pHis against pHis antibody binder conjugates BSA–G-τ-pHis or BSA–G-π-pHis: a) 1:500 (*v*/*v*) τ-pHis isomer specific monoclonal antibody SC56-2 against BSA–G-τ-pHis; b) 1:1000 (*v*/*v*) π-pHis isomer specific monoclonal antibody SC1-1 against BSA–G-π-pHis; c) 1:1000 (*v*/*v*) π-pHis isomer specific monoclonal antibody SC50-3 against BSA–G-π-pHis.

Generation of selective τ-pHis and π-pHis polyclonal antibodies

The combination of carrier protein keyhole limpet hemocyanin (KLH), a glutaraldehyde linker and pHis analogue has previously been used in pHis antibody generation.[19,22] Haptens KLH–G-**8**, and KLH–G-**13** were prepared using standard conditions[37] and administered into individual sheep. In a post immunization ^{31}P NMR spectroscopy analysis of KLH–G-**13**, a sharp signal at 5.02 ppm was observed (Figure S6). This preliminary finding suggests that in future studies, ^{31}P NMR spectroscopy could also be used to quantify the number of phosphorylated analogues conjugated to a carrier protein, as we have shown for BSA–G-τ-pHis, and BSA–G-π-pHis conjugates.

Sheep bleeds were collected monthly prior to boost injections, with a total of three bleeds. Analysis of the pre-immune bleed with bleeds 1–3 by sandwich ELISA indicated that both haptens had elicited an immune response with bleeds from the sheep immunized with KLH–G-**8** and KLH–G-**13** detecting BSA–G-π-pHis, and BSA–G-τ-pHis respectively (Figure S7).

To isolate selective pHis antibodies from the antisera (bleeds 3) generated with KLH–G-**8**, and KLH–G-**13**, BSA–G-amino acid conjugates were used. As we have shown previously, to remove linker (glutaraldehyde) and cross-reactive antibodies, bleed 3 antisera generated with KLH–G-**8** or KLH–G-**13** were each separately affinity depleted with a BSA–G-pTyr (Figure S8) Sepharose conjugate resin.[15,22] Subsequently both antiserums where individually affinity depleted using BSA–G-(τ or π)pHis Sepharose conjugate resin to give two antibody fractions generated with KLH–G-**8** (τ-pHis-FT2 and τ-pHis-W) and KLH–G-**13** (π-pHis-FT2 and π-pHis-W). Competitive ELISA of τ-pHis-FT2 and τ-pHis-W showed the presence of selective τ-pHis antibodies (Figure 6a and b). Similarly, competitive ELISA of π-pHis-

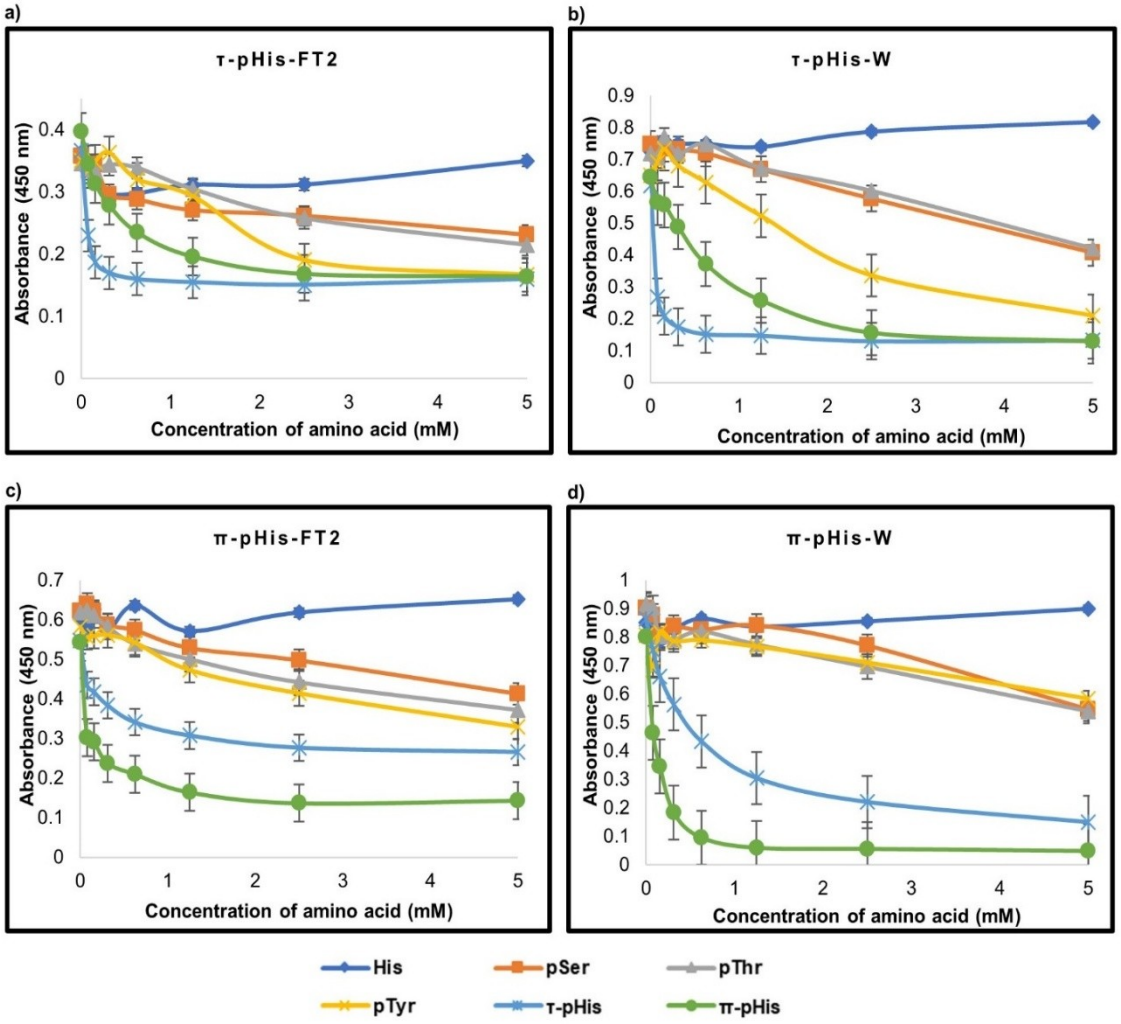

Figure 6. Competitive ELISA of polyclonal pHis antibodies with His, pSer, pThr, pTyr, τ-pHis and π-pHis against pHis antibody binder conjugates BSA–G-τ-pHis or BSA–G-π-pHis: a) 1 : 18 (*v*/*v*) τ-pHis-FT2 against BSA–G-τ-pHis; b) 1 : 4 (*v*/*v*) τ-pHis-W against BSA–G-τ-pHis; c) 1 : 5 (*v*/*v*) π-pHis-FT2 against BSA–G-π-pHis; d) 1 : 5 (*v*/*v*) π-pHis-W against BSA–G-π-pHis

FT2 and π-pHis-W showed the presence of selective π-pHis antibodies (Figure 6c and d). Our results also demonstrate that a 6 membered phosphopyridine can be used to mimic the 5 membered aromatic ring of π-pHis in antibody generation. Importantly, the competitive ELISA results strongly indicate that matching electronic features (with minor compromises in structure) was likely important in designing pHis analogues. On the basis of the competitive ELISA data, one could argue that the polyclonal antibodies generated using pyrazolyl ethylamine **8** and 3,4-disubstituted pyridine amino amide **13** might be more selective and sensitive for τ-pHis and π-pHis respectively, than the commercially available monoclonal antibodies which were generated using a randomised combination of alanine glycine peptides containing triazolyl side chain **3** or **6** to give τ-pHis and π-pHis antibodies respectively. However, a direct comparison cannot be made between the competitive ELISA of Figures 5 and 6 because pHis antibodies used in Figure 5 are monoclonal and those in Figure 6 are polyclonal. Nevertheless, the data would suggest that the pHis analogue design used in this study was effective and shows how a relatively small chemical change can have a significant effect in the generation of selective τ-pHis and π-pHis polyclonal antibodies.

Application of τ- and π-pHis antibodies in cell biology studies

With selective τ- and π-pHis antibodies in hand, the application of these antibodies in western blots and immunofluorescence was explored. Airway epithelia express His phosphorylated proteins and the 16HBE14o- or HBE cell line is a well-known airway epithelia cell line.[38,39] Using pHis stabilizing western blot conditions (see methods) many pHis protein bands were detected from the 16HBE14o-cell lysate using the pHis antibodies (Figure 7). All pHis antibodies, τ-pHis-FT2, τ-pHis-W, π-pHis-FT2, and π-pHis-W antibodies detected a broad molecular weight range of pHis proteins (see Figure S9 for western blots with SC56-2, SC50-3, and SC1-1 monoclonal antibodies; only a few pHis protein bands were detected by these antibodies).

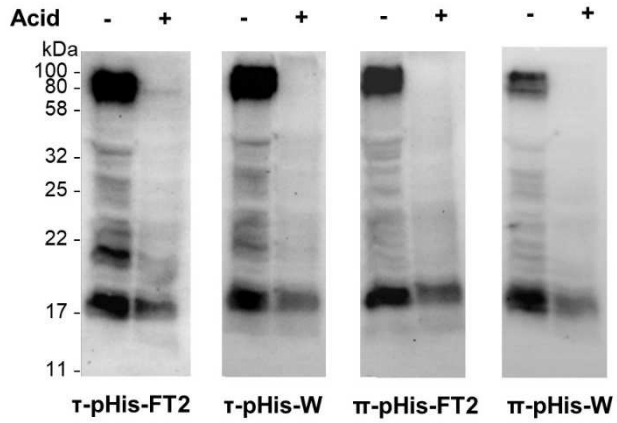

Figure 7. Western blot of 16HBE14o- cell lysate probed with pHis polyclonal antibodies τ-pHis-FT2, τ-pHis-W, π-pHis-FT2, and π-pHis-W. Left lanes: untreated; right lanes: after treatment with acetic acid to pH ~ 4, at 90 °C for 45 min.

Pre-acidification (pH ~ 4) and heating (90 °C, 45 min) of the 16HBE14o- cell lysate reduced the pHis western blot signal suggesting selective detection of pHis.[18,22] The presence of pHis in other cell types including HuVEC and RFPEC was explored by western blots (Figure S10 and S11). Similarly, both τ-pHis-FT and π-pHis-FT antibodies detected a broad molecular weight range of pHis proteins in HuVEC and RFPEC cells. Additionally, in a qualitative analysis, immunofluorescence stained images of 16HBE14o- cells using τ-pHis-W and π-pHis-W antibodies show that pHis proteins are present throughout 16HBE14o- cells and are in higher concentrations in the nucleus than the cytoplasm, which was more pronounced with the cells probed with the τ-pHis-W antibody (Figure 8a).

Having established that τ-pHis-W and π-pHis-W antibodies could be used in immunofluorescence imaging we also used the antibodies on other cells, namely neurons, in which pHis remains understudied. The presence of pHis has not been reported in healthy human neurons, although it has been reported in neuroblastoma cells[40] and there is some indirect evidence for pHis in neurons.[41–43] Immunofluorescence stained images of human induced pluripotent stem cells (iPSCs) derived neurons probed with τ-pHis-W and π-pHis-W antibodies shows pHis to be present throughout the cell (Figure 8b). However, in contrast to the 16HBE14o- cells, only weak pHis immunofluorescence staining was observed in the neuron nucleus (see Figure S12 for τ-pHis-W and π-pHis-W antibody dilution experiment). Due to paucity of material, it was not possible to analyse the pHis proteins from the neurons by western blots. The presence of pHis proteins in human iPSCs derived neurons is especially interesting because protein phosphorylation (O-linked) is implicated/involved in many neurological disease states.[44] This therefore raises some important questions: what is the biological function of pHis in neurons; is pHis involved in neurological diseases; what is the biological relevance of little/no pHis in the neuron nucleus compared to 16HBE14o- and Müller cells (see below)? Nucleoside diphosphate kinase B (NDPK–B) is a known mammalian His kinase[45] which is involved

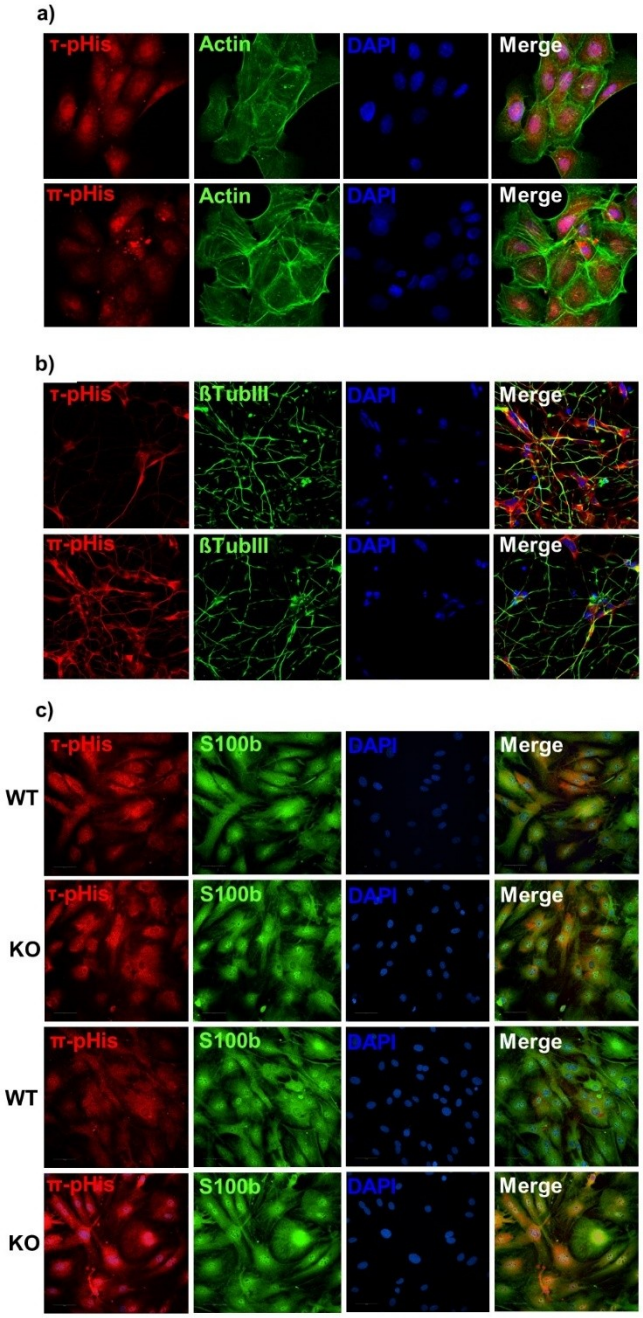

Figure 8. Immunofluorescence cell images using τ-pHis-W and π-pHis-W antibodies of: **a**) 16HBE14o- cells stained with τ-pHis-W and π-pHis-W antibodies (red, Alexa Fluor™ 594 secondary antibody), Alexa Fluor™ 488 Phalloidin (green; this stains actin filaments and was used as a marker that defines the boundary of cells) and DAPI (blue, for nuclear staining). b) Neurons derived from human induced pluripotent stem cells (iPSCs) were stained with τ-pHis-W and π-pHis-W antibody (red, Alexa Fluor™ 594 secondary antibody), TubβIII (green, Alexa Fluor™ 488 secondary antibody) – a classical neuronal marker – and DAPI (blue). c) Müller WT and Müller NDPK–B KO cells stained with τ-pHis-W and π-pHis-W antibody (red, Alexa Fluor™ 594 secondary antibody), S100b (green, Alexa Fluor™ 488 secondary antibody) – a glial cell marker – and DAPI (blue).

in many cellular functions.[46] To see if there is a difference in immunofluorescence pHis signal on cells without NDPK–B, Müller wild-type (WT) and Müller NDPK–B knockout (KO) cells[47]

were stained using τ-pHis-W and π-pHis-W antibodies (Figure 8c). There was no manifestly clear difference between the immunofluorescence intensities of Müller WT and NDPK–B KO cells probed with the τ-pHis-W antibody (Figure S13a).

However, there was a noticeable increase in immunofluorescence intensity of Müller NDPK–B KO cells compared to Müller WT cells probed with the π-pHis-W antibody (Figure S13b). These observations were also reflected in western blots of the Müller WT vs Müller NDPK–B KO cells (Figure S14). The π-pHis-W antibody can detect *in vitro* phosphorylated NDPK–A, B, and C (Figure S15),[48] and thus one might have expected a weaker π-pHis immunofluorescence signal from the NDPK–B KO Müller cells compared to the WT because NDPK–B is thought to be phosphorylated on a His residue to give π-pHis.[3] A plausible explanation could be the upregulation of some π-pHis proteins to compensate for the absence of NDPK–B.

To demonstrate that the pHis antibodies could potentially be used to enrich pHis proteins from a complex mixture, the τ-pHis-FT2 antibody was used in immunoprecipitation of pHis proteins from the 16HBE14o- cell lysate. The τ-pHis-FT2 antibody was chosen as it was available in the largest quantities. Phosphopyrazolyl butane **14** (see Figure S16 for synthesis scheme) was used as a competitor to release proteins bound to the τ-pHis-FT2 antibody. Pyrazolyl ethylamine **8** was not used because primary amines are known to dephosphorylate pHis derivatives.[49] τ-pHis or π-pHis were considered not suitable for use as competitors due to their tendency to transfer the phosphoryl group to other nucleophilic residues which could give rise to false positives. The immunoprecipitation fractions were examined by colloidal gold protein stain and western blot using the τ-pHis-FT2 antibody (Figure S17). Reduced colloidal gold staining and pHis western blot signal was observed in the competitor fractions when the proteins from the 16HBE14o- cell lysate was treated with acid prior to immunoprecipitation. The data here would suggest pHis proteins were indeed being immunoprecipitated by the τ-pHis-FT2.

Conclusions

This study demonstrates the importance of chemical design in the identification of τ-pHis and π-pHis analogues and their use in τ-pHis and π-pHis antibody generation. The application of τ-pHis and π-pHis polyclonal antibodies in ELISA, western blot, immunofluorescence cell imaging and immunoprecipitation demonstrated their broad applicability in common biological experiments. The results of these preliminary biological experiments suggested that pHis is a significant and widespread PTM in mammalians cells and requires further investigation. Consequently, the pHis analogues developed in this study including the BSA–G-τ-pHis and BSA–G-π-pHis conjugates will be very useful in monoclonal antibody generation and selection as well as other biological studies. The application of pyrazolyl ethylamine **8** and pyridyl amino amide **13** in the generation of τ-pHis and π-pHis monoclonal antibodies, and the synthesis for the incorporation these side chains into peptides is currently underway. The design of other pHis analogues is also being explored.

Experimental Section

Electrostatic surface potential maps and density calculations: The calculations were all carried out using Gaussian 2009 software with the B3LYP density functional and with the PCM solvation model to mimic an aqueous environment. The phosphonates were modelled as anions, since this is their likely state under physiological conditions. We chose a large basis set (aug-cc-pTVZ) because anions require the use of diffuse basis functions. The geometries of the molecules were optimised with the chosen basis, and the vibrational frequencies were checked to ensure that a minimum had been achieved. The three-dimensional electrostatic potential (ESP) grids were calculated using the Gaussian cubegen software. Two different kinds of electrostatic potential diagrams were calculated: a) The ESP was mapped onto a surface defined by a constant electron density value of 0.004, that value being chosen to approximate a Connelly molecular surface. The red areas are mostly negative in electrostatic potential, with the most negative being at −0.400 a.u. (equivalent to $-1050\ kJ\,mol^{-1}$). The green areas represent values of electrostatic potential around 0.0 a.u. b) We displayed the ESP surface itself as an isocontour drawn at a potential of −0.285 a.u. (equivalent to $-748\ kJ\,mol^{-1}$). This display method was found to give more useful information about the *extent* of the areas of negative ESP around the molecule. The value of ESP is exactly −0.285 a.u. on the surface, and more *negative* inside the surface.

General chemical synthesis experimental: Reagents were purchased from Fluorochem, Sigma Aldrich, Alpha Aeser, Manchester Organics, VWR International, Fisher Scientific, and Acros Organics. Distilled or ultrapure H_2O was used, and all solvents were of HPLC grade or distilled. 4-Iodo-1*H*-pyrazole,[50] *tert*-butyl *N*-(2-bromoethyl)carbamate,[51] methyl (2*R*)-2-[(*tert*-butoxycarbonyl)amino]-3-iodopropanoate,[52,53] amino(potassiooxy)phosphinic acid (potassium phosphoramidate)[54] were prepared following reported procedures. Pet. ether refers to the petroleum ether fraction that boils in the range 40–60 °C. Dry dimethylformamide, tetrahydrofuran, toluene and dichloromethane was obtained from the in-house Grubbs system. Ethanol was dried by refluxing over iodine activated magnesium for 24 h. The ethanol was then distilled and stored over 4 Å molecular sieves under nitrogen for up to 6 months. All reactions were stirred using a Teflon coated stirrer bar. N,N-diisopropylethylamine was distilled from calcium hydride and stored under nitrogen. All moisture and air sensitive reactions were performed under a positive pressure of nitrogen or argon in flame dried glassware. All other reagents were used without further purification. Flash column chromatography was done manually under positive 0.34 atmospheres of pressure. Silica gel 40–60 μm from VWR International was used. Merck TLC Silica gel 60 F254 TLC plates were used, and compounds were visualized by UV light (254 nm), 5% (*w*/*v*) ninhydrin in methanol, or basic potassium permanganate. Optical rotations were measured using an Optical Activity Ltd. AA-10 Series Automatic polarimeter at 589 nm. Specific rotations were measured to the nearest 0.1 degrees, and concentration are given to the nearest $0.1\ mg\,mL^{-1}$. The melting points were determined using Linkam HFs91 heating stage used in conjunction with a TC92 controller and are uncorrected. The infrared spectra were taken using a Perkin Elmer Paragon 100 FTIR spectrophotometer as thin films using sodium chloride plates or attenuated total reflectance (ATR). Only selected peaks are reported, and the absorption maxima are given to the nearest

wavenumber (cm^{-1}). All NMR spectra were recorded at room temperature unless stated otherwise. ^1H NMR spectra were recorded using a Bruker Avance 400 operating at 400.13 MHz, or Bruker Avance III HD 400 spectrometer operating at 400.23 MHz. ^{13}C NMR spectra were recorded using a Bruker Avance 400 operating at 100.61 MHz, or Bruker AVANCE III HD 400 spectrometer operating at 100.64 MHz. ^{31}P NMR spectra were recorded using a Bruker Avance III HD 400 spectrometer operating at 162.02 MHz. Chemical shifts were measured relative to the residual solvent and expressed in parts per million (δ). The multiplicities are defined as s = singlet, d = doublet, t = triplet, q = quartet, quint. = quintet, sex. = sextet, m = multiplet, br = broad. Coupling constant (*J*) are given in Hertz and the measured values are rounded to the nearest 0.5 Hz and are rationalized. High-resolution mass spectra were measured using an Agilent Technologies 653 Accurate-Mass Q-TOF LC/MS operating in electrospray mode.

Diethyl (1*H*-pyrazol-4-yl)phosphonate was synthesised using Hirao cross coupling conditions described by Bessmertnykh *et al.*[29] 4-Iodo-1*H*-pyrazole (1.94 g, 10.0 mmol, 1.0 equiv.), triphenylphosphine (0.79 g, 3.0 mmol, 0.3 equiv.) and palladium(II) acetate (0.242 g, 1.0 mmol, 0.1 equiv.) were added to a round bottom flask equipped with a condenser. A vacuum was applied followed by a positive pressure of nitrogen (×3). Ethanol (35 mL), triethylamine (2.02 g, 2.8 mL, 20.0 mmol, 2.0 equiv.) and diethyl phosphite (3.04 g, 2.8 mL, 22.0 mmol, 2.2 equiv.) were then added, and the mixture was stirred at reflux for 6 h. The mixture was allowed to cool to room temperature and then diluted with saturated aqueous ammonium chloride (200 mL). The aqueous phase was extracted with ethyl acetate (3 × 200 mL), and the combined organic phases were washed with brine (100 mL), dried over anhydrous magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography over silica eluting with ethyl acetate/petroleum ether (1:9) to give diethyl (1*H*-pyrazol-4-yl)phosphonate (2.01 g, 98%), as a white solid: R_F = 0.11 (EtOAc); m.p. = 57-59 °C (lit.[55] 85–87 °C); ν_{max}(ATR)/cm^{-1} 3150, 2902, 1543, 1478, 1378, 1294, 1213, 1185, 1154, 1014, ^1H NMR (400 MHz, $CDCl_3$) 1.34 (t, *J* = 7.0 Hz, 6H), 4.04-4.19 (m, 4 H), 7.92 (s, 2H), 12.65 (br, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) 16.2 (d, *J* = 6.5 Hz), 62.3 (d, *J* = 5.5 Hz), 106.5 (d, *J* = 222.5 Hz), 138.2 (br); ^{31}P NMR (162 MHz, $CHCl_3$) 15.06; *m/z* (ESI+): 205.0737 (MH^+, 100% $C_7H_{14}N_2O_3P$ requires 205.0700). The characterization data are comparable to a previous report, however the melting point observed was significantly lower than reported.[55]

[1-(2-Aminoethyl)-1*H*-pyrazol-4-yl]phosphonic acid (8) was synthesised similar to a reported method.[19] The method described below was designed to prepare a pure sample of the final compound, and yield optimization was not done. Diethyl 1*H*-pyrazol-4-ylphosphonate (0.500 g, 2.45 mmol, 1.0 equiv.) *tert*-butyl *N*-(2-bromoethyl)carbamate (0.75 g, 3.35 mmol, 1.4 equiv.) caesium carbonate (2.73 g, 8.38 mmol, 3.5 equiv.) and acetonitrile (9.6 mL) were stirred under nitrogen at room temperature for 6 h. The mixture was diluted with ethyl acetate (50 mL), filtered through a silica plug (1 cm × 2 cm) and the filtered solid was washed with ethyl acetate (200 mL). The filtrate was concentrated under reduced pressure and the residue was dissolved in acetonitrile under nitrogen (24 mL). Bromotrimethylsilane (3.66 g, 3.2 mL, 23.9 mmol, 10 equiv.) was added and the mixture was stirred at 50 °C, overnight. After cooling to room temperature, the mixture was concentrated under reduced pressure and the residue was triturated using propan-2-ol (15 mL). The suspension was transferred to a centrifuge vial, centrifuged (3220 relative centrifugal force, 4 °C, 5 min) and the supernatant was discarded. The solid was loaded onto a pre-equilibrated (ethanol/water 8:2) flash chromatography silica gel column. The column was eluted with a 80:18:2→65:22:8 gradient of ethanol/water/32% (*w/w*) aqueous ammonia. The combined product fractions were concentrated under reduced pressure and then lyophilized. The remaining residue was dissolved in water (2.5 mL) and an aliquot was diluted in deuterated water for quantification by ^1H NMR using 1,4-dioxane as the internal standard. [1-(2-aminoethyl)-1*H*-pyrazol-4-yl]phosphonic acid **8** (0.025 g, 5%): R_F = 0.47 (EtOH/H_2O/32% (*w/w*) NH_3 (aq.), 65:22:8); ^1H NMR (400 MHz, D_2O) 3.40 (t, *J* = 5.5 Hz, 2H), 4.40 (t, *J* = 5.5 Hz, 2H), 7.62 (m, 1H), 7.72 (d, *J* = 2.5 Hz, 1 H); ^{13}C NMR (101 MHz, D_2O) 39.3 (s), 48.3 (s), 117.5 (d, *J* = 197.0 Hz), 134.3 (d, *J* = 20.5 Hz), 142.6 (d, *J* = 13.0 Hz); ^{31}P NMR (162 MHz, D_2O) 5.72; *m/z* (ESI+): 192.1000 (MH^+, 100% $C_5H_{11}N_3O_3P$ requires 192.0500). The characterization data are comparable to the literature.[19]

Methyl (2*S*)-3-(3-bromopyridin-4-yl)-2-[(*tert*-butoxycarbonyl)amino]propanoate (11). A round bottom flask containing rapidly stirred zinc dust (0.980 g, 15.0 mmol, 3.0 equiv.) was flame dried under vacuum, which was followed by a positive pressure of nitrogen (×3). Dimethylformamide (4.0 mL) was added followed by iodine (0.380 g, 1.5 mmol, 0.3 equiv.); the solution changed from yellow to colourless. Methyl (2*R*)-2-[(*tert*-butoxycarbonyl)amino]-3-iodopropanoate (1.72 g, 5.2 mmol, 1.0 equiv.) was added followed by the immediate addition of iodine (0.380 g, 1.5 mmol, 0.3 equiv.). A strong exotherm was observed and the mixture was stirred at room temperature for 15 minutes. The supernatant was withdrawn using a cotton wool filled syringe and decanted into a flask containing bis(triphenylphosphine)palladium(II) dichloride (0.350 g, 0.5 mmol, 0.1 equiv.), and 3,4-dibromopyridine (1.18 g, 5.0 mmol, 1.0 equiv.) at 50 °C under nitrogen. The remaining zinc was washed with dry dimethylformamide (2.0 mL), and the supernatant was transferred as above. After 3 h, a second equivalent of zinc reagent was added, and the mixture was stirred for a further 3 h. The mixture was diluted with ethyl acetate (60 mL) and filtered through a bed of Celite (1 cm). The Celite was washed with ethyl acetate until the filtrate was colourless. The combined filtrates were washed with water (250 mL), and then brine (250 mL). The organic phase was dried over anhydrous magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography over silica eluting with ethyl acetate/petroleum (3:7) ether to give methyl (2*S*)-3-(3-bromopyridin-4-yl)-2-[(*tert*-butoxycarbonyl)amino]propanoate **11** (0.895 g, 50% yield) as a pale yellow oil: R_F = 0.33 (EtOAc/pet. ether 3:7); $[\alpha]_D^{25}$ = +10.0 (*c* 1.0, $CHCl_3$); ν_{max}(ATR)/cm^{-1} 3350, 2978, 2952, 2936, 1745, 1705, 1588 1516, 1434, 1395, 1362, 1278, 1252, 1212, 1160, 1088, 1046, 1023; ^1H NMR (400 MHz, $CDCl_3$) 1.39 (s, 9H), 3.10 (dd, *J* = 8.5, 14.0 Hz, 1H), 3.33 (dd, *J* = 5.5, 14.0 Hz, 1H), 3.76 (s, 3H), 4.65-4.74 (m, 1H), 5.18 (d, *J* = 8.0 Hz, 1H), 7.19 (d, *J* = 5.0 Hz, 1H), 8.44 (d, *J* = 5.0 Hz, 1H), 8.70 (s, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) 28.2 (s), 38.1 (s), 52.7 (s), 80.3 (s), 123.6 (s), 125.9 (s), 145.1 (s), 148.2 (s), 152.0 (s), 154.9 (s), 171.7 (s); *m/z* (ESI+): 359.0602 (MH^+, 100% $C_{14}H_{20}BrN_2O_4$ requires 359.0600).

Methyl (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-[3-(diethoxyphosphoryl)pyridin-4-yl]propanoate (12). Palladium(II) acetate (0.024 g, 0.11 mmol, 0.1 equiv.), triphenylphosphine (0.084 g, 0.32 mmol, 0.2 equiv.), were added to a round bottom flask equipped with a condenser. A vacuum was applied followed by a positive pressure of nitrogen (×3). Sequentially, ethanol (4 mL), triethylamine (0.162 g, 0.22 mL, 1.6 mmol, 1.2 equiv.), diethyl phosphite (0.220 g, 0.21 mL, 1.6 mmol, 1.2 equiv.) and methyl (2*S*)-3-(3-bromopyridin-4-yl)-2-[(*tert*-butoxycarbonyl)amino]propanoate (**11**; 0.467 g, 1.3 mmol, 1.0 equiv.) were added and the mixture was stirred at reflux for 48 h. The mixture was concentrated under reduced pressure and the residue was purified by flash column chromatography over silica, eluting with 15:85 petroleum ether/ethyl acetate to give methyl (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-[3-(diethoxyphosphoryl)pyridin-4-yl]propanoate (**12**; 0.295 g, 54% yield) as a yellow oil: R_F = 0.20 (15:85 pet. ether/EtOAc); $[\alpha]_D^{25}$ = +

17.0 (*c* 1.0, $CHCl_3$); ν_{max}(ATR)/cm^{-1} 2980, 2929, 2906, 1742, 1712, 1582, 1520, 1445, 1364, 1244, 1218, 1160, 1044, 1017; ^{1}H NMR (400 MHz, $CDCl_3$) 1.33 (s, 9H), 1.38 (t, J=7.0 Hz, 3 H), 1.42 (t, J=7.0 Hz, 3 H), 3.33 (dd, J=4.5, 13.5 Hz, 1H), 3.45 (dd, J=11.5, 13.5 Hz, 1 H), 3.78 (s, 3H), 4.15-4.34 (m, 4H), 4.50-4.58 (m, 1H), 6.21 (d, J=8.5 Hz, 1H), 7.37 (t, J=5.0 Hz, 1H), 8.70 (dd, J=2.0, 5.0 Hz, 1H), 8.94 (d, J=7.5 Hz, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) 16.2-16.4 (m), 28.2 (s), 35.3 (s), 52.5 (s), 54.8 (s), 62.7-63.2 (m), 79.7 (s), 124.4 (d, J=185.5 Hz), 125.4 (d, J=11.5 Hz), 150.6 (d, J=9.5 Hz), 153.1 (s), 153.3 (d, J=11.5 Hz), 155.6 (s), 172.2 (s); ^{31}P NMR (162 MHz, $CDCl_3$) 16.48; *m/z* (ESI+): 417.1792 (MH^+, 100% $C_{18}H_{30}N_2O_7P$ requires 417.1800).

4-[(2*S*)-2-Amino-2-carbamoylethyl]pyridin-3-ylphosphonic acid (13). Methyl (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-[5-(diethoxyphosphoryl)pyridin-3-yl]propanoate (**12**) (1.25 g, 3.0 mmol, 1.0 equiv.) was dissolved in methanol (24 mL). Ammonium hydroxide [32% (*w/w*), 12 mL] was added, and the mixture was stoppered and stirred at room temperature for 4 h. The mixture was concentrated under reduced pressure. The residue was dissolved in acetonitrile (28 mL) under nitrogen. Bromotrimethylsilane (4.24 g, 3.7 mL, 27.7 mmol, 10.8 equiv.) was added and the mixture was stirred at 50 °C overnight. The solvent was removed under reduced pressure and the residue was loaded on to a pre-equilibrated (ethanol/water 8:2) flash chromatography silica gel column. The column was eluted with a 80:18:2 to 65:22:8 gradient of ethanol/water/32% (*w/w*) aqueous ammonium. The combined product fractions were concentrated under reduced pressure and then lyophilized. The remaining solid residue was dissolved in water (2.5 mL) and an aliquot was diluted in deuterated water for quantification by ^{1}H NMR using 1,4-dioxane as the internal standard. 4-[(2*S*)-2-amino-2-carbamoylethyl]pyridin-3-ylphosphonic acid (**13**; 0.110 g, 15% yield): R_F = 0.58 (EtOH/H_2O 65:35); $[\alpha]_D^{23}$ = −8.0 (*c* 1.0, H_2O); m.p.=200 °C (decomp.); ν_{max}(ATR)/cm^{-1} 3011, 2970, 2285, 1741, 1667, 1587, 1439, 1399, 1370, 1218, 1130, 1040; ^{1}H NMR (400 MHz, D_2O) 3.39 (dd, J=7.0, 14.0 Hz, 1H), 3.55 (dd, J=5.5, 14 Hz, 1H), 4.17-4.23 (m, 1H), 7.18-7.23 (m, 1H), 8.27-8.31 (m, 1H), 8.63 (d, J=7.0 Hz, 1H); ^{13}C NMR (101 MHz, D_2O) 34.9 (d, J=4.0 Hz), 52.5, 126.3 (d, J=9.0 Hz), 135.9 (d, J=158.5 Hz), 147.7 (d, J=7.0 Hz), 148.0, 149.9 (d, J=11.0 Hz), 171.5; ^{31}P NMR (162 MHz, D_2O) 4.86; *m/z* (ESI+): 246.0642 (MH^+, 100% $C_8H_{13}N_2O_4P$ requires 246.0600).

Diethyl 1-butylpyrazol-4-ylphosphonate. Diethyl 1*H*-pyrazol-4-ylphosphonate (1.83 g, 9.0 mmol, 1.0 equiv.), 1-bromobutane (1.73 g, 1.35 mL, 12.6 mmol, 1.4 equiv.), caesium carbonate (10.2 g, 31.3 mmol, 3.5 equiv.) and acetonitrile (35 mL) were stirred under nitrogen at room temperature for 6 h. The precipitate was filtered off, washed with ethyl acetate (2 × 50 mL) and the combined filtrate and washings were concentrated under reduced pressure. The residue was diluted with ethyl acetate (100 mL) and washed with saturated aqueous ammonium chloride (100 mL). The aqueous phase was extracted with ethyl acetate (2 × 200 mL) and the combined organic phases were washed with brine (200 mL), dried over magnesium sulfate, filtered and concentrated under reduced pressure to give diethyl 1-butylpyrazol-4-ylphosphonate, (1.76 g, 75%) as a pale yellow oil; ν_{max}(ATR)/cm^{-1} 3480, 3104, 2964, 2937, 2879, 1525, 1244, 1136, 1061, 1025; ^{1}H NMR (400 MHz, $CDCl_3$) 0.91 (t, J=7.5 Hz, 3H), 1.24-1.35 (m, 8H), 1.84 (quint., J=7.5 Hz, 2H), 4.00-4.16 (m, 6H), 7.69 (s, 1H), 7.73 (d, J=2.0 Hz, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) 13.5 (s), 16.3 (d, J=6.5 Hz), 19.7 (s), 32.0 (s), 52.2 (s), 61.9 (d, J=5.5 Hz), 107.3 (d, J=221.0 Hz), 134.4 (d, J=23.5 Hz), 142.0 (d, J=13.0 Hz); ^{31}P NMR (162 MHz, $CDCl_3$) 14.95 (s); *m/z* (ESI+): 261.1366 (MH^+, 100% $C_{11}H_{22}N_2O_3P$ requires 261.1400).

1-Butylpyrazol-4-ylphosphonic acid 14. Diethyl 1-butylpyrazol-4-ylphosphonate (1.63 g, 6.3 mmol, 1.0 equiv.), was dissolved in acetonitrile (63 mL) under nitrogen. Bromotrimethylsilane (9.64 g, 8.3 mL, 63 mmol, 10.0 equiv.) was added and the mixture was stirred at 50 °C overnight. After cooling to room temperature, the mixture was concentrated under reduced pressure. The residue was purified by flash chromatography over silica eluting with a 75:4:10 mixture of ethanol/water/32% (*w/w*) aqueous ammonia. The combined product fractions were concentrated under reduced pressure and then lyophilized to give 1-butylpyrazol-4-ylphosphonic acid (**14**; 1.00 g, 78%) as a white solid: R_F=0.51 (EtOH/H_2O/32% (*w/w*) NH_3 (aq.), 65:22:8); m.p.= 135–137 °C; ν_{max}(ATR)/cm^{-1} 3519, 3363, 2957, 2865, 1527, 1452, 1370, 1136, 1112, 1068, 1032; ^{1}H NMR (400 MHz, D_2O) 0.72 (t, J=7.5 Hz, 3H), 1.08 (sex., J=7.5 Hz, 2H), 1.64 (quint., J=7.0 Hz, 2H), 4.01 (t, J=7.0 Hz, 2H), 7.51 (s, 1H), 7.65 (d, J=2.5 Hz, 1H); ^{13}C NMR (101 MHz, D_2O) 12.7 (s), 18.9 (s), 31.5 (s), 51.3 (s), 114.1 (d, J=204.0 Hz), 133.8 (d, J=21.5 Hz), 141.1 (d, J=13.5 Hz); ^{31}P NMR (162 MHz, D_2O) 7.22; *m/z* (ESI−): 203.0596 (M-H^-, 100% $C_7H_{12}N_2O_3P$ requires 203.0600).

2-amino-3-(3-phosphonoimidazol-4-yl)propanoic acid (π-pHis). L-Histidine (0.251 g, 1.62 mmol, 1.0 equiv.), potassium phosphoramidate (0.57 g, 4.21 mmol, 2.6 equiv.) and water (4.5 mL) were stirred at 25 ° C for 40 min. π-pHis was chromatographically purified see below for detailed procedure. The combined product fractions were concentrated by rotary evaporation at 25 °C to approximately 4 mL whilst maintaining a pH between 10–12 using 2 molar aqueous sodium hydroxide and 1 molar aqueous hydrochloric acid. The remaining solution was aliquoted and snap frozen and stored at −80 °C. An aliquot was diluted in deuterated water for quantification by ^{1}H NMR using 1,4-dioxane as the internal standard. π-pHis (0.0084 g, 2% yield): R_F=0.44 (EtOH/32% (*w/w*) NH_3 (aq.)/H_2O, 65:8:22); ^{1}H NMR (400 MHz, D_2O) 2.92 (dd, J=8.0, 15.5 Hz, 1H), 3.14 (dd, J=5.0, 15.5 Hz, 1H), 3.81-3.89 (m, 1H), 6.70 (s, 1H), 7.70 (s, 1H); ^{13}C NMR (101 MHz, D_2O) 30.6 (s), 55.3 (s), 126.2 (d, J=8.0 Hz), 130.4 (d, J=3.5 Hz), 140.4 (d, J=5.0 Hz), 181.9 (s); ^{31}P NMR (162 MHz, D_2O) −5.62; *m/z* (ESI+): 236.0431 (MH^+, 100% $C_6H_{11}N_3O_5P$ requires 236.0400). The characterization data are comparable to the literature.[30]

2-amino-3-(1-phosphonoimidazol-4-yl)propanoic acid (τ-pHis). L-Histidine (0.251 g, 1.62 mmol, 1.0 equiv.), potassium phosphoramidate (0.57 g, 4.21 mmol, 2.6 equiv.) and water (4.5 mL) were stirred at 25 ° C for 16 h. τ-pHis was chromatographically purified, see below for detailed procedure. The combined product fractions were concentrated by rotary evaporation at 25 °C to approximately 8 mL whilst maintaining a pH between 10–12 using 2 molar aqueous sodium hydroxide and 1 molar aqueous hydrochloric acid. The remaining solution was aliquoted and snap frozen and stored at −80 °C. An aliquot was diluted in deuterated water for quantification by ^{1}H NMR using 1,4-dioxane as the internal standard. τ-pHis (0.103 g, 27% yield): R_F=0.40 (EtOH/32% (*w/w*) NH_3 (aq.)/H_2O, 65:8:22); ^{1}H NMR (400 MHz, D_2O) 2.53 (dd, J=9.0, 14.5 Hz, 1H), 2.79 (dd, J=4.5, 14.5 Hz, 1H), 3.32-3.38 (m, 1H), 6.86 (s, 1H), 7.56 (s, 1H); ^{13}C NMR (101 MHz, D_2O) 32.6 (s), 56.0 (s), 117.8 (d, J=5.5 Hz), 137.0 (d, J=8.5 Hz), 139.0 (d, J=5.0 Hz), 181.2 (s); ^{31}P NMR (162 MHz, D_2O) −4.78; *m/z* (ESI+): 236.0431 (MH^+, 100% $C_6H_{11}N_3O_5P$ requires 236.0400). The characterization data are comparable to the literature.[30] (§) The following flash chromatography procedure was carried out at 4 °C and a positive pressure of 0.68 atmospheres. The sample was loaded onto a pre-equilibrated (ethanol/32% (*w/w*) aqueous ammonia/water, 85:4:1) silica gel column (4 cm diameter column with 27 cm of silica) and eluted with a mixture of ethanol, 32% (*w/w*) aqueous ammonia and water gradient (Initially 300 mL of an 85:4:1 mixture was added to the solvent reservoir, once 150 mL was eluted off, 150 mL of a 75:4:10 mixture was added to the solvent reservoir). Once a further 150 mL was eluted off an additional 150 mL of this solvent was added to the solvent reservoir. Subsequently the same cycle was repeated with solvent mixture ratio of 70:4:15 (300 mL), and 60:4:25, (300 mL) until the compound was eluted off. The first 750 mL was

run off and 15 mL fractions were collected. The product fractions were collected such that 5 fractions between the product and the first (for π-pHis) and last (π-pHis) co-eluted π-pHis and τ-pHis mixture were discarded as analysed by TLC.

General biology experimental: All reagents and consumables used were purchased from Fluorochem, MilliporeSigma, Sigma-Aldrich, Alpha Aeser, VWR International, Biolegend, Bio-Rad, Abcam or Thermo Scientific. Ultrapure water was used to make buffers and solutions unless stated otherwise. All solvents were of HPLC grade. Blue Pre-stained Protein Standard, Broad Range (11-190 kDa) was purchased from New England BioLabs. Sartorius Vivaspin 6/20's (3 and 10 kDa MWCO) were used for buffer exchange and protein concentration. Thermo Scientific Snakeskin dialysis tubing, 16 mm dry diameter (3.5 Da MWCO) was used for buffer exchange of purified antibodies. NHS-Activated Sepharose 4 Fast Flow was purchased from GE Healthcare Life Sciences. Sigma Trypsin-EDTA solution (1 ×, sterile; sterile-filtered, BioReagent, suitable for cell culture, 0.5 g porcine trypsin and 0.2 g EDTA•4Na per litre of Hanks' Balanced Salt Solution with phenol red) was used to trypsinise adhered cells. Sigma Aldrich hemocyanin from *Megathura crenulata* (keyhole limpet) in phosphate-buffered saline (PBS) was used. BIO-RAD Colloidal Gold Total Protein Stain was used to stain electro-blotted proteins. Corning® 96 Well EIA/RIA clear flat bottom polystyrene high bind microplate was used for ELISA assays. Nunc™ Cell Culture/Petri Dishes (56.7 cm^2) from Thermo Scientific™ were used for cell culture. Pierce™ BCA protein assay kit was used to quantify proteins. Thermo Scientific SuperSignal™ West Pico PLUS chemiluminescent substrate was used to develop blotted membranes. Ultra Pure AccuGel™ 29:1, 30% (*w*/*v*) 29:1 acrylamide: bis-acrylamide solution (gas stabilised) or ULTRA PURE AccuGel™ 19:1, 40% (*w*/*v*) 19:1 acrylamide: bis-acrylamide solution (gas stabilised) was used to make SDS-PAGE gels. Interchim TMB ELISA substrate standard solution was used to develop ELISA plates. Millipore Immobilon-P PVDF Membrane (0.45 μm pore size) was used for western blot membranes. Western blotted membranes were visualised using a BIO-RAD ChemiDoc™ XRS+ with image Lab™ software. ELISA absorbance was measured using a Varioskan flash spectrometer. Conjugate concentrations were determined using NanoDrop™ 2000/c Spectrophotometers by Thermo Fisher Scientific. Specific details of commercial antibodies used are given in experimental sections using antibodies. Specific consumables used for human iPSCs cell culture are provided under the "Human induced pluripotent stem cell (iPSCs) cell culture and neuronal differentiation" section.

Conjugation of amino acid to BSA via glutaraldehyde. Following procedure described by Hermanson with minor modification.[37] Coupling buffer: 0.1 M sodium carbonate, 0.15 M sodium chloride, pH 8.5. Storage buffer: 0.1 M sodium carbonate/bicarbonate, pH 10.8. Bovine serum albumin (50 mg, 0.0007 mmol, 1.0 equiv.) was added to a 50 mL centrifuge tube and dissolved in coupling buffer (20 mL). The mixture was cooled on ice. The amino acid (0.03 mmol, 40 equiv.) was added followed by fresh 50 *w*.% (aq.) glutaraldehyde solution (0.5 mL) to give a 1% (*v*/*v*) working concentration after dilution (see below). The solution was topped up to 25 mL with ice cold coupling buffer. The tube was sealed and left on a rotor at 4 °C for 3.5 h. 1% (*w*/*v*) sodium borohydride (0.25 g) was added to the mixture, and a small hole was pierced in the lid before centrifugation (4 °C, 3220 RCF, 60 min). The mixture was concentrated down ~2 mL using a Vivaspin 20 (10 kDa MWCO) by centrifugation (4 °C, 3220 RCF). The solution was diluted with ice cold storage buffer to 10 mL and concentrated down again to 2 mL (the solution was aspirated every 10 min). This cycle was repeated two more times. After a final concentration to ~1 mL, the sample was aliquoted, snap frozen and stored at −80 °C. The protein concentration was determined by bicinchoninic acid assay (BCA) or NanoDrop with absorbance at 280 nm, 1 cm path length, BSA = 66.463 kDa and $\varepsilon = 43.824\ M^{-1}\ cm^{-1}$.[31] ^{31}P NMR spectra were acquired using a Bruker AVANCE III or Avance III HD spectrometer, operating at 162 MHz or 202.5 MHz respectively. Quantitative spectra were typically acquired using a 30° excitation pulse and a relaxation delay of 8 s, which was determined as sufficiently long to enable full relaxation on one sample with external standard early on in the study. External standard: 2 mM triphenylphosphine oxide, 20 mM chromium(III) acetylacetonate, in 150 μL of deuterated chloroform. Norell(tm) high throughput 3 mm NMR sampling tubes length: 203 mm; inner diameter: 2.41 mm was used as the internal standard capillary. Norell® Standard Series™ 5 mm NMR tubes length, 7 inches; inner diameter: 4.2 mm was used. All other samples were assumed to have similar T1 relaxation profiles. Transients acquired typically ranged between 6k and 9k to obtain enough signal-to-noise in most cases. For BSA–G-amino acid conjugates, protein concentration between 20–40 mg mL^{-1} were used. Acquisition windows ranged between 6.9 kHz and 20 kHz, using 32k acquisition points.

Conjugation of amino acids to KLH via glutaraldehyde for immunisation. Following a procedure described by Hermanson with minor changes.[37] Coupling buffer: 0.1 M sodium carbonate, 0.15 M sodium chloride, pH 8.5. PBS: 1.8 mM monopotassium phosphate, 10 mM disodium phosphate, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4. Keyhole limpet hemocyanin (10 mg, 0.002 μM, 1.9 mL, 1.0 equiv.) in PBS solution was added to a centrifuge tube (15 mL) containing coupling buffer (3 mL) and the mixture was cooled on ice. Either [1-(2-aminoethyl)-1*H*-pyrazol-4-yl]phosphonic acid **8** or 4-[(2*S*)-2-amino-2-carbamoylethyl]pyridin-3-ylphosphonic acid **13** (0.004 mmol, 2000 equiv.) was added followed by 50 *w*% (aq.) fresh glutaraldehyde solution (0.1 mL) to give a 1% (*v*/*v*) working concentration after dilution (see below). The solution was topped up to 5 mL with ice-cold coupling buffer. The tube was sealed and left on the rotor at 4 °C for 4 h. 1% (*w*/*v*) sodium borohydride (0.05 g) was added to the mixture, and a small hole was pierced in the lid before centrifugation (4 °C, 3220 RCF, 1.5 h.). The mixture was transferred to a Vivaspin 6 (10 kDa MWCO) and concentrated down to 1 mL by centrifugation (4 °C, 3220 RCF). The solution was diluted with ice cold PBS to 2 mL and concentrated down again to 1 mL during which the solution was aspirated every 10 min. This cycle was repeated four more times. The protein concentration was determined by NanoDrop with absorbance at 280 nm, 1 cm path length, and $\varepsilon_{1\%} = 15.7\ M^{-1}\ cm^{-1}$. The final KLH concentration was made up to 2 mg mL^{-1} using PBS, aliquoted, subsequently snap frozen and stored at −80 °C before being shipped on dry ice to Orygen Antibodies LTD for immunisation of sheep.

Coupling of BSA-G-amino acid conjugates to NHS-activated Sepharose 4 Fast Flow. Sepharose BSA–G-amino conjugate resins were synthesized following the manufacturer's instructions with modifications. Wash solution: 1 mM hydrochloric acid. Coupling buffer: 0.2 M sodium bicarbonate, 0.5 M sodium chloride, pH 8.3. Protein coupling solution: 3 mg mL^{-1} BSA–G-amino acid, 0.5 M sodium chloride, 0.2 M sodium bicarbonate, pH 8.3. Fresh NHS activated Sepharose 4 fast flow resin suspension was added to a column with a sintered frit and drained. The resin was washed with ice cold wash solution (3 × resin bed volume) and then with ice cold coupling buffer (2 × resin bed volume). The column was caped, and the resin was suspended in protein coupling solution (1/2 × resin bed volume). The suspension was transferred to a centrifuge vial and mixed end over end at room temperature for 1 h. A second equivalent of protein coupling buffer solution was added, and the mixture was turned end over end for another 1 h. The suspension was transferred back to the column. The solution was drained off and the resin was washed with coupling buffer (3 ×

resin bed volume). When the resin was not immediately used it was stored in coupling buffer at 4 °C and used within 24 h.

Crude antiserum serial dilution sandwich ELISA. Coating buffer: 0.1 M bicarbonate/carbonate, pH 10.8. PBS: 1.8 mM monopotassium phosphate, 10 mM disodium phosphate, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4. Wash buffer: 0.05 % (*v*/*v*) Tween 20, PBS, pH 7.4. Blocking buffer: 0.05 % (*v*/*v*) Tween 20, 0.2 % (*w*/*v*) gelatine, PBS, pH 7.4. Secondary antibody: Dako rabbit anti-goat immunoglobulins/ horseradish peroxidase diluted in 1 : 2000 (*v*/*v*) blocking buffer. To a 96-well ELISA plate (labelled plate 1) BSA–G-τ-pHis or BSA–G-π-pHis (100 μL, 20 μg/mL) diluted in coating buffer was added to each well. The plate was wrapped in foil and stored at 4 °C overnight. The wells were emptied and washed with wash buffer (×3). The wells were incubated in blocking buffer (150 μL), wrapped in foil and left at 37 °C for 2 h. The wells were emptied and washed with wash buffer (×3). To a separate 96-well ELISA plate (labelled plate two), blocking buffer (60 μL) was added to each well except for the top row. Sheep antiserum diluted 1 : 50 (*v*/*v*) in blocking buffer was added in duplicate to the top wells (120 μL). 60 μL from the top wells was removed and serially diluted down the plate. Blocking buffer (60 μL) was added to each well. 100 μL from each well of plate two was added to the corresponding wells of plate 1. Plate 1 was wrapped in foil and left at 37 °C for 1.5 h. The wells were emptied and washed with wash buffer (×3). Dako rabbit anti-goat immunoglobulins/ horseradish peroxidase diluted 1 : 2000 (*v*/*v*) in blocking buffer (100 μL) was added to each well, and the plate was wrapped in foil and left at 37 °C for 1 h. The wells were emptied and washed with wash buffer (×3) and finally with distilled water. 3,3′,5,5′-tetramethylbenzidine (50 μL) ELISA substrate was added to each well and incubated at room temperature between 0.5-5 min (until pale blue coloured solution was observed). 2 molar sulfuric acid (50 μL) was added to each well to quench the reaction. The absorbance was measured immediately at 450 nm.

Competitive ELISA. Coating buffer: 0.1 M bicarbonate/carbonate, pH 10.8. PBS: 1.8 mM monopotassium phosphate, 10 mM disodium phosphate, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4. Wash buffer: 0.05 % (*v*/*v*) Tween 20, PBS, pH 7.4. Blocking buffer: 0.05 % (*v*/*v*) Tween 20, 0.2 % (*w*/*v*) gelatine, PBS, pH 7.4. To a 96-well ELISA plate (labelled plate 1); BSA–G-τ-pHis or BSA–G-π-pHis (100 μL, 20 μg/mL) diluted in coating buffer was added to each well. This plate was wrapped in foil and left at 4 °C overnight. The wells were emptied and washed with wash buffer (×3). Blocking buffer (150 μL) was added to each well and the plate was wrapped in foil and left at 37 °C for 2 h. The wells were emptied and washed with wash buffer (×3). To a separate 96-well plate (labelled plate two) blocking buffer (60 μL) was added to each well except for the top row. Each amino acid (120 μL, 10 mM) was added in duplicate to the top wells. 60 μL from each top row wells was drawn and serially diluted down the plate until the second last row. pHis antibody diluted (see Table 1 for antibody dilutions) in blocking buffer (60 μL) was added to each well. Plate two can be prepared the day before and stored wrapped at 4 °C. 100 μL from each well of plate two was added to the corresponding wells of plate 1. Plate 1 was wrapped in foil and left at 37 °C for 1.5 h. The wells were emptied and washed with wash buffer (×3). Secondary antibody diluted in blocking buffer (100 μL) was added to each well. The plate was wrapped in foil and left at 37 °C for 1 h. The wells were emptied and washed with wash buffer (×3) and finally with distilled water. 3,3′,5,5′-tetramethylbenzidine ELISA substrate (50 μL) was added to each well and incubated at room temperature between 0.5-5 min (until pale blue coloured solution was observed). 2 M sulfuric acid (50 μL) was added to each well to quench the reaction. The absorbance was measured immediately at 450 nm (Table 1).

Table 1. Antibodies and dilutions used for competitive ELISA.

pHis antibody	pHis antibody dilution (v/v)	Secondary antibody	Secondary antibody dilution (v/v)
τ-pHis-FT	1/18	Dako rabbit anti-goat immunoglobulins/ horseradish peroxidase	1/2000
τ-pHis-W	1/4	Dako rabbit anti-goat immunoglobulins/ horseradish peroxiase	1/2000
π-pHis-FT	1/5	Dako rabbit anti-goat immunoglobulins/ horseradish peroxidase	1/2000
π-pHis-W	1/5	Dako rabbit anti-goat immunoglobulins/ horseradish peroxidase	1/2000
SC56-2[II]	1/500	Dako goat anti-rabbit immunoglobulins/ horseradish peroxidase	1/2000
SC50-3[II]	1/1000	Dako goat anti-rabbit immunoglobulins/ horseradish peroxidase	1/2000
SC1-1[II]	1/1000	Dako goat anti-rabbit immunoglobulins/ horseradish peroxidase	1/2000

Culturing of human bronchial epithelial (16HBE14o−) cell line. Full serum medium: Medium 199 (Medium 199 with Earle's salts and sodium bicarbonate, without L-glutamine, liquid, sterile-filtered, suitable for cell culture including phenol red), supplemented with 10 % (*v*/*v*) foetal bovine serum, 1.3 mM of L-glutamine, 80 μg/mL streptomycin, 80 units/mL penicillin. Phosphate buffered saline (PBS): 1.8 mM monopotassium phosphate, 10 mM disodium phosphate, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4. 16 HBE14o- cell line was cultured in 56.7 cm^2 dishes in full serum medium (10 mL) and incubated at 37 °C in a 5 % CO_2 atmosphere. The medium was changed every 2–3 days by discarding the old medium, washing the cells with PBS (3 mL) and then replacing with fresh full serum medium (10 mL). Once the 16HBE14o- cells were between 70–90 % confluency, they were passaged or harvested

Culturing of rat fat pad endothelial cell (RFPEC) line. Full serum medium: DMEM (DMEM, with high glucose, L-glutamine, phenol red; without sodium pyruvate and HEPES) supplemented with 10 % (*v*/*v*) foetal bovine serum, 80 μg/mL streptomycin, 80 units/mL penicillin. Phosphate buffered saline (PBS): 1.8 mM monopotassium phosphate, 10 mM disodium phosphate, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4. RFPEC cell line was cultured in 56.7 cm^2 dishes in full serum medium (10 mL) and incubated at 37 °C and a 5 % CO_2 atmosphere. The medium was changed every 2–3 days by discarding the old medium, washing the cells with PBS (3 mL) and then replacing with fresh full serum medium (10 mL). Once the RFPEC cells were between 70–90 % confluency, they were passaged or harvested

Isolation and culturing of HUVEC cells. HUVEC cells were kindly provided by Dr. Anupriya Chatterjee and were isolated and cultured as described previously.[56]

Isolation and culturing of Müller wild-type (WT) and NDPK–B knockout (KO) cells. Müller WT and NDPK B KO cells were provided by Dr Yuxi Feng and Professor Thomas Wieland. The cells were isolated and cultured as previously described.[57–59]

Human induced pluripotent stem cell (iPSCs) cell culture and neuronal differentiation. Human iPS cells used in this study named control MIFF1[60] was kindly provided by Professor Peter Andrews and Dr Ivana Barbaric (Centre for Stem Cell Biology, the University of Sheffield). iPSCs were maintained in complete TeSR™-E8™ Medium (#05990, StemCell Technologies) in Matrigel® Growth Factor Reduced-coated plates (Corning®, cat.: 356230) according to the manufacturer's recommendations. Cultures were replenished with fresh medium every day. The passage of cells was performed when the cells reached around 75 % confluency, every four to six

days as clumps using Versene an EDTA solution for use as a gentle non-enzymatic cell dissociation reagent (15040066, ThermoFisher) according to the manufacturer's recommendations. For all the experiments in this study, iPSCs were used between passage 20 and 25, all iPSCs were cultured in 5% oxygen, 5% CO_2 at 37 °C. Human iPSCs were differentiated into cortical neurons for 75 days according to the protocol from Shi et al.[61] after 75 days cortical neurons was processed for immunofluorescence.

Cell lysis. Lysis buffer: Modified radio-immunoprecipitation assay (RIPA) lysis buffer: 150 mM sodium chloride, 0.5% (*w/v*) sodium deoxycholate, 1% (*w/v*) Triton X-100, 0.1% (*w/v*) sodium dodecyl sulfate, 10 mM sodium fluoride, 5 mM sodium orthovanadate, 10 mM sodium pyrophosphate, Roche complete protease inhibitor cocktail, 50 mM Tris, pH 9.0. Phosphate buffered saline (PBS): 1.8 mM monopotassium phosphate, 10 mM disodium phosphate, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4. The cell culture medium was discarded, and the cells were washed PBS (5 mL). The cell culture plates were cooled on ice and residual wash was discarded. Lysis buffer (100 μL) was added and the cells were scrapped into the lysis buffer. The lysate was briefly centrifuged, and the supernatant was aliquoted, snap frozen using liquid nitrogen, and stored at −80 °C. Protein concentrations were determined by BCA assay.

SDS-PAGE preparation. Resolving gel: 12% (*v/v*) acrylamide, 0.1% (*w/v*) sodium dodecyl sulfate, 0.1% (*w/v*) ammonium persulfate, 0.001% (*v/v*) tetramethylenediamine, 0.38 M Tris·HCl, pH 8.8. Stacking gel: 4% (*v/v*) acrylamide, 0.1% (*w/v*) sodium dodecyl sulfate, 0.1% (*w/v*) ammonium persulfate, 0.001% (*v/v*) tetramethylenediamine, 0.125 M Tris·HCl, pH 8.8. Freshly prepared resolving gel solution was immediately poured between 10 cm × 10 cm (1 mm spacers) and 10 cm × 10 cm (notched) glass plates. Isopropanol (~200 μL) was slowly added to the top of the gel solution. The gel was left to polymerise at room temperature for 30 min. The isopropanol was poured away, and the top of the gel was washed with water (×3). Excess water was soaked up with a filter paper. Freshly prepared stacking gel solution was poured over the resolving gel to fill the cavity. A 10 well comb (~50 μL capacity per well) was inserted into the solution and the gel was allowed to polymerise at room temperature for 2 h. Gels were used immediately or wrapped in plastic with a damp tissue (running buffer) and stored for up to 3 days.

Coomassie staining. Fixer solution: 25% (*v/v*) isopropanol, 10% (*v/v*) acetic acid. Coomassie blue stain solution: 0.1% (*w/v*) Coomassie blue, 40% (*v/v*) MeOH (aq.), 10% (*v/v*) acetic acid. Destaining solution: 10% (*v/v*) acetic acid. The following steps were carried out using an orbital shaker at room temperature. The gel containing proteins was mixed with fixer solution for 10 min. The fixer solution was discarded, and the gel was mixed with Coomassie blue stain solution for 1 h. The stain solution was discarded, and the stained gel was mixed with destaining solution until minimal background stain remained. The destaining solution was changed several times.

Western blot using pHis antibodies. 5× (concentrated) sample buffer: 10% (*w/v*) lithium dodecyl sulfate, 40% (*w/v*) glycerol, 0.02% (*w/v*) bromophenol blue, 50 mM EDTA, 500 mM dithiothreitol, 300 mM Tris, pH 9.0. 1× sample buffer was made by diluting 5× sample buffer in water.

Resolving buffer: 0.1% (*w/v*) sodium dodecyl sulfate, 192 mM glycine, 25 mM Tris, pH 8.3. Transfer buffer: 10% (*v/v*) methanol, 192 mM glycine, 25 mM Tris, pH 8.3. Wash buffer: 165 mM sodium chloride, 0.05% (*v/v*) Tween 20, 10 mM Tris·HCl, pH 8.0. Blocking buffer: 0.2% (*v/v*) gelatine from cold water fish skin, 165 mM sodium chloride, 0.05% (*v/v*) Tween 20, 10 mM Tris·HCl, pH 8.0. Cell lysates (80-100 μg of protein) were treated with 5× sample buffer to give a 1× final concentration and then made up to 30 μL with 1× sample buffer. The sample buffer treated samples were shaken at room temperature for 25 min and then loaded on to SDS-PAGE gels. The samples were resolved with stirring using resolving buffer with the tank immersed in ice, at 120 V for the first 10 min and then gradually increased to 180 over 5 min. The resolved proteins were immediately electro blotted onto a methanol activated PVDF membrane using transfer buffer with stirring and the tank immersed in ice at 100 V for 1 h. The following steps were carried out at room temperature. Blocking buffer (75 mL) was added over the membrane (~8×8 cm) in a tray (~10 cm × 14 cm) and left mixing on an orbital shaker for 1 h. The membrane was transferred to a 50 mL centrifuge vial containing pHis antibody diluted (see Table 2 for antibody dilutions) in blocking buffer (5 mL), and left mixing on a roller mixer for 1 h. The membrane was transferred to a covered tray (~10 cm × 14 cm) and the membrane was washed (6× 5 min) with wash buffer (50 mL). The membrane was transferred to a 50 mL centrifuge vial containing secondary antibody diluted in blocking buffer (5 mL), and left mixing on a roller for 1 h. The membrane was transferred to a covered tray (~10 cm × 14 cm), and the membrane was washed (6×5 min) with wash buffer (50 mL). The membrane was covered with chemiluminescence solution for 1 min (no mixing). After draining of the excess chemiluminescence solution, the membrane was imaged (Table 2).

Table 2. Antibodies and dilutions used for western blots.

pHis antibody	pHis antibody dilution (v/v)	Secondary antibody	Secondary antibody dilution (v/v)
τ-pHis-FT	1/1000	Dako rabbit anti-goat immunoglobulins/ horseradish peroxidase	1/2000
τ-pHis-W	1/100	Dako rabbit anti-goat immunoglobulins/ horseradish peroxidase	1/2000
π-pHis-FT	1/1000	Dako rabbit anti-goat immunoglobulins/ horseradish peroxidase	1/2000
π-pHis-W	1/100	Dako rabbit anti-goat immunoglobulins/ horseradish peroxidase	1/2000
SC56-2[‖]	1/100	Dako goat anti-rabbit immunoglobulins/ horseradish peroxidase	1/2000
SC50-3[‖]	1/1000	Dako goat anti-rabbit immunoglobulins/ horseradish peroxidase	1/2000
SC1-1[‖]	1/1000	Dako goat anti-rabbit immunoglobulins/ horseradish peroxidase	1/2000

[‖]Commercially available pHis monoclonal antibody available at the time of the study.

Acid treatment of cell lysates. Cell lysates were either treated with glacial acetic acid or 1 M hydrochloric acid to pH 3–4 and then heated at 90 °C for 45 min. Some precipitation was observed for all cell lysates. The lysate was allowed cool to room temperature and the pH was raised to pH ~8–9 using 2 M sodium hydroxide and any precipitate went back into solution. Lysates were stored at −80 °C or used immediately.

Immunofluorescence staining. PBS: 1.8 mM monopotassium phosphate, 10 mM disodium phosphate, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4. Incubation buffer: 0.3% (*v/v*) Triton X-100, PBS, pH 7.4. Blocking buffer: 5% (*v/v*) donkey serum, PBS. For immunostaining, cells were washed with PBS and fixed with 4% (*w/v*) paraformaldehyde for 15 min at room temperature. After fixation, samples were washed with PBS (3×), and incubation buffer was added, and the cells were left for 5 min at room temperature. The solution was discarded and blocking buffer was added over the cells and left for 1 h at room temperature. The solution was discarded, and the primary antibody diluted (see Table 3 for antibody dilutions) in 1% (*v/v*) donkey serum in PBS was added over the cells and left overnight at 4 °C. Cells were washed

Table 3. Antibodies and dilutions used for immunofluorescence staining.

Primary antibody	Primary antibody dilution (*v*/*v*)
τ-pHis-W	1:20
π-pHis	1:10
Abcam S100b immunoglobulin (ab868)	1:1000
Thermofisher Alexa Fluor™ 488 Phalloidin	1:200
BioLegend Beta III tubulin immunoglobulin (801201)	1:1000
Secondary antibody	Secondary antibody dilution (*v*/*v*)
Thermofisher donkey anti-goat IgG Alexa Fluor™ 555 (A-21432)	1:400
Thermofisher donkey anti-rabbit IgG Alexa Fluor™ 488 (A-21206)	1:400
Thermofisher donkey anti-mouse IgG Alexa Fluor™ 488 (A-21202)	1:400
Thermofisher donkey anti-goat IgG Alexa Fluor™ 594 (A-11058)	1:400

with PBS (3×) and the secondary antibodies diluted in blocking buffer was added and cells were left at 1 h at room temperature. The cells were washed with PBS (3×) and 4,6-diamidino-2-phenylindole (DAPI; 1.0 mg mL^{-1}) was added. Cells were analysed with confocal (Leica SP8) fluorescence microscope or with Opera Phenix® High Content Screening System. Images were processed with using the Harmony Image analysis system or Photoshop CS (Adobe) software. All experiments included cultures where the primary antibodies were not added, unspecific stained was not observed in such negative controls (Table 3).

Immunoprecipitation of proteins from the 16HBE14o- cell lysate using the τ-pHis-FT2 antibody. Wash buffer 1: 165 mM sodium chloride, 10 mM Tris·HCl, pH 8.0. Wash buffer 2: 165 mM sodium chloride, 0.05% (*v*/*v*) Tween 20, 10 mM Tris·HCl, pH 8.0. Wash buffer 3: 500 mM sodium chloride, 0.3% (*v*/*v*) Tween-20, 20 mM Tris·HCl, pH 7.5. Elution buffer 1: 165 mM sodium chloride, 0.05% (*v*/*v*) Tween 20, 40 mM phosphopyrazolyl butane **14**, 10 mM Tris·HCl, pH 8.0. Elution buffer 2: 0.1 M glycine, pH 2.0. Centrifugation conditions: 1000 RCF, 2 min, 4 °C. Protein G Sepharose slurry (~ 80 μL bed volume) was added to a 1.5 mL centrifuge vial and washed with wash buffer 1 (4×400 μL; note: all washes herein were followed by centrifugation to pellet the resin and removal of the supernatant). Wash buffer 1 (960 μL) and τ-pHis-FT2 (40 μL) was added to the resin. The vial was added to a 50 mL centrifuge tube and mixed on a roller mixture at 4 °C for 2 h. The suspension was centrifuged, and the supernatant was discarded. The resin wash washed with wash buffer 2 (3×1 mL) and then suspended in wash buffer 2 (600 μL). The mixture was aspirated, centrifuged and the supernatant was discarded. Freshly prepared 16HBE14o- cell lysate (0.42 mg), made up to 1 mL using wash buffer 2 was added. The vial was closed and added to a 50 mL centrifuge tube and left on the roller mixture at 4 °C for 45 min. The resin was centrifuged, and the supernatant was removed and kept for later analysis. The resin was then washed with wash buffer 3 (4×1 mL). The suspension was treated with elution buffer 1 (80 μL) and mixed using a thermomixer (550 rpm) for 15 min at room temperature. The resin was pelleted and 60 μL of eluent was removed using gel loading tips. The elution step was repeated (3×) and the resin was washed with wash buffer 3 (2×1 mL). The resin was finally treated with elution buffer 2 (80 μL) and mixed using a thermomixer (550 rpm) for 15 min at room temperature. The resin was pelleted, and the eluent (60 μL) was removed and immediately neutralised with 1 molar Tris, pH 8.0 (1:10 volume of elution buffer 2).

Colloidal gold staining. Wash buffer: 500 mM sodium chloride, 0.3% (*v*/*v*) Tween-20, 20 mM Tris·HCl, pH 7.5. The following steps were carried out at room temperature using an orbital shaker. The electroblotted proteins on PVDF membrane was washed with wash buffer for 20 min (×3). The membrane was washed with water for 2 min (×3). The membrane was covered with Colloidal Gold Total Protein stain for 1 h or until protein were visible. The membrane was washed with water for 1 min (×3) prior to visualisation.

***In vitro* phosphorylated nucleoside diphosphate kinase A, B and C**. *In vitro* phosphorylated nucleoside diphosphate kinase (NDPK) A, B and C,full length and mutants were biosynthesised as previously described.[48]

Supporting Information

Further references are cited in the Supporting Information.[19,48]

Acknowledgements

We thank Patrik Niesobski for preliminary synthetic work; Bezaleel Mambwe for technical assistance; Sandra van Meurs and Mike P. Williamson for ^{31}P NMR spectroscopy assistance; Anupriya Chatterjee for providing HUVEC cells, and EPSRC for support (DTA studentship to M.V.M.).

Conflict of Interests

Two of the authors are named on relevant patents (WO/2015/033120; EU - 20160207948 and USA – US 10,053,476).

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: phosphohistidine · phosphorylation · antibodies · cell-signalling · biochemistry

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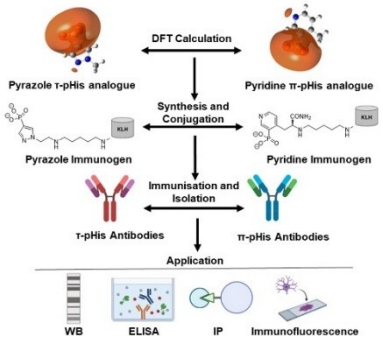
Manuscript received: March 7, 2023
Revised manuscript received: May 11, 2023
Accepted manuscript online: May 14, 2023
Version of record online: ■■, ■■

RESEARCH ARTICLE

Histidine phosphorylation is a post translation modification that occurs in all cells and is currently understudied. Using DFT calculations, we report the synthesis of a pyrazole τ-pHis and a pyridine π-pHis analogue. The pyrazole and pyridine analogues were used to generate isomer-selective τ-pHis and π-pHis antibodies, respectively. The antibodies were applicable in many immunological techniques.



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Chemical Tools for Studying Phosphohistidine: Generation of Selective τ-Phosphohistidine and π-Phosphohistidine Antibodies