A Journal of the Gesellschaft Deutscher Chemiker

Angewandte International Edition GDCh Chemie

www.angewandte.org

Accepted Article

Title: Chemical genetics reveals a role of dCTP pyrophosphatase 1 in Wnt signaling

Authors: Herbert Waldmann, Alexandra Friese, Shobhna Kapoor, Tabea Schneidewind, Srinivasa Rao Vidadala, Juhi Sardana, Alexandra Brause, Tim Förster, Matthias Bischoff, Jessica Wagner, Petra Janning, and Slava Ziegler

To be cited as: *Angew. Chem. Int. Ed.* 10.1002/anie.201905977
Angew. Chem. 10.1002/ange.201905977

Link to VoR: http://dx.doi.org/10.1002/anie.201905977
http://dx.doi.org/10.1002/ange.201905977

WILEY-VCH

10.1002/anie.201905977

Chemical genetics reveals a role of dCTP pyrophosphatase 1 in Wnt signaling

Alexandra Friese[1,#], Shobhna Kapoor[1,2,#], Tabea Schneidewind[1], Srinivasa Rao Vidadala[1,3], Juhi Sardana[1], Alexandra Brause[1], Tim Förster[1], Matthias Bischoff[1], Jessica Wagner[1,4], Petra Janning[1], Slava Ziegler[1], and Herbert Waldmann[1,4,*]

Affiliations:

[1]Department of Chemical Biology, Max Planck Institute of Molecular Physiology, Otto-Hahn-Straße 11, 44227 Dortmund, Germany

[2]Present address: Department of Chemistry, Indian Institute of Technology Bombay, Powai, Mumbai - 400076, Maharashtra, India

[3]Present address: Syngene International Limited, Bengaluru, Karnataka 560099, India

[4]Faculty of Chemistry and Chemical Biology, Technische Universität Dortmund, Otto-Hahn Straße 6, 44227 Dortmund, Germany

[#]These authors contributed equally to this work.

*Correspondence to: herbert.waldmann@mpi-dortmund.mpg.de

10.1002/anie.201905977

Abstract

Cell-based screening is a powerful approach to identify novel chemical modulators and biological components of relevant biological processes. The canonical Wnt pathway is essential for normal embryonic development and tissue homeostasis, and its deregulation plays a crucial role in carcinogenesis. Therefore, the identification of new pathway members and regulators is of significant interest. By means of a cell-based assay monitoring Wnt signaling we identified the pyrrolocoumarin Pyrcoumin as inhibitor of canonical Wnt signaling. Target identification and validation revealed that Pyrcoumin is a competitive inhibitor of dCTP pyrophosphatase 1 (dCTPP1). We demonstrate a yet unknown interaction of dCTPP1 with ubiquitin carboxyl-terminal hydrolase (USP7) that is counteracted by dCTPP1 inhibitors. These findings indicate that dCTPP1 plays a role in regulation of Wnt/β-catenin signaling most likely through a direct interaction with USP7.

10.1002/anie.201905977

Canonical Wnt/β-catenin signaling is a fundamental growth control pathway essential for e.g. embryonic development, stem cell maintenance, cell proliferation and tissue homeostasis[1]. Upon activation the central Wnt pathway component β-catenin is no longer phosphorylated and degraded by the so-called destruction complex[2], but rather stabilized and translocated to the nucleus, where it activates the expression of Wnt target genes[3]. Hyperactivated Wnt signaling has been associated with carcinogenesis, in particular colorectal cancer[4]. Thus, the discovery of new canonical Wnt pathway members and new small-molecule modulators of Wnt signaling is of major interest[5].

We report the identification of the pyrrolocoumarin Pyrcoumin, which modulates Wnt signaling by competitive inhibition of dCTP pyrophosphatase 1 (dCTPP1). dCTPP1 is a positive regulator of canonical Wnt signaling, interacts with Ubiquitin carboxyl-terminal hydrolase (USP7) in Wnt signal-dependent manner, and this interaction is weakened by dCTPP1 inhibitors. These findings reveal a yet unknown interaction between dCTPP1 and USP7 and a role for dCTPP1 in the Wnt/β-catenin pathway.

Screening of 16,574 in-house compounds at 10 μM concentration in a Wnt-responsive reporter gene assay in HEK293 cells stably transfected with the human Frizzled-1 receptor and a TOPFlash reporter plasmid[6] identified pyrrolocoumarins as promising inhibitor class (Figure S1a, Table S1). Activity was confirmed in a manual reporter gene assay using HEK293T cells transiently transfected with the SuperTOPflash reporter (Figure S1b). Compound **1** (termed Pyrcoumin, Figure 1a) inhibited Wnt signaling with a half-maximal inhibitory concentration (IC_{50}) of 8.4 μM in the SuperTOPFlash reporter gene assay (Figure 1b) and was selected for further characterization. Pyrcoumin dose-dependently decreased the expression of Wnt target genes *AXIN2*, *CCND1* and *LEF1* in Wnt-activated HEK293 cells (Figure 1c) and inhibited Wnt pathway activity also in HCT116 cells (Figure 1b), harboring a Ser45del mutation in β-catenin that constitutively activates the Wnt pathway[7]. Pyrcoumin did not affect the abundance of β-catenin (Figure 1d, Figure S1c), or other pathway components

10.1002/anie.201905977

such as Axin2 and phosphorylated (Ser33/Ser37/Thr41) β-catenin (Figure 1e). These results suggest a mode of action downstream of β-catenin.

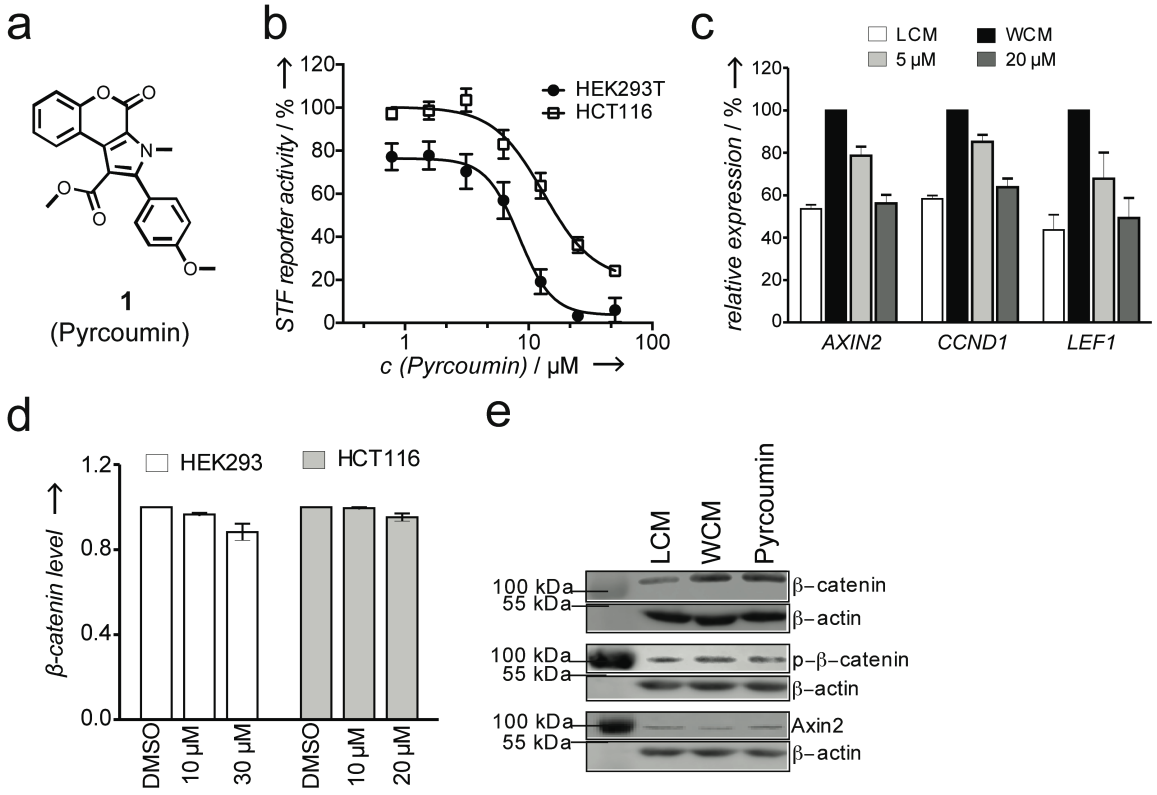


Figure 1: Pyrcoumin antagonizes Wnt signaling downstream of β-catenin. (a) Structure of Pyrcoumin (**1**)**.** (b) Wnt-responsive reporter gene assays in HEK293T and HCT116 cells transiently transfected with SuperTOPFlash (STF) plasmid upon treatment with Pyrcoumin or DMSO in Wnt-3a conditioned medium (WCM) for 22 h (mean ± S.D., n = 6 for HEK293T, n = 3 for HCT116). (c) mRNA expression levels of the Wnt target genes *AXIN2*, *LEF1* and *CCND1* in HEK293 cells treated with L-cell conditioned medium (LCM) and compound or DMSO in WCM for 24 h as determined using RT-qPCR (mean ± S.D., n = 3). (d) In-Cell-Western in HEK293 and HCT116 cells treated with Pyrcoumin in WCM (for HEK293) or DMEM (for HCT116) to quantify β-catenin levels (mean ± S.D., N = 4, n = 3). (e) Representative immunoblots for β-catenin, Axin2, phosphorylated β-catenin and β-actin, used as loading control, using HEK293 cells treated with LCM, WCM or 10 µM Pyrcoumin in WCM for 24 h. Uncropped blots in Figure S5a.

For target identification by affinity-based chemical proteomics we synthesized active probe **2** (Figure 2a) (IC_{50} = 14.8 μM for the corresponding azide analog (Figure S1d-e)) and a control probe (Figure S1f; the corresponding azide analog was inactive (Figure S1d-e)). As bulky substituents at the nitrogen of the pyrrole moiety impaired inhibitory activity (Figure S1a), the linker was attached to the carboxylic acid. To allow quantification of enriched proteins, we employed stable isotope labeling of amino acids in cell culture (SILAC)[8]. To this end, probes were immobilized on solid support and the active probe **2** was incubated with normal ("light") HCT116 cell lysate, whereas the control probe was exposed to ^{13}C- and ^{15}N-labeled ("heavy") HCT116 cell lysate (and vice versa). Beads enriched with proteins by **2** and the control probe were mixed prior to identification of bound proteins by means of mass spectrometry. Probe **2**, but not the control probe significantly enriched deoxycytidine-triphosphate (dCTP) pyrophosphatase 1 (dCTPP1) and AIFM2 (Data Sheets S1-2) with SILAC ratios of >13:1. dCTPP1 is an all-α-nucleoside triphosphate (NTP) pyrophosphatase that eliminates non-canonical NTPs, thus acting as a house-keeping nucleotide hydrolase, which preserves genomic integrity[9]. It catalyzes the hydrolysis of dNTPs to their corresponding monophosphates and displays higher affinity towards 5-modified non-canonical dNTPs (e.g., 5-I-dCTP>5-Br-dCTP>5-Me-dCTP>dCTP), thereby decreasing their intracellular levels and hence subsequently their insertion into nascent DNA during replication[9-10]. dCTPP1 is considered an attractive anti-cancer target, because it is overexpressed in many carcinomas (e.g., breast and gastric)[11].

Reversible binding of dCTPP1 to probe **2** was confirmed by means of concentration-dependent competitive pull-down using immunoblotting for dCTPP1 (Figure 2b). Physical engagement of dCTPP1 by Pyrcoumin in a complex cellular matrix was proven by means of the cellular thermal shift assay (CETSA) in HCT116 and HEK239T cell lysates[12]. Pyrcoumin induced thermal stabilization of dCTPP1 with a shift in melting temperature of 6 °C (Figure 2c-d, and Figure S2). In isothermal mode at 52 °C, Pyrcoumin dose-dependently stabilized dCTPP1 with a half-maximal effective concentration (EC_{50}) of 5.9 μM (Figure 2e-f). These results demonstrate engagement of dCTPP1 by the compound in cellular lysate.

10.1002/anie.201905977

In contrast, AIFM2 enrichment by **2** could not be confirmed using immunoblotting after affinity pull-down, and binding of Pyrcoumin to AIFM2 could not be detected by differential scanning fluorimetry (DSF) and CETSA in HCT116 lysates (Figure S3). Thus, AIMF2 was not further investigated as potential target.

dCTPP1 has not been linked to Wnt signaling. To analyze the role of dCTPP1, we depleted dCTPP1 by means of short interfering RNA (siRNA) in HEK293T cells by approx. 98% (Figure 2g) which led to a 50% reduction in the Wnt-responsive reporter gene activity (Figure 2h). Thus, depletion of dCTPP1 phenocopies the effect of Pyrcoumin and further confirms dCTPP1 as novel positive regulator of the Wnt/β-catenin pathway.

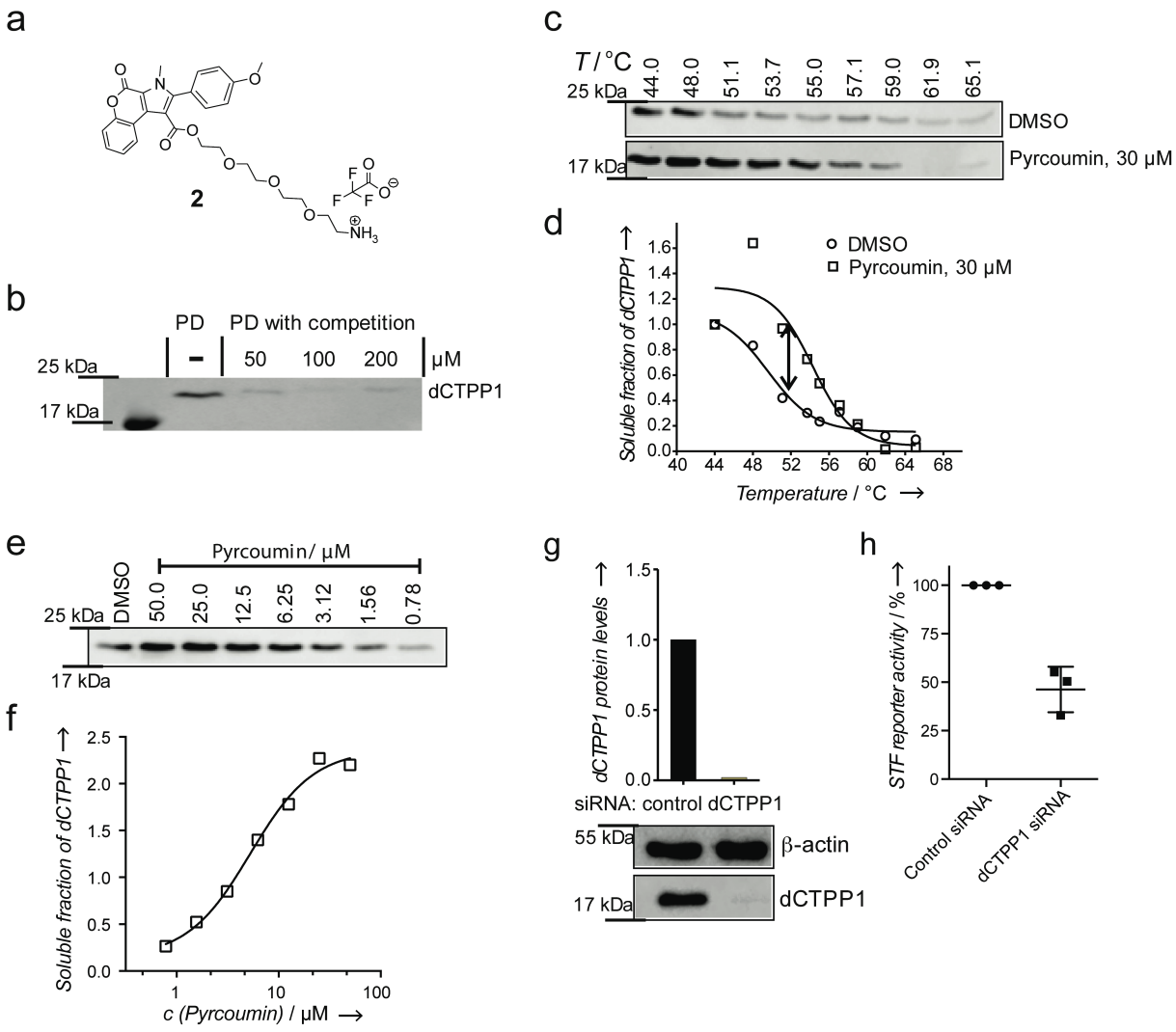


Figure 2: dCTPP1 is the target of Pyrcoumin. (a) Structure of the active probe **2** used in the affinity pull-down (PD) in HCT116 cells. (b) Immunodetection of dCTPP1

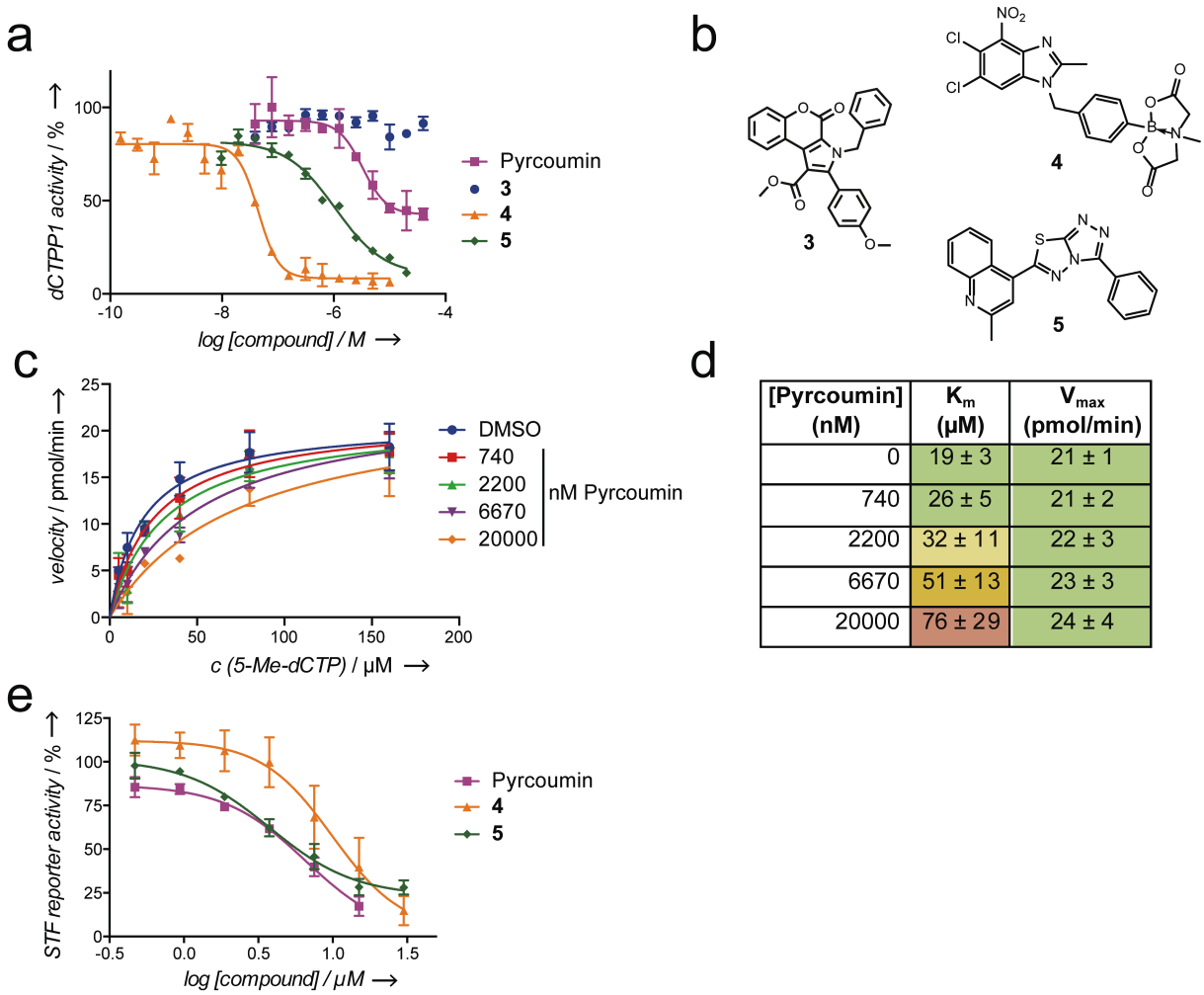
10.1002/anie.201905977

binding to immobilized probe **2** using HCT116 cell lysates pre-incubated with Pyrcoumin. Uncropped blot in Figure S5b. (c) Representative immunoblots of CETSA for dCTPP1 in HCT116 cell lysates treated with 30 μM of Pyrcoumin or DMSO (n = 3). Uncropped blots in Figure S5c. (d) Quantification of band intensities from (c), which were normalized to the intensity of the band at 44 °C. (e) Representative immunoblots of CETSA-based isothermal dose-response for dCTPP1 in HCT116 cell lysates treated with Pyrcoumin or DMSO and incubated at 52 °C (n = 3). Uncropped blot in Figure S5d. (f) Quantification of band intensities from (e), which were normalized to the band intensity of the control cells. (g) Quantification of dCTPP1 knockdown. HEK293T cells were transiently transfected with siRNA targeting dCTPP1 or control siRNA for 48 h, and 22 h later harvested for immunoblotting (n = 3). Uncropped blots in Figure S5e. (h) Wnt-responsive reporter gene assay in HEK293T cells transiently transfected with SuperTOPFlash (STF) plasmid and siRNA targeting dCTPP1 or control siRNA (mean ± S.D., n = 3).

Investigation of dCTPP1 inhibition by Pyrcoumin revealed that the compound partially inhibits (43% residual activity) the enzymatic activity with an IC_{50} value of 3.3 μM (Figure 3a, Table S1, Figure S4a). Inactive derivative **3** (Figure 3b) does not inhibit dCTPP1. dCTPP1 inhibition by the pyrrolocoumarin hits correlated with inhibition of Wnt signaling (Figure S4b, Table S1). Pyrcoumin is a competitive inhibitor of dCTPP1, as it increased the Michaelis-Menten constant K_m of dCTPP1 for its substrate 5-Me-dCTP by four fold without altering the maximum reaction velocity V_{max} (Figure 3c-d).

Different dCTPP1 inhibitors have been developed recently, e.g. benzimidazole **4**[11a] (Figure 3b) (IC_{50} = 44 nM in this study), and triazolothiadiazoles[13] like **5** (Figure 3b) (IC_{50} = 1.1 μM in this study) (Figure 3a). Both compounds inhibited the Wnt-responsive reporter gene activity in HEK293T cells with IC_{50} values of 10.1 μM and 4.1 μM, respectively (Figure 3e, Figure S4c). These findings demonstrate that dCTPP1 inhibitors with different chemotypes inhibit Wnt signaling and indicate that dCTPP1 may play a role in Wnt pathway regulation.

10.1002/anie.201905977



[Pyrcoumin] (nM)	K_m (µM)	V_{max} (pmol/min)
0	19 ± 3	21 ± 1
740	26 ± 5	21 ± 2
2200	32 ± 11	22 ± 3
6670	51 ± 13	23 ± 3
20000	76 ± 29	24 ± 4

Figure 3: Pyrcoumin is a competitive inhibitor of dCTPP1. (a) dCTPP1 enzymatic assay in presence of Pyrcoumin, the inactive derivative **3**, benzimidazole **4** and triazolothiadiazole **5** (mean ± S.D., n = 3). IC_{50} values are 3.3 ± 0.3 µM for Pyrcoumin, 44 ± 3 nM for **4** and 1.07 ± 0.03 µM for **5**. (b) Structures of pyrrolocoumarin **3** and dCTPP1 inhibitors **4** and **5**. (c) Michaelis-Menten plot of dCTPP1 in presence of Pyrcoumin or DMSO determined with dCTPP1 enzymatic assay (mean ± S.D., n = 3). (d) Michaelis-Menten constant K_m and maximum reaction velocity V_{max} of dCTPP1 in presence of Pyrcoumin or DMSO calculated from (c). (e) Wnt-responsive reporter gene assay in HEK293T cells transiently transfected with Wnt-3a and SuperTOPFlash (STF) plasmids, treated with Pyrcoumin, **4** or **5** for 24 h (mean ± S.D., n = 3).

10.1002/anie.201905977

dCTPP1 has not been linked to Wnt signaling before and a STRING analysis for known interaction partners[14] did not reveal a link either. To establish a connection between dCTPP1 and Wnt signaling, we performed co-immunoprecipitation coupled to mass spectrometry using HCT116 cells that expressed FLAG-tagged dCTPP1. Ubiquitin carboxyl-terminal hydrolase 7 (USP7) and 3-hydroxyacyl CoA dehydrogenase type-2 (HSD17B10) co-purified with dCTPP1 (Data Sheets S3-4). HSD17B10 has not been linked to Wnt signaling either. However, the deubiquitinating enzyme (DUB) family member USP7 mediates deubiquitination and prevents proteasomal degradation of tumor suppressors and oncogenes[15], and is involved directly or indirectly in Wnt signaling. Thus, USP7 acts as positive feedback regulator of Wnt by enhancing the stability of β-catenin, and its knockdown impairs Wnt signaling[16].

Co-immunoprecipitation in HCT116 cell lysates using anti-dCTPP1 antibody-coated beads confirmed the interaction between dCTPP1 and USP7 (Figure 4a). In addition, pull-down and gel filtration shift assays using recombinant proteins confirmed a direct dCTPP1-USP7 interaction (Figure 4b-d). Fluorescence polarization measurements revealed a K_d of 1.2 μM for binding of fluorescein-labeled dCTPP1 to USP7 (Figure 4e), and Pyrcoumin concentration dependently weakened this interaction (K_d = 10.5 μM for 20 μM Pyrcoumin and 5.5 μM for 3 μM Pyrcoumin). By analogy, dCTPP1 inhibitors with different chemotypes impaired the dCTPP1-USP7 interaction (Figure 4f, K_d = 19.1 μM for 20 μM **4** and 9.5 μM for 20 μM **5**), whereas the inactive pyrrolocoumarin **3** had no effect on dCTPP1-USP7 complex formation (Figure S4d). Notably, the dCTPP1 substrate 5-Me-dCTP also compromised dCTPP1 binding to USP7 (Figure 4f, K_d = 5.9 μM for 100 μM 5-Me-dCTP), hence, the binding event as such and its consequences, and not primarily the inhibition itself may be at the heart of the inhibition of the protein-protein interaction, e.g. by modulation of the protein conformation. These findings suggest that the dCTPP1-USP7 interaction may be functionally relevant to Wnt signaling and that it is amenable to small-molecule perturbation.

10.1002/anie.201905977

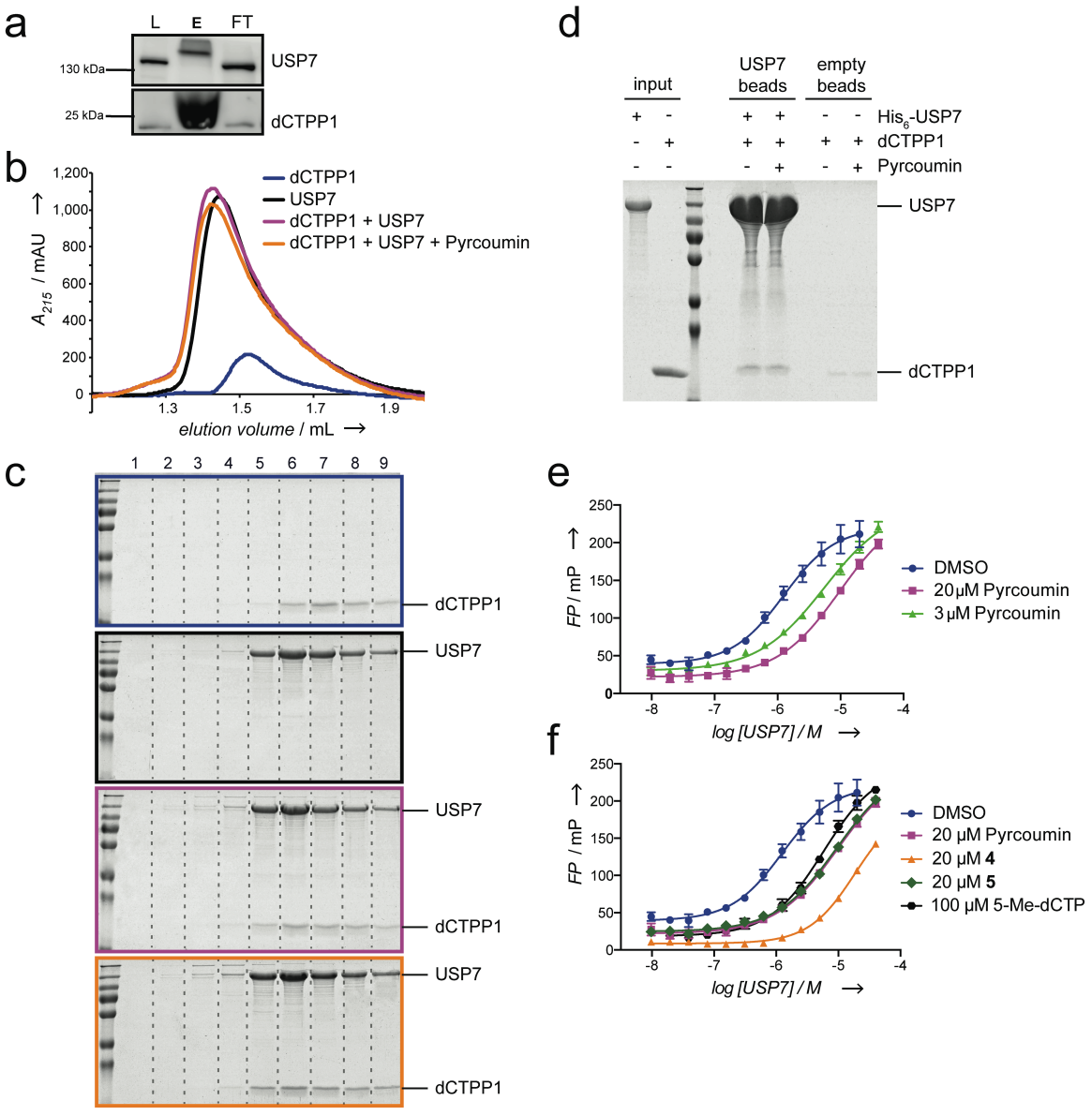


Figure 4: Pyrcoumin impairs the direct interaction of dCTPP1 and USP7. (a) Co-immunoprecipitation of dCTPP1 and USP7 in HCT116 cell lysate using anti-dCTPP1 antibody-coated beads (n = 3). L: HCT116 lysate, E: eluate with dCTPP1-bound proteins, FT: flow-through. Uncropped blots in Figure S5f. (b) dCTPP1 co-elutes with USP7 in gel filtration shift assays with recombinant USP7 and dCTPP1 in presence of 20 µM Pyrcoumin or DMSO. Representative gel filtration chromatograms. (c) Representative gel images of respective gel filtration runs from (b). (d) Pull-down assays with recombinant His_6-USP7 and dCTPP1 in presence of 20 µM Pyrcoumin or DMSO (n = 3). (e) Fluorescence polarization assays using 20 nM fluorescein-labeled dCTPP1 and USP7 in presence of Pyrcoumin or DMSO (mean ± S.D., n = 3). K_d

values are 1.2 ± 0.1 μM for DMSO control, 10.5 ± 2.8 μM for 20 μM Pyrcoumin, 5.5 ± 0.6 μM for 3 μM Pyrcoumin. (f) Fluorescence polarization assays using 20 nM fluorescein-labeled dCTPP1 and USP7 in presence of Pyrcoumin, **4**, **5**, 5-Me-dCTP or DMSO (mean ± S.D., n = 3). K_d values are 1.2 ± 0.1 μM for DMSO control, 10.5 ± 2.8 μM for 20 μM Pyrcoumin, 19.1 ± 2.7 μM for 20 μM **4**, 9.5 ± 0.5 μM for 20 μM **5**, 5.9 ± 0.9 μM for 100 μM 5-Me-dCTP.

Through a cell-based assay and subsequent target identification and validation we have discovered the new Wnt pathway inhibitor Pyrcoumin and shown that it targets the nucleotide pyrophosphatase dCTPP1. dCTPP1 is a novel positive regulator of Wnt signaling. Pyrcoumin partially inhibits the enzymatic activity of dCTPP1 in a substrate competitive manner and both Pyrcoumin, and structurally unrelated dCTPP1 inhibitors, as well as 5-Me-dCTP modulate the interaction of dCTPP1 with its newly identified binding partner USP7. These findings suggest that impaired complex formation with USP7 may result from a conformational change of dCTPP1 upon inhibitor binding and that this indirect interference with the dCTPP1-USP7 interaction might be at the heart of Wnt pathway inhibition by dCTPP1 inhibitors.

Acknowledgments

This research was supported by the Max Planck Gesellschaft and the European Research Council under the European Union's Seventh Framework Programme (FP7/2007-2013) / ERC Grant agreement n°268309. We thank Dr. Sonja Sievers and the Compound Management and Screening Center (COMAS), Dortmund, Germany, for screening; the Dortmund protein facility for cloning of nFLAG-tagged dCTPP1; Andreas Brockmeyer and Malte Metz for mass spectrometry; Jana Flegel and Michele Saretzki for WST assays; Hacer Karatas for help with chemical synthesis; Dennis Schade for the protocol for the STF reporter gene assay and Beate Schölermann and Christine Nowak for technical assistance.

References

[1] R. Nusse, H. Clevers, *Cell* **2017**, *169*, 985-999.

[2] J. L. Stamos, W. I. Weis, *Cold Spring Harb Perspect Biol* **2013**, *5*, a007898.

[3] B. T. MacDonald, K. Tamai, X. He, *Dev Cell* **2009**, *17*, 9-26.

[4] X. Cheng, X. Xu, D. Chen, F. Zhao, W. Wang, *Biomed Pharmacother* **2019**, *110*, 473-481.

[5] J. N. Anastas, R. T. Moon, *Nat Rev Cancer* **2013**, *13*, 11-26.

[6] S. Park, J. Gwak, M. Cho, T. Song, J. Won, D. E. Kim, J. G. Shin, S. Oh, *Mol Pharmacol* **2006**, *70*, 960-966.

[7] P. J. Morin, A. B. Sparks, V. Korinek, N. Barker, H. Clevers, B. Vogelstein, K. W. Kinzler, *Science* **1997**, *275*, 1787-1790.

[8] S. E. Ong, B. Blagoev, I. Kratchmarova, D. B. Kristensen, H. Steen, A. Pandey, M. Mann, *Mol Cell Proteomics* **2002**, *1*, 376-386.

[9] a) C. E. Requena, G. Perez-Moreno, L. M. Ruiz-Perez, A. E. Vidal, D. Gonzalez-Pacanowska, *Biochem J* **2014**, *459*, 171-180; b) F. F. Song, L. L. Xia, P. Ji, Y. B. Tang, Z. M. Huang, L. Zhu, J. Zhang, J. Q. Wang, G. P. Zhao, H. L. Ge, Y. Zhang, Y. Wang, *Oncogenesis* **2015**, *4*, e159.

[10] R. Holliday, T. Ho, *Somat Cell Mol Genet* **1991**, *17*, 537-542.

[11] a) S. Llona-Minguez, A. Hoglund, S. A. Jacques, L. Johansson, J. M. Calderon-Montano, M. Claesson, O. Loseva, N. C. K. Valerie, T. Lundback, J. Piedrafita, G. Maga, E. Crespan, L. Meijer, E. B. Moron, P. Baranczewski, A. L. Hagbjork, R. Svensson, E. Wiita, I. Almlof, T. Visnes, F. Jeppsson, K. Sigmundsson, A. J. Jensen, P. Artursson, A. S. Jemth, P. Stenmark, U. W. Berglund, M. Scobie, T. Helleday, *J Med Chem* **2016**, *59*, 1140-1148; b) Y. Zhang, W. Y. Ye, J. Q. Wang, S. J. Wang, P. Ji, G. Y. Zhou, G. P. Zhao, H. L. Ge, Y. Wang, *Eur J Histochem* **2013**, *57*, e29.

[12] D. M. Molina, R. Jafari, M. Ignatushchenko, T. Seki, E. A. Larsson, C. Dan, L. Sreekumar, Y. Cao, P. Nordlund, *Science* **2013**, *341*, 84-87.

[13] S. Llona-Minguez, A. Hoglund, E. Wiita, I. Almlof, A. Mateus, J. M. Calderon-Montano, C. Cazares-Korner, E. Homan, O. Loseva, P. Baranczewski, A. S. Jemth, M. Haggblad, U. Martens, B. Lundgren, P. Artursson, T. Lundback, A. Jenmalm Jensen, U. Warpman Berglund, M. Scobie, T. Helleday, *J Med Chem* **2017**, *60*, 2148-2154.

[14] D. Szklarczyk, A. L. Gable, D. Lyon, A. Junge, S. Wyder, J. Huerta-Cepas, M. Simonovic, N. T. Doncheva, J. H. Morris, P. Bork, L. J. Jensen, C. V. Mering, *Nucleic Acids Res* **2019**, *47*, D607-D613.

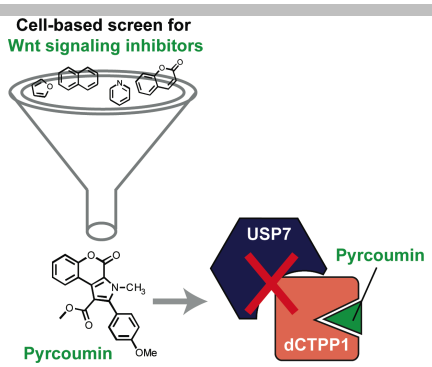
[15] a) K. W. Cheon, K. H. Baek, *Int J Oncol* **2006**, *28*, 1209-1215; b) M. Li, C. L. Brooks, N. Kon, W. Gu, *Mol Cell* **2004**, *13*, 879-886.

[16] P. Ma, X. Yang, Q. Kong, C. Li, S. Yang, Y. Li, B. Mao, *Mol Cell Biol* **2014**, *34*, 4355-4366.

10.1002/anie.201905977

COMMUNICATION

A cell-based screen for inhibitors of canonical Wnt signaling identified the pyrrolocoumarin Pyrcoumin, a new competitive inhibitor of dCTP pyrophosphatase 1 (dCTPP1). Pyrcoumin counteracts the yet unknown interaction of dCTPP1 with ubiquitin carboxyl-terminal hydrolase (USP7).



*Alexandra Friese, Shobhna Kapoor, Tabea Schneidewind, Srinivasa Rao Vidadala, Juhi Sardana, Alexandra Brause, Tim Förster, Matthias Bischoff, Jessica Wagner, Petra Janning, Slava Ziegler, and Herbert Waldmann**

Chemical genetics reveals a role of dCTP pyrophosphatase 1 in Wnt signaling

Keywords: natural products, protein-protein interactions, small-molecule inhibitors, target identification, Wnt pathway