Human Peroxisomal Coenzyme A Diphosphatase NUDT7 (NUDT7)

A Target Enabling Package (TEP)

Gene ID / UniProt ID / EC	826884 / P0C024 / EC 3.6.1.-
Target Nominator	SGC Internal Nomination
SGC Authors	Srikannathasan Velupillai, Laura Díaz Sáez, Tobias Krojer, James Bennett, Gian Filippo Ruda, Tamas Szommer, Verena Straub, Graciela Nunez Alonso, Paulina Siejka, Anthony Bradley, Romain Talon, Michael Fairhead, Jon Elkins, Frank von Delft, Oleg Fedorov, Paul Brennan, Kilian Huber
Collaborating Authors	Nir London[1], John Spencer[2]
Target PI	Dr Kilian Huber (SGC Oxford)
Therapeutic Area(s)	Metabolic diseases
Disease Relevance	NUDT7 is linked to regulation of CoA homeostasis
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SUMMARY OF PROJECT

In an effort to characterise the human NUDIX family SGC Oxford has expressed recombinant human NUDT7 as part of the SGC chemical probe programme and solved the first crystal structure of this enzyme. This enabled a crystallographic fragment screen which in conjunction with a separate covalent fragment approach yielded a first-in-class small molecule inhibitor of NUDT7 with activity in the single-digit micromolar range in a catalytic assay. This compound paves the way for chemical probe development and further functional exploration of NUDT7 in physiological and disease contexts.

SCIENTIFIC BACKGROUND

NUDT7 is part of a protein family characterised by a 23-amino acid motif referred to as the 'NUDIX box'. These proteins have been reported to hydrolyse a diverse range of substrates including (d)NTPs, nucleotide sugars, diadenosine polyphosphates as well as capped RNA and dinucleotide coenzymes (1). NUDT7 is a peroxisomal CoA pyrophosphohydrolase with additional distinct sequence features such as a CoA-binding motif (NUDIX CoA signature, UPF0035) and a C-terminal peroxisomal targeting signal (PTS) (2,3). Expression of NUDT7 is highest in liver, with NUDT19 likely acting as the complementary CoA and CoA ester hydrolase in kidney (4). Leptin double knockout mice, which display alterations in CoA homeostasis and exhibit a diabetic phenotype, have been reported to express reduced levels of Nudt7 with a concomitant increase in pantothenate kinase activity (5). Conversely, neuronal overexpression of a cytosolic version of Nudt7 in mice leads to a reduction in motor coordination reminiscent of pantothenate kinase-associated neuro-degeneration (PKAN), a neurodegenerative disease linked to reduced CoA levels (6).

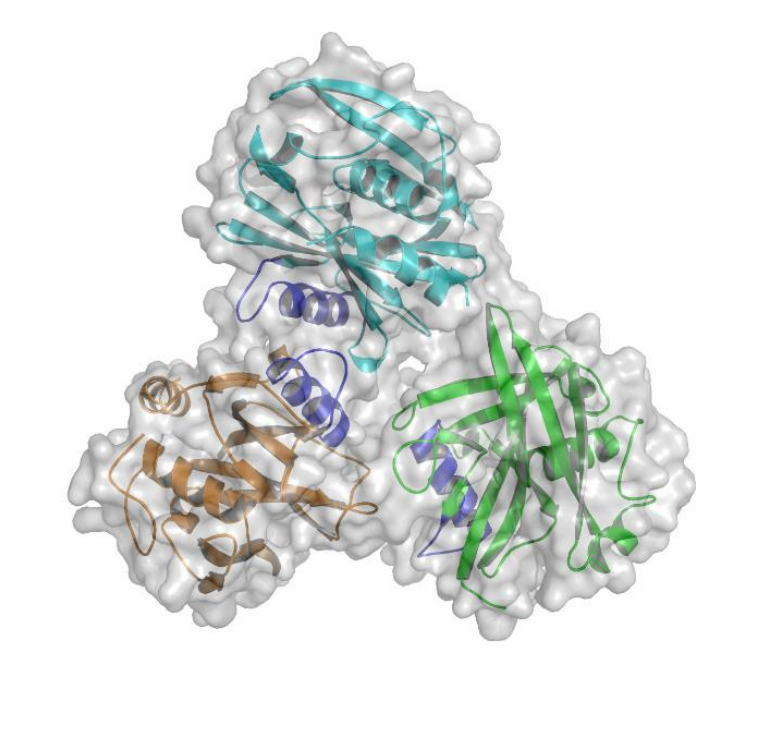
RESULTS – THE TEP

Protein Production

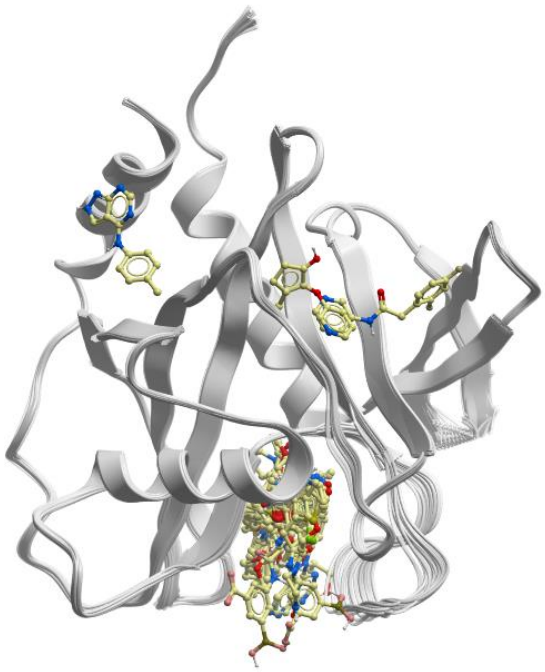
Recombinant human NUDT7 (residues 14-235) was expressed in *E. coli*.

Structures

Several high resolution crystal structures (1.52-2.21 Å) of human NUDT7 including apo and fragment-bound structures have been obtained and deposited in the PDB.



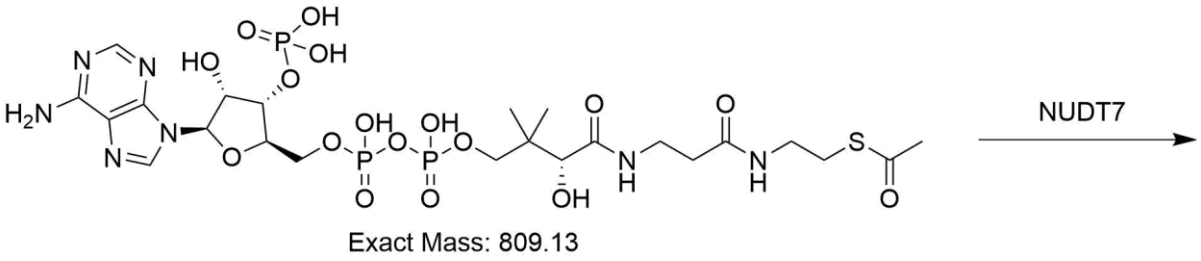
First crystal structure of hNUDT7 (2.03 Å, PDB: 5T3P)



Overview of NUDT7 subunit with bound fragments

Assays

To confirm activity of the recombinant protein and enable chemical probe development we established a mass spectrometry-based assay to monitor acetyl-CoA hydrolysis by NUDT7.



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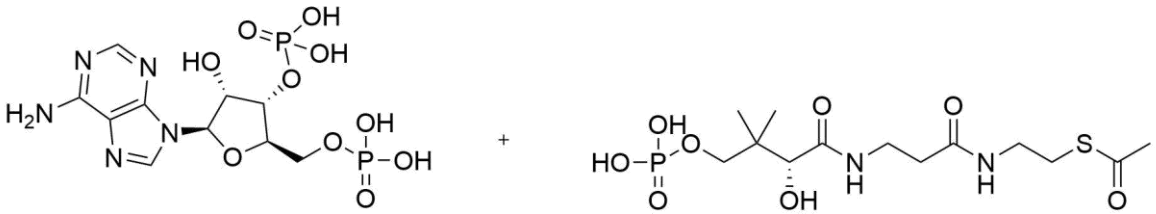
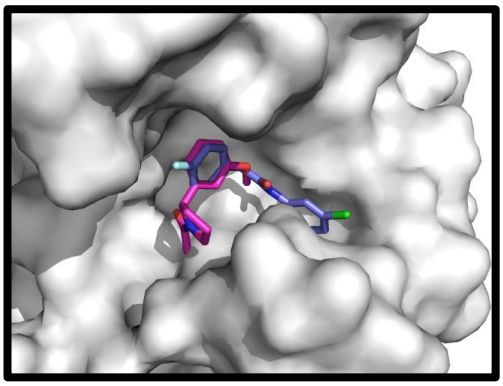
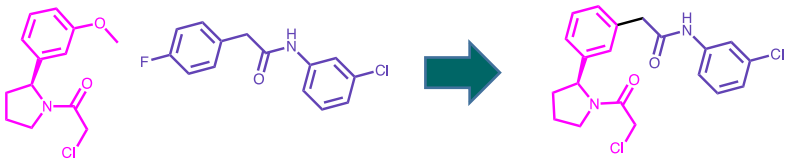


Exact Mass: 427.03 + Exact Mass: 400.11

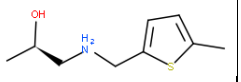
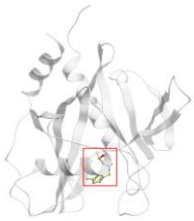
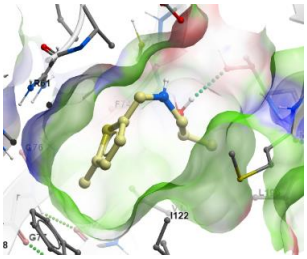
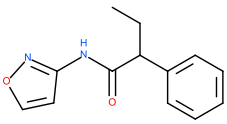
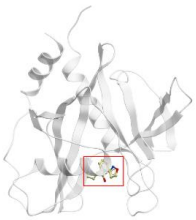
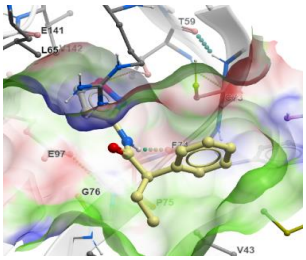
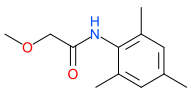
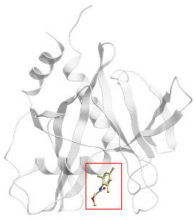
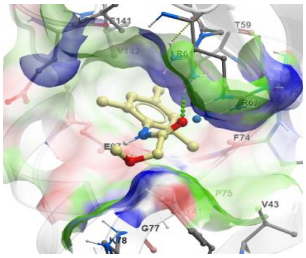
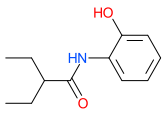
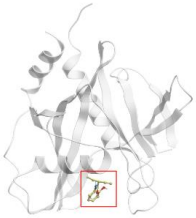
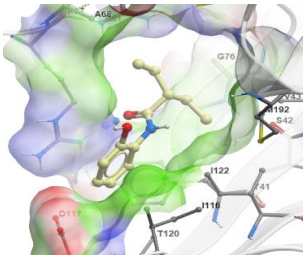
Chemical Matter

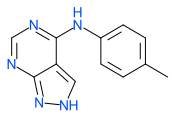
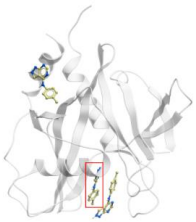
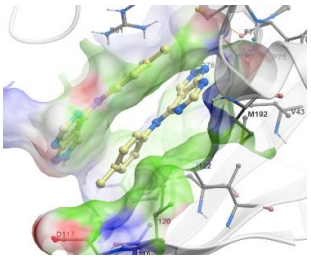
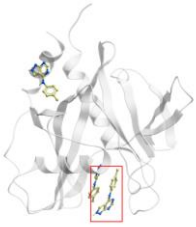
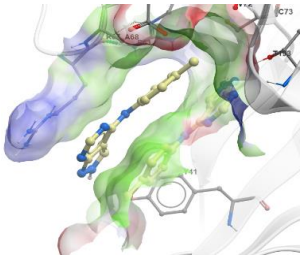
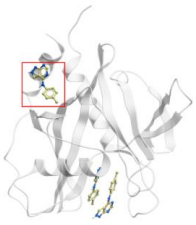
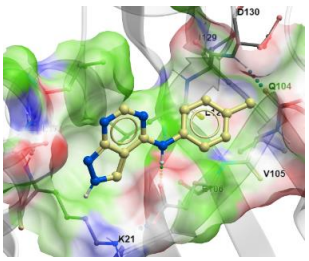
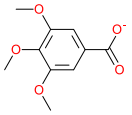
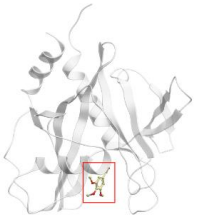
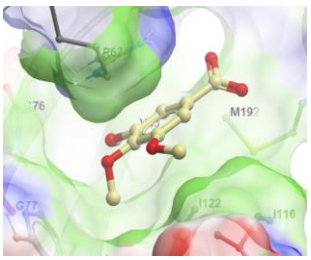
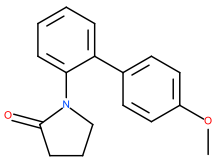
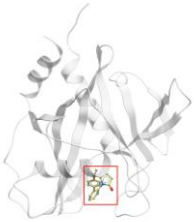
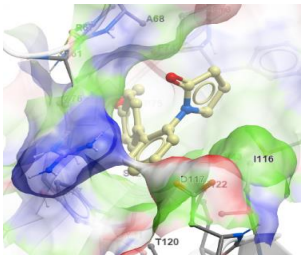
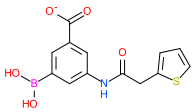
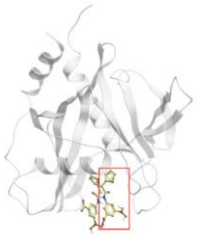
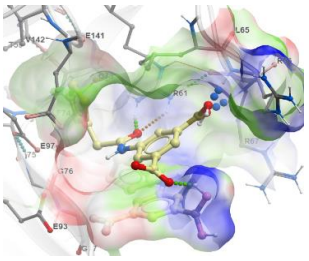
A crystallographic fragment soaking campaign using XChem delivered several hits from chemical different libraries which were optimised to compounds with single-digit micromolar activity.

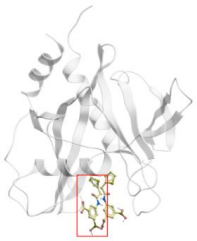
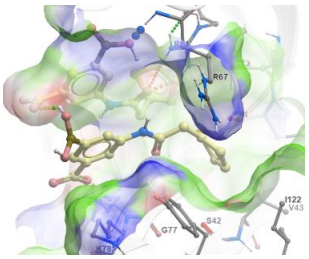
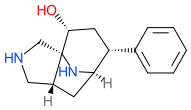
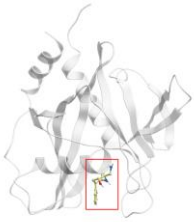
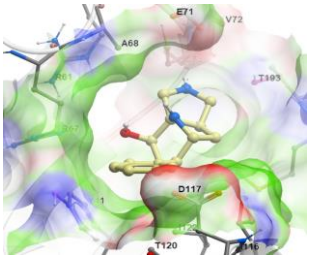
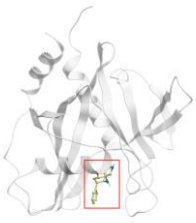
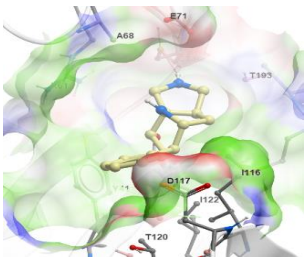
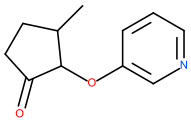
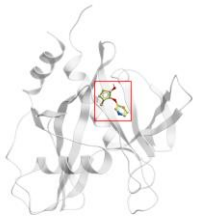
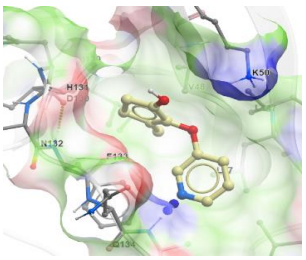
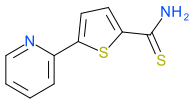
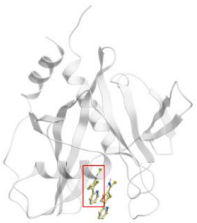
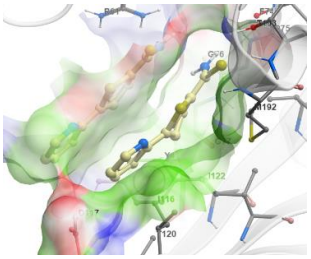
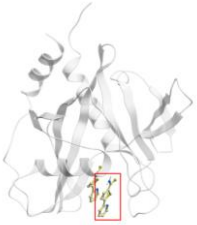
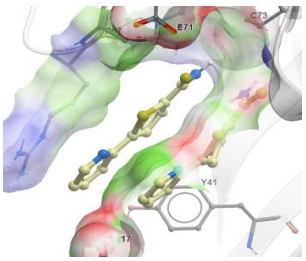


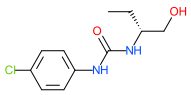
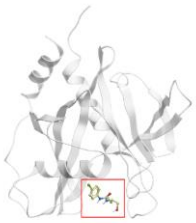
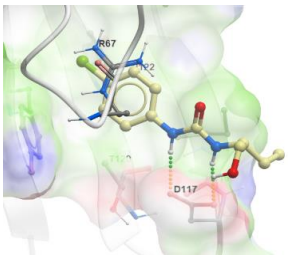
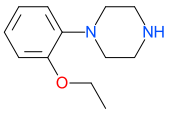
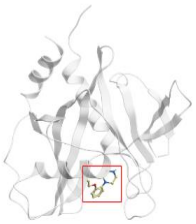
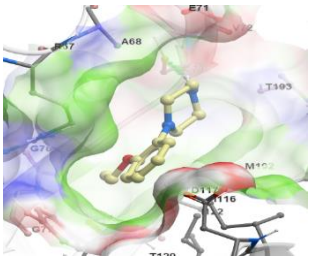
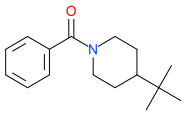
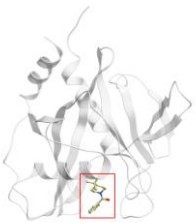
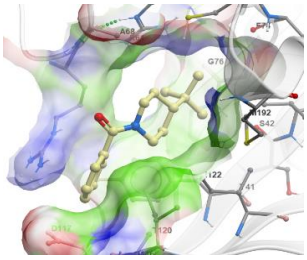
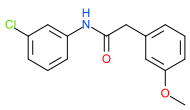
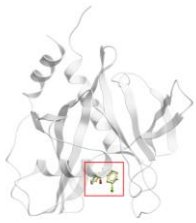
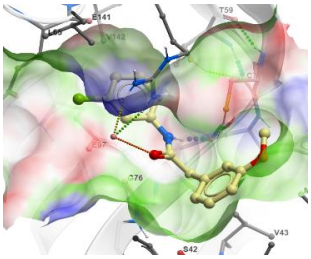
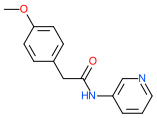
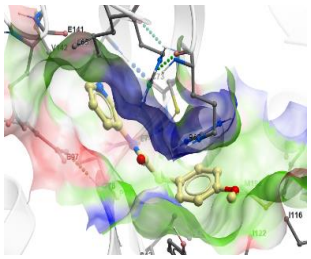
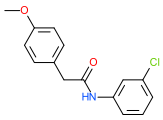
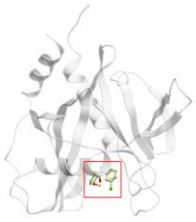
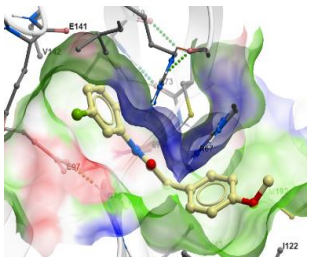
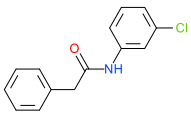
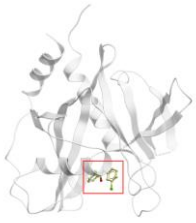
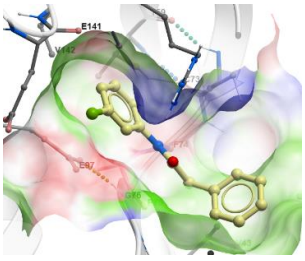


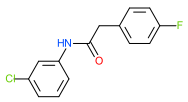
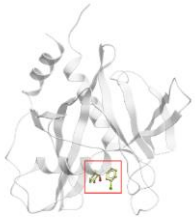
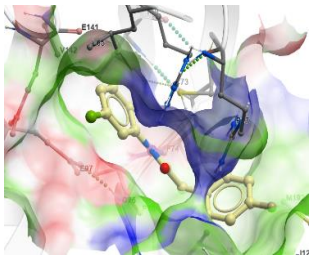
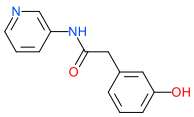
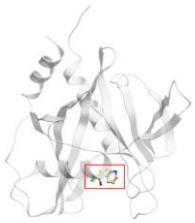
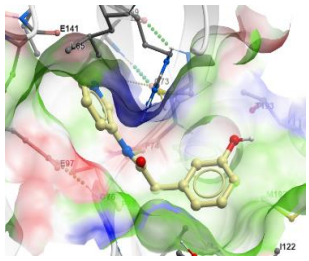
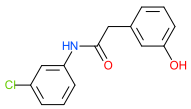
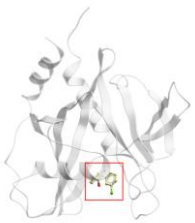
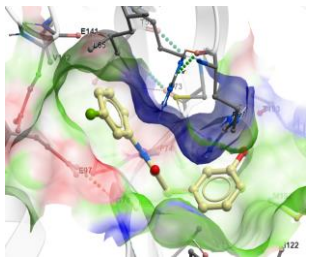
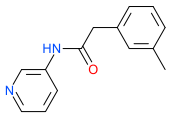
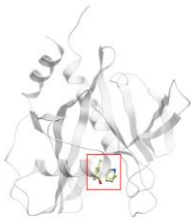
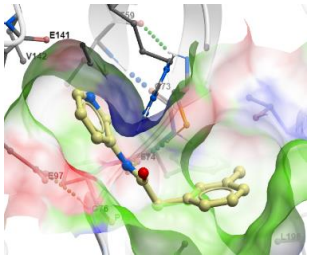
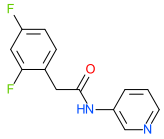
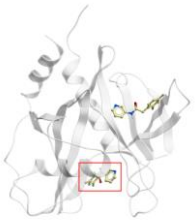
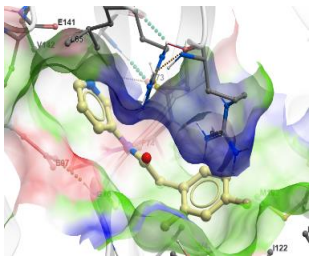
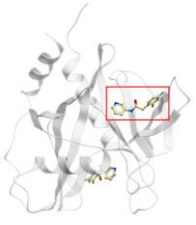
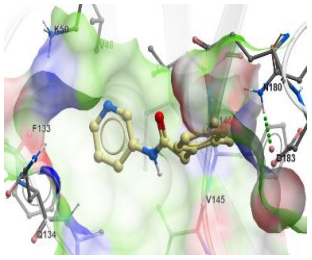
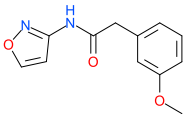
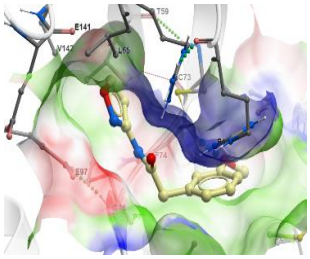
Superimposition of fragment crystal structures and covalent inhibitor lead derived from fragment merging (IC_{50} value 1-3 µM, LE value 0.29-0.32)

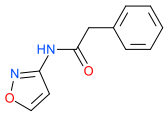
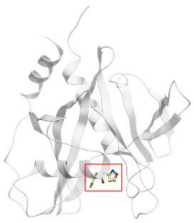
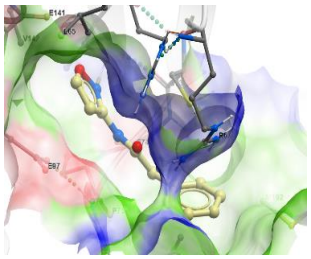
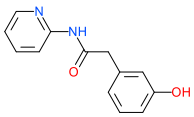
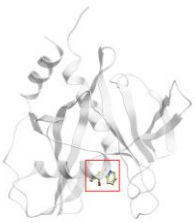
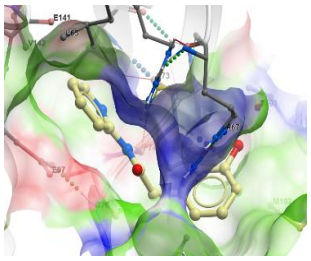
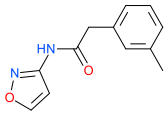
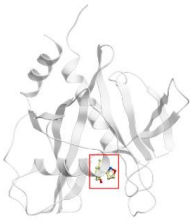
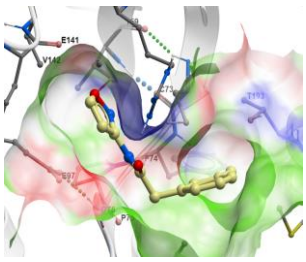
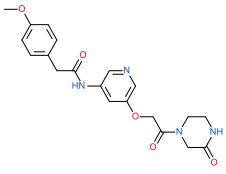
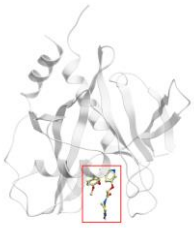
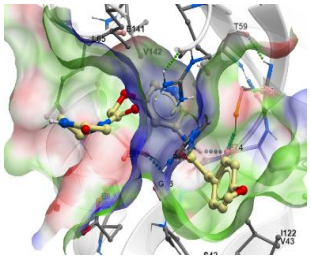
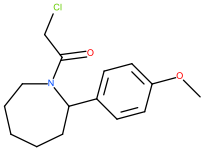
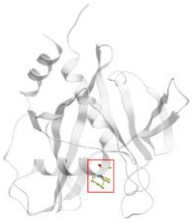
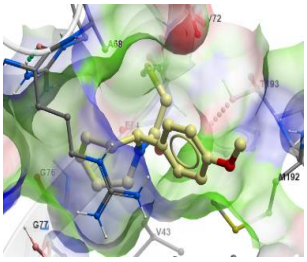
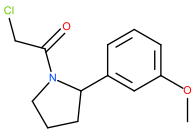
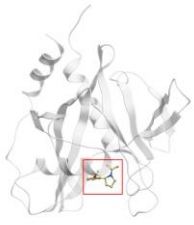
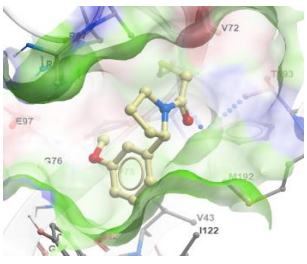
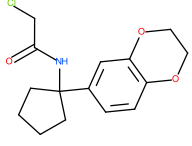
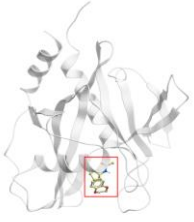
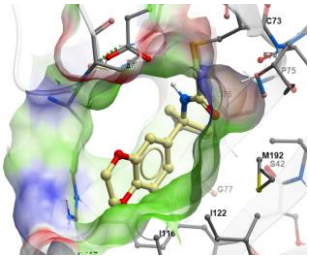
PDBID	Ligand	Binding Location	Binding pocket	Resolution (Å)
5QGG	 FM002318a			1.91
5QGH	 FM010069a			1.82
5QGI	 FM002204a			1.95
5QGJ	 FM002197a			1.95

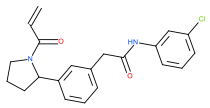
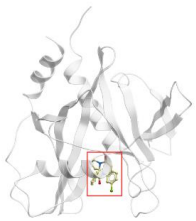
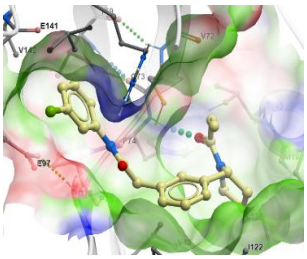
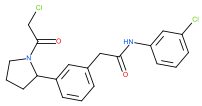
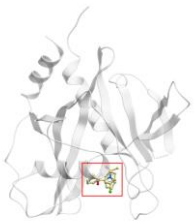
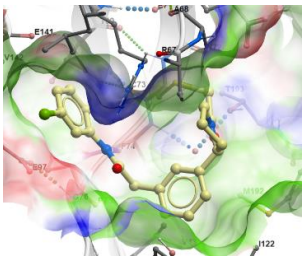
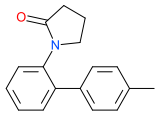
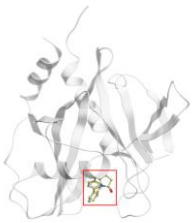
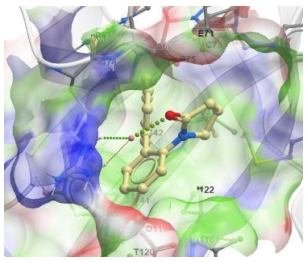
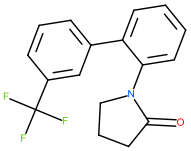
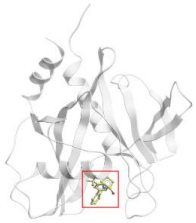
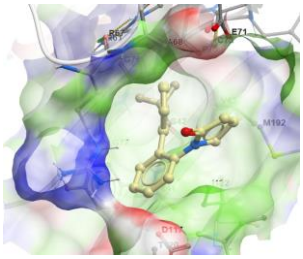
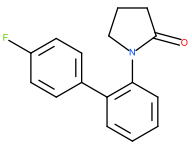
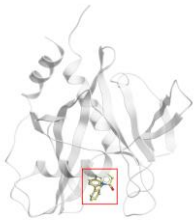
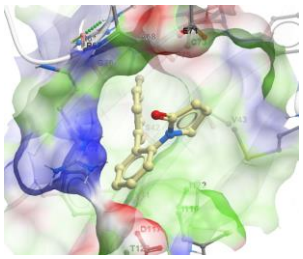
5QGK	 FM001984a	Location 1 		1.81
		Location 2 		
		Location 3 		
5QGL	 XS022626c			2.27
5QGM	 FM010686a			1.95
5QGN	 FM010687a	Location 1 		1.95

		Location 2 		
5QGO, 5QGP	 NU000598b, NU000598c	5QGO 		1.82
		5QGP 		2.09
5QGQ	 FM010688a			1.95
5QGR	 FM001790a	Location 1 		1.96
		Location 2 		

5QGS	 FM001898a			1.55
5QGT	 FM001898a			1.97
5QGU	 FM002048a			1.71
5QGV	 XS081083b			1.59
5QGW	 NU000082a			1.94
5QGX	 NU000083a			1.61
5QGY	 NU000090a			1.72

5QGZ	 NU000098a			1.65
5QH0	 NU000135a			1.57
5QH1	 NU000087a			1.65
5QH2	 NU000088a			1.74
5QH3	 NU000154a	Location 1 		1.65
		Location 2 		
5QH4	 NU000057a			1.67

5QH5	 NU000073a			1.85
5QH6	 NU000056a			1.57
5QH7	 NU000160a			1.74
5QHF	 NU000004a			1.67
5QH8	 NU000792a			1.75
5QH9	 NU000795a			1.72
5QHA	 NU000794a			1.57

5QHG	 NU000442a			1.92
5QHH	 NU000443a			1.52
5QHB	 NU000610a			1.57
5QHC	 NU000612a			2.21
5QHE	 NU000621a			1.74

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

Future plans

Looking forward, we plan to develop a chemical probe for NUDT7 based on the current lead molecule.

Collaborations

We would like to thank our collaborators Nir London (The Weizmann Institute of Science) and John Spencer (University of Sussex) on their work on NUDT7 inhibitor development.

CONCLUSION

We have generated active recombinant protein, developed a functional assay, and solved the first crystal structures of human NUDT7 in apo form and in complex with first-in-class chemical starting points enabling further inhibitor development and functional exploration of NUDT7.

TEP IMPACT

Publications arising from this work

Shumar, S. A.; Kerr, E. W.; Geldenhuys, W. J.; Montgomery, G. E.; Fagone, P.; Thirawatananond, P.; Saavedra, H.; Gabelli, S. B.; Leonardi, R., Nudt19 is a renal CoA diphosphohydrolase with biochemical and regulatory properties that are distinct from the hepatic Nudt7 isoform. *J. Biol. Chem.* **2018,** *293* (11), 4134-4148.

This publication describes the development of the NUDT7 inhibitor lead reported in the TEP using a combination of crystallographic and intact MS-based fragment screening.

- Resnick, E.; Bradley, A.; Gan, J.; Douangamath, A.; Krojer, T.; Sethi, R.; Geurink, P. P.; Aimon, A.; Amitai, G.; Bellini, D.; Bennett, J.; Fairhead, M.; Fedorov, O.; Gabizon, R.; Gan, J.; Guo, J.; Plotnikov, A.; Reznik, N.; Ruda, G. F.; Díaz-Sáez, L.; Straub, V. M.; Szommer, T.; Velupillai, S.; Zaidman, D.; Zhang, Y.; Coker, A. R.; Dowson, C. G.; Barr, H. M.; Wang, C.; Huber, K. V. M.; Brennan, P. E.; Ovaa, H.; von Delft, F.; London, N., Rapid Covalent-Probe Discovery by Electrophile-Fragment Screening. *J. Am. Chem. Soc.* **2019**, 141 (22), 8951-8968.

Kahn Tareque, R.; Hassell-Hart, S.; Krojer, T.; Bradley, A.; Velupillai, S.; Talon, R.; Fairhead, M.; Day, I. J.; Bala, k.; felix, R.; kemmitt, P.; Brennan, P.; von Delft, f.; Díaz Sáez, L.; Spencer, J.; Huber, K., Deliberately Losing Control of C-H Activation Processes in the Design of Small Molecule Fragment Arrays Targeting Peroxisomal Metabolism. *ChemMedChem* **2020** (in press).

FUNDING INFORMATION

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ADDITIONAL INFORMATION

Structure Files

PDB ID	Structure Details	Library/ Series
5T3P	Structure of Human Peroxisomal coenzyme A diphosphatase NUDT7	
5QGG	NUDT7 in complex with fragment	DSPL
5QGH	NUDT7 in complex with fragment	DSPL
5QGI	NUDT7 in complex with fragment	DSPL
5QGJ	NUDT7 in complex with fragment	DPSL
5QGK	NUDT7 in complex with fragment	DSPL
5QGL	NUDT7 in complex with fragment	DSPL
5QGM	NUDT7 in complex with fragment	OxXChem
5QGN	NUDT7 in complex with fragment	OxXChem
5QGO	NUDT7 in complex with fragment	OxXChem
5QGP	NUDT7 in complex with fragment	OxXChem
5QGQ	NUDT7 in complex with fragment	DSPL
5QGR	NUDT7 in complex with fragment	DSPL
5QGS	NUDT7 in complex with fragment	DSPL
5QGT	NUDT7 in complex with fragment	DSPL
5QGU	NUDT7 in complex with fragment	DPSL
5QGV	NUDT7 in complex with fragment	3-chloro-phenylamino
5QGW	NUDT7 in complex with fragment	3-chloro-phenylamino
5QGX	NUDT7 in complex with fragment	3-chloro-phenylamino
5QGY	NUDT7 in complex with fragment	3-chloro-phenylamino
5QGZ	NUDT7 in complex with fragment	3-chloro-phenylamino
5QH0	NUDT7 in complex with fragment	3-chloro-phenylamino
5QH1	NUDT7 in complex with fragment	3-chloro-phenylamino
5QH2	NUDT7 in complex with fragment	3-chloro-phenylamino
5QH3	NUDT7 in complex with fragment	3-chloro-phenylamino
5QH4	NUDT7 in complex with fragment	3-chloro-phenylamino
5QH5	NUDT7 in complex with fragment	3-chloro-phenylamino
5QH6	NUDT7 in complex with fragment	3-chloro-phenylamino
5QH7	NUDT7 in complex with fragment	3-chloro-phenylamino
5QHF	NUDT7 in complex with fragment	3-chloro-phenylamino
5QH8	NUDT7 in complex with fragment	London
5QH9	NUDT7 in complex with fragment	London
5QHA	NUDT7 in complex with fragment	London
5QHG	NUDT7 in complex with fragment	FU covalent
5QHH	NUDT7 in complex with fragment	FU covalent
5QHB	NUDT7 in complex with fragment	Pyrrolidine
5QHC	NUDT7 in complex with fragment	Pyrrolidine
5QHE	NUDT7 in complex with fragment	Pyrrolidine

Materials and Methods

Expression and purification of recombinant human NUDT7

Human NUDT7 (residues 14-235) was cloned into pNIC28-Bsa4 with a TEV-cleavable N-terminal hexahistidine tag. After transformation into *E. coli* (BL21(DE3)-R3), expression was performed in TB auto induction medium (FroMedium), supplemented with 20 g/L glycerol, 50 µg/mL kanamycin and 34 µg/mL chloramphenicol. Cultures were grown for four hours at 37 °C, then the temperature was decreased to 20 °C and the cultures were grown for another 20 hours. Cells were spun at 5000 rpm for 10 min, then resuspended in 0.5 mg/mL

lysozyme, 1 µg/mL benzonase, 20 mM imidazole and stirred for 2 hours at room temperature. 1% Triton X-100 was added and the cells were frozen at -80°C. On thawing, cells were centrifuged for 1 hour at 4000 × g and the supernatant applied to a His GraviTrap column (GE healthcare) equilibrated with binding buffer (10 mM HEPES, 5% glycerol, 500 mM NaCl, 0.5 mM TCEP, pH 7.5). After washing with binding buffer supplemented to 20 mM imidazole, NUDT7 was eluted with buffer supplemented to 500 mM imidazole. The eluted protein was applied to a PD-10 desalting column (GE Healthcare) and eluted with binding buffer supplemented to 20 mM imidazole. The N-terminal affinity tag was removed by TEV cleavage overnight and uncleaved protein was removed by applying it again to a His GraviTrap column. The flow-through was concentrated and purified further by size exclusion chromatography using a YARRA SEC-2000 PREP column (Phenomenex) equilibrated with binding buffer. Fractions containing protein were pooled, concentrated and stored at -80°C.

Crystallization

NUDT7 crystals were obtained by mixing 100 nL of 30mg/mL protein in 10 mM Na-HEPES pH 7.5, 500 mM NaCl, 5% glycerol with 50 nL of reservoir solution containing 0.1M BisTris pH 5.5, 0.1M ammonium acetate and 6%(w/v) PEG10.000. Compact, hexagon-shaped crystals with typical dimensions between 50 – 100 µm appeared within several days from sitting drop plates at 293K. An ECHO 550 acoustic liquid handler (Labcyte) was used to transfer individual fragments from the DSPL (7) and OxXChem (8) fragment library to crystal drops. Briefly, 38 nL of compound solution was added to each crystallisation drop resulting in a final compound concentration of 100 mM with 20% DMSO, calculated based on the initial drop volume. Compounds from the 3-chloro-phenylamino series were soaked into NUDT7 crystals by adding a mixture containing 600 nL of 100 mM compound in DMSO and 1200 nL of reservoir solution containing 0.1 M BisTris pH 5.5, 0.1M ammonium acetate and 10% (w/v) PEG10.000. Crystals were incubated overnight at room temperature and then harvested (without further cryoprotection) and flash cooled in liquid nitrogen. Crystals of NUDT7 with covalent binders were grown by mixing 100 nL of 30 mg/mL protein in 10 mM Na-HEPES pH 7.5, 500 mM NaCl, 5% glycerol with 30 nL of 20 mM compound in DMSO in sitting-drop crystallization plates containing 0.1 M BisTris pH 5.5, 0.1M ammonium acetate and 4 - 16% (w/v) PEG 10.000 in the reservoir at 293 K. After overnight incubation of protein and compound, 100 nL of reservoir solution and 30 nL of a crystal seed solution obtained from a previous crystallisation experiment, diluted 1:4 from the stock in 0.1 M BisTris pH 5.5, 0.1 M ammonium acetate and 9% (w/v) PEG 10.000 were added to the drop. Hexagon-shaped crystals appeared within several days. Prior to data collection, all crystals were transferred to a solution consisting of the precipitation buffer supplemented with 25% ethylene glycol and subsequently flash cooled in liquid nitrogen. All X-ray diffraction data were collected on the beamline I04-1 at Diamond Light Source (Harwell, UK) unless stated otherwise.

Structure determination

Diffraction data were automatically processed by software pipelines at the Diamond Light Source (9). Initial refinement and map calculation was carried out with DIMPLE (10). PanDDA (11) was used for hit identification and further refinement and model building was performed with REFMAC (12) and COOT (13), respectively. All structure determination steps were performed within the XChemExplorer (14) data management and workflow tool.
Coordinates and structure factors for all data sets are deposited in the RCSB Protein Data Bank under group deposition ID G_1002045. Data collection and refinement statistics are available from the PDB pages. The complete PanDDA analysis and processed data from all crystals (including information about soaked compounds) that were prepared as part of the NUDT7 project can be accessed via the ZENODO data repository under DOI 10.5281/zenodo.1244111.

NUDT7 activity assay

Mass spectrometry assays monitoring acetyl-CoA hydrolysis by NUDT7 were performed on a Agilent 6530 RapidFire QTOF Mass Spectrometer in a 384-well plate format using polypropylene plates (Greiner, code

781280) and an assay buffer containing 20 mM HEPES pH 7.5, 200 mM NaCl and 5 mM MgCl2. All bulk liquid handling steps were performed using a multidrop combi reagent dispenser (Thermo Scientific, Code 5840300) equipped with a small tube plastic tip dispensing cassette (Thermo Scientific, Code 24073290). For inhibitor IC_{50} determinations an 11-point and 2-fold serial dilution in was prepared from a 50 mM stock solution in DMSO which was transferred to give four replicates using an ECHO 550 acoustic dispenser (Labcyte). The transferred volume was 400 nL giving a final DMSO concentration of 0.4%. In addition, a DMSO control (400 nL) was transferred into alternate wells in columns 12 and 24 and 50 mM EDTA (NUDT7 inhibitor) was dispensed into alternate wells of column 24 as the background control. 80 µL assay buffer was added to all wells and NUDT7 was prepared to 500 nM (10 X final concentration in assay buffer) and acetyl-CoA was prepared to 200 µM (10 X final concentration in assay buffer). 10 µL NUDT7 was dispensed into half of the assay plate (for two of the compound replicates) and the plate was incubated at room temperature for 30 minutes. 10 µL NUDT7 was then dispensed into the remaining half of the assay plate (for the remaining two compound replicates). 10 µL acetyl-CoA was immediately dispensed into all wells of the assay plate to initiate the reaction and the enzyme reaction was allowed to proceed for 15 min. The enzyme reaction was stopped by addition of 10 uL of 50 mM EDTA and the plate was transferred to a RapidFire RF360 high throughput sampling robot. Samples were aspirated under vacuum and loaded onto a C4 solid phase extraction (SPE) cartridge equilibrated and washed for 5.5 sec with 1 mM octylammonium acetate in LCMS grade water to remove non-volatile buffer components. After the aqueous wash, analytes of interest were eluted from the C4 SPE onto an Agilent 6530 accurate mass Q-TOF in an organic elution step (85% acetonitrile in LC-MS grade water). Ion data for the acetyl-CoA and hydrolysed product were extracted and peak area data integrated using RapidFire integrator software (Agilent). % conversion of substrate to product was calculated in Excel and IC_{50} curves generated using Graphpad Prism version 7.0. The assay had a Z score of 0.79 with the 30 minute pre-incubation and 0.75 without pre-incubation.

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