Human DNA Cross-Link Repair 1A (DCLRE1A, SNM1A)

A Target Enabling Package (TEP)

Gene ID / UniProt ID / EC	DCLRE1A / Q6PJP8 / -
Target Nominator	Peter McHugh (Oxford)
SGC Authors	Joseph Newman, Opher Gileadi, Hazel Aitkenhead
Collaborating Authors	Sook Lee[1], Peter McHugh[2], Christopher Schofield[1].
Target PI	Opher Gileadi (SGC Oxford)
Therapeutic Area(s)	Cancer
Disease Relevance	DCLRE1A is important in repair of DNA inter-strand crosslinks. Inhibition would enhance sensitivity to DNA-damaging agents (chemotherapeutics), and could hypothetically kill cancers with synthetic-lethal genetic background.
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SUMMARY OF PROJECT

Cancer cells experience genomic instability, probably through a combination of excessive replicative activity and the loss of function of checkpoint and DNA repair pathways that may have contributed to the oncogenic transformation. Chemotherapy by DNA-damaging agents such as cisplatin and nitrogen mustards create DNA interstrand crosslinks (ICL), which can lead to double-strand breaks and cell death when the cells replicate their DNA. Genotoxic drugs are counteracted by the cell's DNA damage response. Hence, it is expected that inhibiting DNA repair proteins would sensitise cells to chemotherapy.

Here we address an enzyme that participates in the repair of ICLs, DCLRE1A. The TEP includes expression clones and methods for producing the catalytic domain and high-throughput activity assays. Furthermore, we provide a crystallization system that generates thousands of reproducible crystals that allow soaking of small-molecule ligands. We provide crystal structures of several small molecule fragments and inhibitors, opening the way to development of more potent and selective inhibitors.

SCIENTIFIC BACKGROUND

DCLRE1A is a DNA exonuclease involved in the repair of DNA damage. DCLRE1A, and the related DCLRE1B, have the unique capability to digest through crosslinked nucleotides (1,2). This may underlie the observations that knocking down DCLRE1A expression sensitizes cells to chemotherapeutic agents such as cisplatins and mitomycin C (3,4). DCLRE1A has complex interactions with other components of the DNA repair machinery and with replication forks (2,5,6), including binding to (ubiquitylated) PCNA. Discovery of selective inhibitors of DCLRE1A may serve three purposes: as research tools, to understand the roles of DCLRE1A in genome maintenance and to identify vulnerability of different cell lines; in combination therapy, to increase the sensitivity of cancer cells to DNA-damaging chemotherapeutic agents; and, possibly, to selectively target cancer cells through synthetic lethal interactions. DCLRE1A is subject to intensive research by groups including our collaborators, P. McHugh and C. Schofield in Oxford. The collaborators have investigated the biology of DCLRE1A and its homologue in yeast, mapping structural and functional interactions with the replication and genome repair machineries.
Previously published work leading to this TEP included a crystal structure of DCLRE1A (7), the development of biochemical assays, and the identification of inhibitory molecules from a library of beta-lactam compounds (1,8). In this TEP, we provide a modified crystallization system that generates thousands of reproducible crystals that allow soaking of small-molecule ligands. We provide crystal structures of several small molecule fragments and inhibitors, opening the way to development of more potent and selective inhibitors.

RESULTS – THE TEP

Proteins purified

The catalytic domain of DCLRE1A (aa 698-1040) is expressed from recombinant baculoviruses and purified from SF9 insect cells.

Antibodies

Most commercial antibodies do not recognize the protein when expressed in native levels. Our collaborators have recently identified a polyclonal antibody from Bethyl, A303-747A, which recog nizes a specific protein band in Western Blots in HEK293 and U2OS cells, which is absent in the corresponding knockout cells (Lonnie Swift and Peter McHugh, manuscript in preparation).

Structural data

Earlier crystal forms were unsuitable for medicinal chemistry because the active site was unavailable; within this TEP, we provide methods for obtaining a new crystal form that routinely generates data with resolution better than 1.8Å, and allows soaking of small molecules. A list of structures deposited in the PDB is provided below.

In vitro assays

In vitro assays were developed together with the McHugh lab. A primary HTS-ready assay detects the hydrolysis of the 5'-nucleotide from an oligonucleotide containing a donor/quencher pair. Secondary assays measure the processive degradation of oligonucleotide and plasmid templates using gel-based

assays. More detailed mechanistic studies are done using DNA substrates containing damages sites. Assay methods and results have been published and are summarized in the methods document.

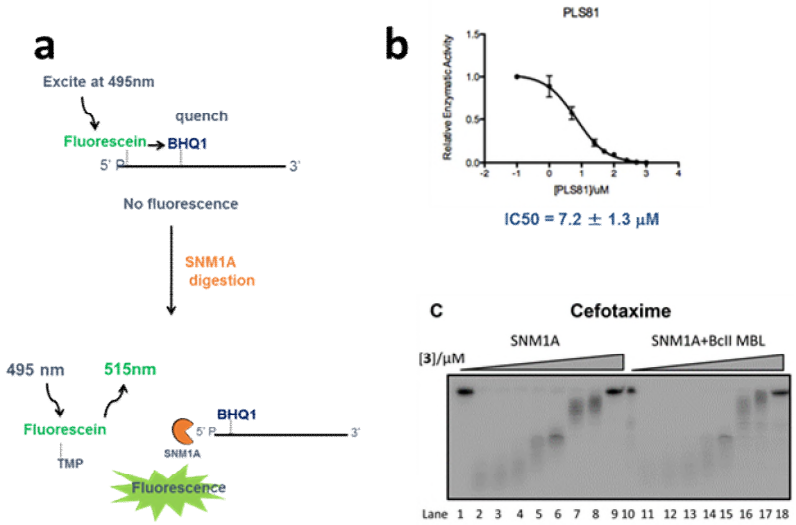
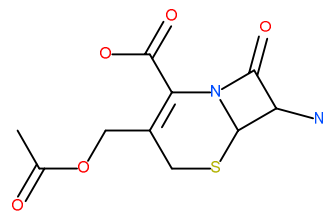
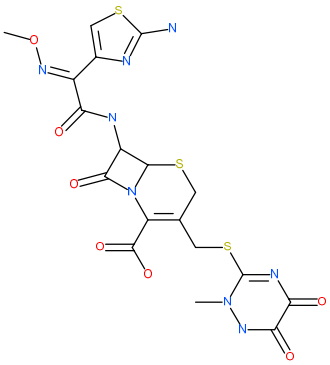
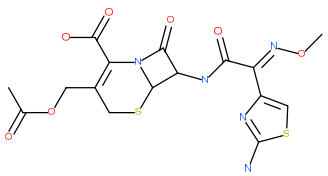


***Fig. 1**: Biochemical assays for DCLRE1A activity. (**A**) Fluorescence-based HTS assay, monitoring the removal of 5'-most nucleotides. (**B**) Inhibition curve. (**C**) Gel-based assays, which also monitor the processivity and substrate-selectivity of DNA degradation.*

Chemical starting points

Chemical starting points emerged from two sources. The first was a library of β-lactamase inhibitors and substrates (based on the relation to bacterial metallo β-lactamases). Three Cephalosporins were identified as inhibitors: 7-ACA, Ceftriaxone, and Cefotaxime, with IC_{50} of 7, 4 and 5 µM, respectively.

		
7-ACA	Ceftriaxone	Cefotaxime
IC_{50} = 7µM	IC_{50} = 4µM	IC_{50} = 5µM
Ligand Efficiency = 0.42	Ligand Efficiency = 0.20	Ligand Efficiency =0.25
Lipophilic Ligand Efficiency= 6.8	Lipophilic Ligand Efficiency = 6.1	Lipophilic Ligand Efficiency = 5.4

Co-crystal structures revealed three binding sites: one site has the Ceftriaxone bound in a position to form a chelate with the active site metal (**Fig.2**). The other binding sites are located at some distance from the catalytic site, but may overlap with DNA-binding regions of the protein (**Fig.2**).

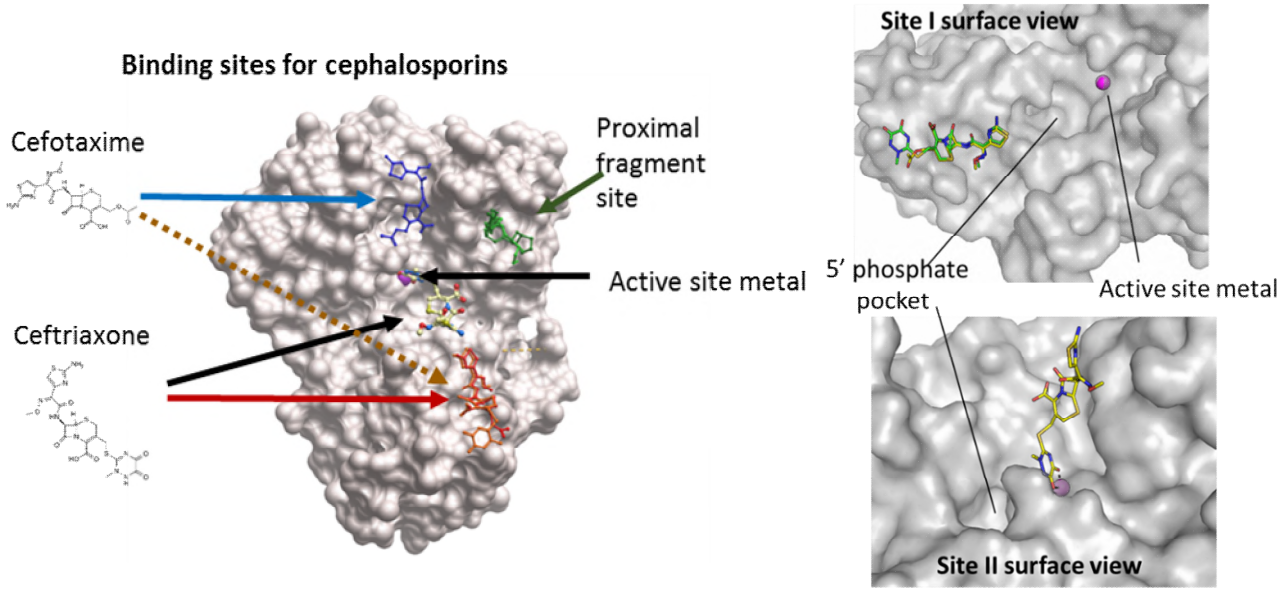


***Fig 2**: **LEFT**: Overlaid structures of cephalospirins bound at three sites. Ceftriaxone can bind directly to the active site metal, or at a proximal site (orange); Cefotaxime can bind at two proximal sites (Orange, Blue). The proximal fragment binding site (from panel A) is shown in green.*
***RIGHT**: detailed views of the proximal and metal-binding sites of Ceftriaxone binding*

A second set of chemical starting points was discovered by X-ray crystallography-based fragment screens, performed at the Diamond Light Source (I04-1). The screen identified 26 small molecules that cluster in two hotspots on the protein (**fig. 3** and **Table 2**) ; a proximal pocket, located ~9 Å from the catalytic centre, and an opposite site, located at the back side of the protein.
Compounds targeting the proximal side may be expanded to improve the selectivity and potency of a chelating "warhead" that would occlude the active site. The utility of the opposite site is less clear, although targeting this allosteric site may affect aspects of SNM1A activity such as processivity on long DNA substrates.

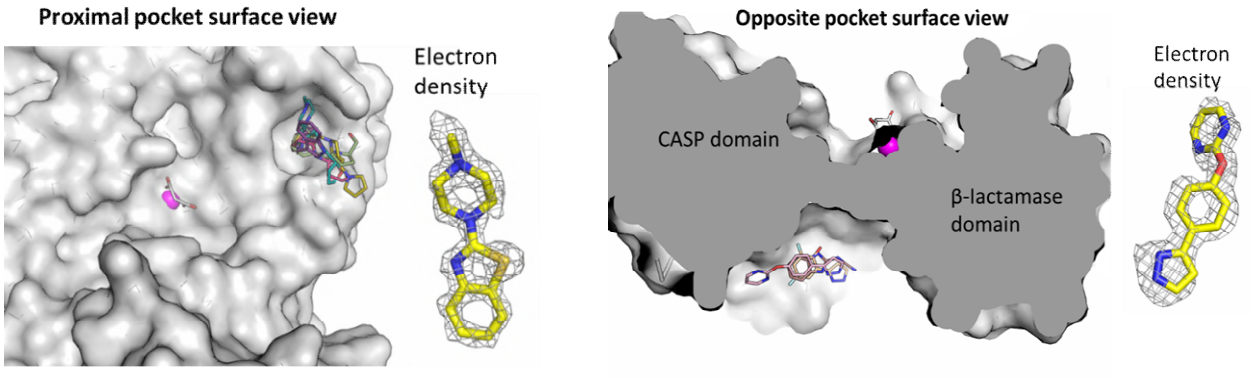


***Fig. 3**: Major binding sites of fragments. **LEFT**: Fragments clustering at a proximal pocket. Electron density map of a representative compound shown on the right. **RIGHT**: The location of the opposite pocket, shown on a cross-sectional view of the protein.*

CONCLUSION

Genetic knockdown experiments have indicated that depletion of the DNA-repair exonuclease DCLRE1A sensitises cells to DNA crosslinking agents used in cancer chemotherapy. This makes DCLRE1A a potential target for cancer therapy, either in combination with chemotherapy, or in a synthetic-lethal genetic background. To investigate and exploit this therapeutic potential, it is essential to develop small-molecule inhibitors of DCLRE1A.

This target enabling package includes high-throughput and secondary assays for DCLRE1A activity, a robust crystallization method, and initial chemical hits from fragment screens as well as from an inhibition screen. Co-crystal structures identify a number of pockets on the protein surface in addition to the Zn-containing catalytic site, which may be used to develop highly specific inhibitors when combined with a metal-chelating moiety.

FUNDING INFORMATION

The work performed at the SGC has been funded by a grant from the Wellcome [106169/ZZ14/Z].

ADDITIONAL INFORMATION

Structure Files

PDB ID	Structure Details
5NZW	Ceftriaxone (metal site)
5NZX	Ceftriaxone (alternative site)
5NZY	Cefotaxime
5Q1V	Fragment bound at the proximal site
5Q1U	Fragment bound at the proximal site
5Q1T	Fragment bound at the proximal site
5Q1R	Fragment bound at the proximal site
5Q10	Fragment bound at the proximal site
5Q22	Fragment bound at the proximal site
5Q1K	Fragment bound at the opposite site
5Q1W	Fragment bound at the opposite site
5Q1X	Fragment bound at the opposite site

Materials and Methods

Protein Expression and Purification

DCLRE1A catalytic domain
Boundaries: residues 698-1040
Vector: pFB-LIC-Bse [9,10]
Tag and additions: TEV-cleavable His6 tag at the N-terminus
(MGHHHHHHSSGVDLGTENLYFQ*SM)
Expression cell: Sf9
Note that this is a different construct from that used to generate earlier crystal structures (PDB: 5AHR and 4B87) [7].

Baculoviruses were generated using the Bac to Bac system (Life Technologies) and used to infect 2-6 L of SF9 cells in Sf-900™ II SFM medium. The cells were collected after 72 hours at 27°C, suspended in a small volume of lysis buffer (50 mM HEPES, pH 7.5, 0.5 M NaCl, 5% glycerol, 10 mM imidazole, 1 mM TCEP; protease inhibitors were added during cell resuspension but were omitted from the subsequent steps) and frozen. After thawing, the cells were diluted to ~5 volumes (v/w) of lysis buffer, then batch-bound to 5 ml of Ni-loaded chelating sepharose beads (GE) for 1 hour. The beads were washed with lysis buffer containing 30 mM imidazole, and the protein was eluted with lysis buffer containing 300 mM imidazole.
The tag was cleaved by incubating the protein overnight at 4°C with TEV protease (1/40 (w/w)) in a dialysis tube placed in 1 L dialysis buffer (50 mM HEPES, 0.5 M NaCl, 5% glycerol and 1 mM TCEP). The protein was then passed through a 1-mL Ni-sepharose column, and the flowthrough fractions were collected. The protein was concentrated on a centrifugal concentrator (Centricon, MWCO 30 kDa) before loading on a Superdex S200 HR 16/60 gel filtration column in dialysis buffer, and separated at 1.2 mL/min. fractions containing purified DCLRE1A protein were identified by SDS-PAGE, pooled and concentrated to 9-10 mg/mL.

Protein crystallization and compound soaking

Protein crystallization was performed by vapour diffusion in sitting drops at 4°. A protein solution at 9-10 mg/mL was mixed at with an equal volume crystallization solution containing 30% PEG 1000, 0.1M MIB pH 6.0 (MIB is Sodium malonate dibasic monohydrate, Imidazole, Boric acid). The crystals contained malate bound to the active site. For fragment screening, concentrated solutions (0.5 M) of the fragments in DMSO were added to the crystallization drops using an Echo dispenser, up to 10% of the drop volume, at the Diamond light source XChem facility. Crystals were harvested after 1-4 hour of incubation, flash-cooled in liquid N2 without additional cryoprotectant. For the cephalosporin compound soaks, the crystals were first

incubated overnight in malate-free liquor (30% PEG, 0.1 M HEPES, pH 7.0) to remove the malate at the active site; compounds were subsequently soaked overnight at a final concentration of approximately 20 mM before harvesting and cryo-cooling. Data was collected in Diamond light source beamline I04-1 (Fragments), I04 and ESRF beamline BM30B.

Activity assay

Real-time Fluorescence Assays: Real-time fluorescence assays were performed as described [1, 8] using the 20-mer oligonucleotide:

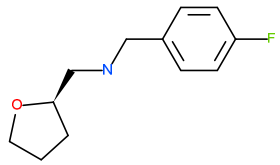
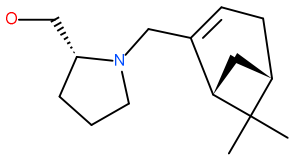
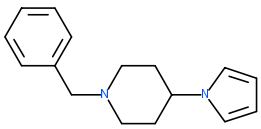
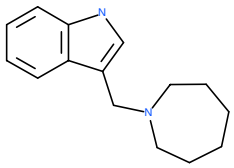
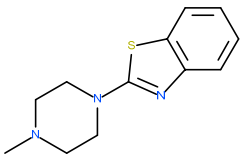
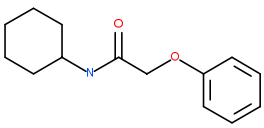
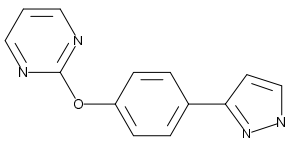
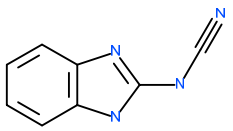
5'-phosphate-A-[fluorescein-T]-AATTTGATCA [BHQ1-T] CTATTAT

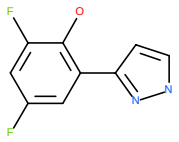
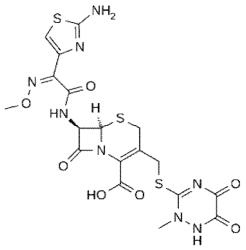
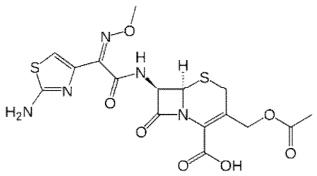
In this oligonucleotide, fluorescence from the fluorescein linked at position 2 is quenched by the BHQ1 group linked to position 13. When the first two nucleotides are hydrolysed, the unquenched fluorescence of the free fluorescein-TMP can be detected in real time (excitation at 495 nm, emission at 525 nm).
Reactions were carried out in black 384-well microplates, and measurements were made using a SpectraMax M2e fluorescent plate reader in fluorescent top read mode, with SoftMaxPro software (Molecular Devices, Sunnyvale, CA, USA) to control the settings. Reactions were performed in a total volume of 15 µL in nuclease buffer (20 mM HEPES pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.05% Triton-X100, 0.1 mg/mL BSA, 5% glycerol) with varying concentrations of compound (for IC50 determinations) or DNA substrate (for kinetics assays) and 0.24 nM DCLRE1A (the Km for ssDNA oligo was < 5 nM). Each reaction was started by the addition of DCLRE1A, and the fluorescein emission spectra measured (ex. 495 nm, em. 525 nm and cutoff at 515 nm) with six readings taken at 7 s intervals for 6 min. The fluorescence intensity of each well was plotted against time, and the rate of increase was determined, plotted against compound or substrate concentration and fitted to a log(inhibitor)- response or Michaelis–Menten curve on Prism software (GraphPad Software, Inc., La Jolla, CA, USA) to determine IC50 or Km and Vmax.
For radioactive gel-based assays, similar reaction conditions were used, with substrate DNAs labelled at the 3' end with α-[^{32}P]-dATP and terminal deoxynucleotidyl transferase. Reactions were stopped by boiling in sample buffer (95% formamide, 10 mM EDTA, bromophenol blue and xylene cyanol) and analysed by electrophoresis on 20% polyacrylamide, 0.5 x TBE and 7M urea.

Gene Knockouts
CRISPR/Cas9 knockout of DCLRE1A has been performed in the McHugh lab. Contact Prof. Peter McHugh, Oxford (peter.mchugh@imm.ox.ac.uk) for collaborative opportunities.

Antibodies
We are not aware of antibodies that can detect the native levels of DCLRE1A in wester/immunofluorescence.

IMPORTANT: Please note that the existence of small molecules within this TEP may only indicate that chemical matter can bind to the protein in a functionally relevant pocket. As such these molecules should not be used as tools for functional studies of the protein unless otherwise stated as they are not potent enough and not characterised enough to be used in cellular studies. A TEP's 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.

PDB ID	Compound structure
Fragments in proximal pocket	
5Q1V	
5Q1U	
5Q1T	
5Q1R	
5Q10	
5Q22	
Fragments in opposite pocket	
5Q1K	
5Q1W	

5Q1X	
Cephalosporin inhibitors	
5NZW, 5NZX: Ceftriaxone	
5NZY: Cefotaxime	

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