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CETSA screening identifies known and novel thymidylate synthase inhibitors and slow intracellular activation of 5-fluorouracil

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Target engagement is a critical factor for therapeutic efficacy. Assessment of compound binding to native target proteins in live cells is therefore highly desirable in all stages of drug discovery. We report here the first compound library screen based on biophysical measurements of intracellular target binding, exemplified by human thymidylate synthase (TS). The screen selected accurately for all the tested known drugs acting on TS. We also identified TS inhibitors with novel chemistry and marketed drugs that were not previously known to target TS, including the DNA methyltransferase inhibitor decitabine. By following the cellular uptake and enzymatic conversion of known drugs we correlated the appearance of active metabolites over time with intracellular target engagement. These data distinguished a much slower activation of 5-fluorouracil when compared with nucleoside-based drugs. The approach establishes efficient means to associate drug uptake and activation with target binding during drug discovery.

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herapeutic efficacy is achieved when drugs bind their relevant molecular targets in the physiologically relevant setting. Despite this known fact, insufficient control of target engagement is surprisingly common and contributes to high failure rates in clinical trials.1-3. Methods that allow for robust measurements of drug target engagement in primary cells, tissues and patient biopsies are thus urgently needed, but have been hard to establish.4,5.

Ligand-induced changes in protein thermal stability are frequently used to monitor binding to isolated proteins in thermal shift assays.6-9. The recently developed cellular thermal shift assay (CETSA; see Supplementary Note 1 for a list of abbreviations) builds on the discovery that ligand induced thermal shifts can also be measured in the context of cell lysates, whole cells or tissues.10. This finding effectively allows for biophysical binding studies in native environments—preserving expression levels, posttranslational modifications and the local environment for the endogenous protein. Whereas the original CETSA study included multiple case studies, recent work extends this method to include melting transitions for a significant portion of the proteome, thus expanding the putative use of the methodology to a large number of protein families.11-13 Of practical importance is that the melting transitions are established for individual proteins by the use of protein affinity reagents10,14 or quantitative mass spectrometry (MS)11–13. As a consequence these measurements are amenable to either high-throughput measurements or proteome-wide multiplexing.

To improve current strategies for drug development, stringent control of target engagement should ideally be established from initial hit identification, through preclinical and clinical development. The same demands apply to the validation of chemical probes discovered in academic settings.2,4,15 To probe the value of CETSA in earlier stages of the discovery process we applied it for primary screening of thymidylate synthase (TS) in live human myelogenous leukemia cells. TS is a pivotal enzyme in production of thymidine monophosphate and a well validated cancer target.16,17 Inhibition of TS leads to thymineless death characterized by DNA-damage, chromosomal fragmentation and concomitant induction of apoptosis. Novel classes of TS inhibitors with improved efficacy and resistance profiles could provide important complements to current TS directed drugs, for which there are reports of resistance.18,19

Here, we show for the first time that a CETSA-based screen for direct physical target engagement constitutes an attractive high throughput screening (HTS) strategy, which allows for the detection of known and novel TS inhibitors with cellular activity. Furthermore, we establish a hit validation strategy, in which time-dependent target engagement is explored in parallel with measurement of intracellular compound concentration. Taken together this provides a sound and efficient strategy to establish control of target engagement from an early stage of the drug discovery process, and which is likely to minimize problems in subsequent stages.

Results

Microplate-based CETSA measuring target engagement of TS.

CETSA is based on measurements of remaining soluble target protein against a background of thermally denatured and precipitated proteins following a heat challenge.10,14. To enable large-scale screening and automation we developed a no-wash immunooassay for TS using AlphaScreen technology in 384-well plates (see Supplementary Figs 1–6 and Supplementary Table 1). As outlined in Fig. 1a the assay workflow starts with a pre-incubation of K562 cells with library compounds or controls to allow cellular uptake, potential compound metabolism and binding to TS. The treated samples in the plates are next transiently heated in a PCR machine, resulting in denaturation and precipitation of intracellular TS unless stabilized by ligand. After cooling to room temperature the cells are lysed and the remaining (stabilized) levels of TS are measured.

We validated the assay by investigating the response to two drugs of structurally different classes, that is, 5-fluorouracil and raltitrexed. Both drugs require intracellular enzymatic conversion prior to high-affinity TS binding.12. Pyrimidine-based inhibitors, such as 5-fluorouracil, bind to TS as the corresponding monophosphate, whereas folate-based drugs, such as raltitrexed, are polyglutamylated and bind TS in a ternary complex with 2′-deoxuryridine 5′-monophosphate (dUMP). Two assay formats were employed for validation. First, the heating was done at a series of different temperatures at a fixed compound concentration to establish aggregation temperature ($T_{agg}$) curves (Fig. 1b). As expected both drugs resulted in substantial shifts of the thermal stability of TS, thus confirming cellular uptake and intracellular enzymatic conversion to the active forms that bind TS. Based on these curves 50°C was selected for further characterization in isothermal dose-response fingerprint (ITDRF_CETSA) experiments. In these experiments the compound concentration is titrated during the pre-incubation, after which all samples are heated to the same temperature (Fig. 1c,d). Both drugs showed dose-dependent stabilization of TS with half maximal effective concentration (EC50) values in the sub-nM range. Data from parallel experiments using quantitative western blots for assessment of stabilized TS confirmed a significant shift in $T_{agg}$ in the presence of 5 μM of either of these drugs as well as potent dose-dependent stabilization (Supplementary Fig. 7). No change in total TS levels was observable at 5 μM concentration following a 2 h pre-incubation time in the K562 cells, demonstrating that the thermal stabilization data were not influenced by drug-induced changes in total protein levels under these conditions.

Small molecule library screening and hit confirmation.

We screened a library of 10,928 compounds at Chemical Biology Consortium Sweden (CBCS; www.cbcs.se) using the TS assay described above. The library includes a structurally-diverse selection of lead-like compounds20, nucleosides and known drugs (see Methods section for details on the library). These latter subsets include folate and nucleoside-based drugs known to act on TS, suppress thymidine incorporation into DNA and reduce cell proliferation.16,21. A schematic outline of the screen logistics is available in Supplementary Fig. 8. Screening was performed at a compound concentration of 50 μM and resulted in a reproducible response to the controls and the appearance of several stabilizing compounds (Fig. 2a). Additional graphs illustrating the screen performance and statistics are available in Supplementary Fig. 9 and Supplementary Table 2, respectively. The campaign involved one day of screening, with the AlphaScreen readings done the following morning to ensure equilibration of the antibody recognition.

The threshold for active solutions was calculated at 11.7% stabilization and resulted in 65 hits (Supplementary Table 3). Solutions for 63 of these were available for cherry-picking from our vial-based compound stores, that is, a different source intended for long-term storage. The activities of these solutions were examined in ITDRF_CETSA experiments to confirm the screen results (Supplementary Table 3). The majority of compounds with an apparent stabilization above 30% in the primary screen confirmed activity. We also found that 12 out of the 15 vial-based solutions that failed to reproduce activity (highlighted at the bottom of Supplementary Table 3) had been contaminated with
highly active compounds because of insufficient tip washing during the transfer from vials to screen library plates (Supplementary Fig. 10). Consequently these hits reproducibly confirmed activity when tested from the contaminated source plates, while the original solutions were inactive. Taken together the confirmation rate was 90% for hits yielding more than 30% apparent thermal stabilization of TS in the original screen.

Fluoropyrimidines, anti-folates and their analogs. The majority of confirmed hits were pyrimidine-based nucleosides and analogs thereof. At the top of the list were substituted 2'-deoxyuridines, including floxuridine (three independent occurrences in the library), 5-trifluoro-2'-deoxythymidine (TFT), and 5-ethyl-2'-deoxyuridine (EdU) (Fig. 2b and Supplementary Table 3). All of these are known to be taken up and metabolized intracellularly to the active monophosphate forms that interact with TS16,17. Novel findings among the nucleosides included the two drugs azacitidine and its deoxyribose analog decitabine, as well as two purine nucleosides (8-bromoadenosine and 8-allyloxy-N2-isobutyryl-2'-deoxyguanosine).

With regards to folate analogs, methotrexate was present at two instances in the library, both as a racemate and as its L form. As expected both appeared as strong hits. The CETSA screen also identified two other marketed drugs, triamterene, a sodium channel inhibitor used to treat hypertension, and pyrimethamine, an inhibitor of dihydrofolate reductase from Plasmodium falciparum that is used to treat malaria. They have related...
structures and can potentially act as folic acid antagonists, but they have not been previously shown to bind TS. Given the scarcity of anti-folates in the hit list we also looked whether there were any obvious false negatives in the screen and confirmed this was not the case (Supplementary Tables 4 and 5).

CBK115334 as a novel TS inhibitor. Besides the pyrimidine- and folate-based inhibitors there were 17 additional weak hits of different chemical classes (Supplementary Table 3). We investigated one of these, CBK115334 or 3-amino-2-benzoyl-4-methylthieno(2,3-b)pyridin-6-ol (1), which was chemically distinct from known TS inhibitors (Fig. 2c and Supplementary Fig. 11). It also appeared for the first time as a hit in our screens (Supplementary Table 6). Confirmatory data were obtained using CETSA on K562 cell lysates, which demonstrated a 3.7 °C shift at 200 μM (Supplementary Fig. 12). When applied to isolated recombinant human TS, 1 showed a 2.6 °C shift at 25 μM and a 5.2 °C shift at 100 μM and confirmed binding in the low μM range using surface plasmon resonance (Supplementary Fig. 12). We tested whether binding affected enzymatic activity of TS in vitro and observed 60% inhibition at 10 μM concentration and near complete inhibition at 100 μM (Supplementary Fig. 12). A crystal structure of TS with 1 revealed that the compound binds the active site of TS occupying the folate-binding pocket (Fig. 2d). The binding involves π–π stacking interactions with the substrate dUMP and polar interactions with residues lining the catalytic cavity (Asn112 and Arg50 in particular). This constitutes a novel mode of binding as compared with other anti-folates occupying this space.

Addressing kinetics of compound transport and metabolism. We performed time-traces of the ITDRF CETSA experiments, that is, by varying the time during which cells were exposed to compound prior to the heating step. ITDRF CETSA data obtained after various pre-incubation times are shown in Fig. 3a,b for 5-fluorouracil (5-FU) and floxuridine, demonstrating several orders of magnitude lower potency for 5-FU. The corresponding data on additional nucleosides are available in Supplementary Fig. 13. To examine whether the observed target engagement coincides with appearance of the active forms of these compounds, we monitored levels of compounds and their anticipated active metabolites using liquid chromatography
Intracellular and extracellular concentrations as a function of preincubation time with floxuridine. The CETSA data are presented as the average and range from two independent experiments. The LC–MS/MS data are provided as the average and s.e.m. from experiments done at three different occasions. (e) The corresponding data for EdU (green) and EdUMP (grey).
whereas its presence at 200 μM completely blunted the ability of decitabine to bind TS (Fig. 4c). However, studies using recombinant DCK to generate decitabine monophosphate resulted in only a marginal stabilization of TS (Fig. 4d). The sample was therefore additionally treated with deoxycytidylate deaminase (DCTD), which is known to deaminate decitabine monophosphate, resulting in a thermal shift of nearly 10°C (Fig. 4d). The inhibitory capacity of these samples mirrored these data, that is, minor inhibition was observed after DCK treatment in the TS enzymatic assay, whereas near full inhibition appeared after treatment with both DCK and DCTD (Fig. 4e). Taken together with the structural analogy to the natural substrate of TS...
these data strongly infer that the TS ligand is 5-aza-2'-deoxyuridine 5'-monophosphate, that is, the expected product of phosphorylation and deamination of decitabine.

**Discussion**

Target engagement is essential for efficacy of targeted therapies and validation of new chemical probes \(^{2,4,13}\). These validating experiments are ideally performed for many representatives within a chemical series to allow comparisons of structure–activity relationships. To push towards the goal of having a procedure amenable to automation and screening we applied CETSA for assessment of intracellular target engagement at the stage of primary screening. Prior to this work the methodology had been applied on a growing number of drugs and chemical probes \(^{10–14}\), but it remained challenging to apply to large chemical libraries.

To achieve this evaluation, we developed a homogeneous CETSA and applied it to screening in live, non-engineered cells expressing thymidylate synthase, which is targeted by several different classes of drugs in clinical use \(^{6,17}\). Importantly, the screen identified all drugs within the test library that act on TS, as well as novel compounds capable of binding and inhibiting this enzyme. Collectively the known drugs and new inhibitors span over a broad range of affinities. Amongst the new hits was 1, a \(\mu\)M inhibitor of the purified enzyme with sufficient cell penetration to result in intracellular target engagement and anti-proliferative effects in the high \(\mu\)M range. The binding mode of 1 to TS is partly new and it thus provides a potential starting point for further chemistry optimization.

Several other marketed drugs not previously known to inhibit TS emerged as hits, including triamterene, pyrimethamine and decitabine. As these are clinically-used compounds it is of interest to understand whether there are instances where the interaction with TS plays a role in either efficacy or toxicity. The identification of decitabine illustrates the relevance of monitoring target binding in live cells as this finding was dependent on active cellular metabolism. Decitabine itself was largely inactive on the protein such that, in analogy to the already known uridine-based inhibitors, enzymatic conversion to an active species is a prerequisite for observation of binding. We showed that this conversion does not take place to a significant extent in cell lysates, but can be reproduced with the *in vitro* application of enzymes for phosphorylation and deamination to yield the substrate analog 5-aza-2'-deoxyuridine 5'-monophosphate. Although our data give strong support that a metabolite of decitabine yield significant cellular inhibition of TS, further studies are required to determine whether this is relevant for polypharmacology or toxicity at typical therapeutic doses.

Time-traces of iTDRC\_CETSA were used to analyze different scenarios for cellular target engagement, thereby integrating aspects of drug transport and metabolism. Combination of these results with measurements of intracellular drug and metabolite concentrations allowed for a comprehensive dissection of cellular drug kinetics. Overall, the intracellular concentrations of the active species of the drugs correlated with the observed target engagement, consistent with the notion that CETSA directly reports on target binding. The combination of high-throughput target engagement studies with LC–MS/MS measurements of intracellular concentrations of drugs and metabolites constitutes a new paradigm for hit validation and optimization in the discovery of chemical probes and drugs. Importantly, CETSA is applicable to studies in native cells and tissue samples \(^{10–14}\). Thus the basic scenarios for compound metabolism and target engagement derived from these cell culture studies should be possible to translate towards studies of activities and resistance development to drugs in man. Of particular interest in this regard was the observation that we nearly missed the identification of 5-FU in the screen because of the relatively slow appearance of target engagement. It will be interesting to extend these experiments to patient cells.

The present work demonstrates that CETSA constitutes a robust high-throughput screening strategy that allows for target proteins to be approached in their natural cellular environment. This is in contrast to the majority of targeted cellular HTS assays as these rely on overexpressed and tagged proteins. Since CETSA does not require engineered cells or compounds, it could be particularly attractive for screening in primary cells, tissues or patient-derived material. Our approach can be applied to a large number of different proteins, with the generic assay development path being established in this work. The work also introduces the combination of time-dependent iTDRC\_CETSA and measurement of intracellular concentrations of metabolites as a stringent approach for hit validation, where the same assay format can be utilized and provide value throughout the drug discovery process.

**Methods**

**Cell culture conditions in AlphaScreen-based experiments.** Human myelogenous leukemia cell line K562 (ATCC no. CCL-243) were cultured in RPMI-1640 (SH30227.21, HyClone) supplemented with 10% fetal bovine serum (SV30160.03, HyClone), 0.3 g l–1 1-glutamine (G7513, Sigma-Aldrich) and 100 units ml–1 Penicillin-Streptomycin (P4333, Sigma-Aldrich). The same cell medium composition was used for all experiments unless otherwise stated.

**Development of an AlphaScreen-based assay for TS.** Measurements of remaining levels of soluble TS in cell lysates were achieved based on an AlphaScreen-based assay. Establishment of this assay required the identification of a pair of antibodies that simultaneously recognize TS (Supplementary Fig. 1). Combinations of four mouse-derived and three rabbit-derived antibodies directed towards different epitopes of TS (see Supplementary Table 1) were tested for this ability. Four different conditions were tested for each pair, with two of those being the absence and presence of target protein. Given our previous experience of ligand induced quenching of protein target recognition by the antibody pair \(^{14}\) we also included a control containing an excess of dUMP with and without the additional presence of raltitrexed, which are known binders to the active sites of TS. Recombinant TS, diluted in 1 × AlphaLISA buffer (AL1000F, PerkinElmer), was preincubated at room temperature in the presence of buffer only, 100 μM dUMP or 100 μM dUMP and 10 μM raltitrexed in a total volume of 4 μl in a ProxiPlate (#6008280, PerkinElmer). After this preincubation all 12 possible combinations of antibody pairs were added to the sample in a volume of 4 μl followed by incubation for 30 min at room temperature. A mix of AlphaScreen acceptor and donor beads was finally added in a volume of 4 μl under subdued light and allowed to incubate at room temperature for 2 h before reading in an Envision plate reader (PerkinElmer). Final concentrations of the inhibitors were the same as in the detection step. Two nM recombinant TS, 2 nM raltitrexed and 40 μg ml–1 AlphaScreen anti-mouse donor beads (#AS104D, PerkinElmer) and 10 μg ml–1 AlphaScreen anti-rabbit acceptor beads (#AL104C, PerkinElmer). The plates were sealed with TopSeal-A PLUS (#609185, PerkinElmer). The data were analyzed using microsoft excel and GraphPad Prism 6.

Four different antibody pair combinations based on sc-376161, WH000298M1, 15047-1-AP and DS83 (Supplementary Table 1) were selected from the antibody screen to study the kinetics of their recognition of TS in cell lysate. Two batches of 7.5 million K562 cells per ml in supplemented cell culture medium were prepared to serve as max and min controls. One culture was left at room temperature and the other was heated to 52 °C for 3 min in a PCR machine (TECHNE TC-PLUS thermal cycler). Both batches of cells were then lysed by the addition of an equal volume of 2X AlphaScreen SureFire Lysis Buffer (TGLRL100ML, PerkinElmer). After thorough mixing, 4 μl aliquots of the lysates were transferred to a ProxiPlate and detected and analyzed as described above except that the bead incubation was performed at 2 h, 6 h and overnight.

Optimization of cell number was achieved by serial dilution of a cell suspension of K562 cells in supplemented cell culture medium. Each sample was then split into two aliquots, which were either kept at room temperature or heated
to 52 °C as described above. Both aliquots were then lysed as described above. After thorough mixing, 3 μl of the lysates were transferred to a ProxiPlate followed by the addition of 6 μl of a mix of antibodies and AlphaScreen acceptor and donor beads in AlphaLISA buffer under subbed light. Detection and analysis was achieved as described above except the antibody concentrations were modified to 1 nM 15047-1-AP and 0.4 nM sc-376161.

The control experiment, in which recombinant TS was seeded to cell lysates was prepared based on a serial dilution of recombinant human TS. Dilutions were done in equal volumes of supplemented cell medium and 2 × AlphaScreen SureFire Lysis Buffer. K562 cells at a cell density of 2 million cells per ml were lysed as described above and split in two samples, which were either kept at room temperature or heated to 52 °C as described above. A 1 μl aliquot of the TS dilution was then added to the same volume of each of the two lysates as well as to the mixture of cell medium and lysis buffer. A 3 μl aliquot of each sample was then transferred to a ProxiPlate and detected and analyzed as described above.

Thermal aggregation experiments using AlphaScreen. Fluoruridine and raltitrexed were diluted from dimethyl sulfoxide (DMSO) stock solutions to concentrations of 30 μM and 20 μM respectively in supplemented cell culture medium (final DMSO content 1%). These solutions were transferred in a volume of 10 μl to a skirted Twin.tec PCR 96-wellplate (0030 128 672, Eppendorf). A suspension of K562 cells in a volume of 10 μl and a density of 10 million cells per ml were then added to all wells. The PCR plates containing the compounds and cells were sealed with a breathable plate seal (3345, Corning) and incubated for 2 h in a humidified atmosphere at 37 °C and 5% CO2. The plates were then transferred to different temperatures ranging from 40 °C to 86 °C for 3 min, followed by a controlled cooling to 20 °C for 1 min using a real-time PCR machine (ProFlex, Applied Biosystems). After the heating step the plate was centrifuged briefly (1,000 × g for 1 min) followed by lysis of the heated cells by the addition of 20 μl of 2 × AlphaScreen SureFire Lysis Buffer using a Flexdrop IV (PerkinElmer). To ensure sufficient lysis the cell lysates were mixed by 10 repetitive aspiration and dispensing cycles using a Bravo liquid handling platform (Agilent). The lysates (3 μl) were then transferred to 384-well ProxiPlates followed by the addition of 6 μl of a mix of antibodies and AlphaScreen acceptor and donor beads in AlphaLISA buffer under subbed light. Final concentrations of the reagents in the detection step were 1 nM rabbit polyclonal anti-TS IgG (15047-1-AP, Proteintech), 0.4 nM mouse monoclonal anti-TS IgG (sc-376161, Santa Cruz), 40 μg ml−1 AlphaScreen anti-mouse donor beads and 10 μg ml−1 AlphaScreen anti-rabbit acceptor beads. The plates were sealed with TopSeal-A PLUS and incubated over night at room temperature prior to detection in an Envision plate reader. The data were analyzed using microsoft excel and GraphPad Prism 6.

Composition and storage of the primary screening set. The library of compounds applied in this screening campaign consists of 10,928 compounds and is part of the primary screening set at CBCS. The majority of these compounds was donated by Biovitrum AB and originates from both in-house and commercial sources. Compounds included in the primary screening set were selected to represent a diverse selection of a larger set of 65,000 compounds, while keeping a certain structure–activity relationship (SAR) studies. The selection was also biased towards lead-like and drug-like profiles with regards to molecular weight, hydrogen bond donors/acceptors and LogP20. The library also includes a nucleoside set from Berry & Associates and a set of approved drugs from Prestwick. Compounds in stock solutions at 10 mM in DMSO are stored frozen at approximately − 20 °C in individual capped tubes in REMP 96 Storage Tube Racks. The racks are stored in a REMP Small-Size Store, which allows cherrypicking while the solutions are still frozen to minimize repetitive freeze-thaw cycles. For screening purposes the compound solutions have been replicated from the REMP racks to Labcyte 384 LDV plates (LP-0206) and then further into Labcyte 1536 HighBase plates (LPV-7360) to enable dispensing using acoustic liquid handling equipment.

Compound handling. Assay ready plates were prepared by transferring 200 nl of the 10 mM DMSO solutions of compounds and controls by means of acoustic dispensing (Echo 550, Labcyte) to 384-well polypropylene plates (784201, Greiner). Compounds were placed in columns 1–22. DMSO controls were placed in columnub 8. The compounds were then diluted with 20 μl supplemented cell medium to achieve a concentration of 10 mM of test compounds in the incubation with cells were ranging from 100 nM–50 μM depending on estimated potency. The concentration of DMSO was 0.5% in all samples. The final concentration of the positive control raltitrexed was 100 nM. The assay ready plates were prepared as outlined above for the screen.

Screening and dose-response characterization by AlphaScreen. The screen procedure started with the addition of 5 μl of a suspension of K562 cells at a density of 33,000 cells per ml to all wells of a 384 well hardshell PCR plate (HSR840, BIORAD) using an electronic multichannel pipette (Biobit). The plates were then heat sealed with Peelable Aluminium seal in a thermal microplate sealer (PlateLoc, Agilent) and allowed to incubate for 2 h in an incubator at 37 °C and 5% CO2. For the time-course experiments the incubation times were altered to include also 10 min, 30 min and 6 h. To allow gas exchange during the longer incubation times these plates were instead sealed with breathable plate seal (3345, Corning). After the incubation step the plates were transferred to a 384 well microplate containing a real-time PCR machine (LightCycler480 system, Roche). Plate handling was then as described above for the thermal aggregation experiments. The data were analyzed using microsoft excel and GraphPad Prism 6.

Screen and ITDRFCETSA data analysis. Screen data were imported into microsoft excel and normalized for each compound based on the negative and positive controls on each plate, that is, with the response in the presence of DMSO defining 0% stabilization and the response in the presence of 100 nM raltitrexed defining 100% stabilization. A calculation of the average and standard error of means for each set of controls was allowed an illustration of how these responses varied over the 32 screening plates. The Z factor28 is commonly used as a measure of how well the assay separates between the controls and this was calculated as described based on the calculated averages and standard deviations of the controls on a per plate basis. For the Z’ factor and the ITDRF CETSA experiments the data were analyzed in GraphPad Prism using the Boltzmann sigmoid equation and the saturation binding curve (rectangular hyperbola binding isotherm) function, respectively. As already discussed14 these methods make use of equilibrium models for data analysis although the methodology depends on the irreversible aggregation of denatured material. For this reason we refer to the observed responses as apparent and irreversible and dispensing temporal and spatial any quantitative interpretations, being well aware of their dependency on experimental conditions. Experiments are on-going to address the quantitative interpretation of CETSA data.

Measurements of identity and purity of test compound solutions. Assessments of identity and purity of the test solutions that were used for hit confirmation purposes, that is, those being stored in REMP vials, was done by means of high-pressure liquid chromatography coupled to mass spectrometry (HPLC–MS). A small aliquot of each test solution (2 μl of a 10 mM solution) was placed in a 96-well plate (267245, Nunc) and diluted with 20 μl of methanol. The plate was then placed in a Agilent 1,100 HPLC UV/MS with electrospray ionization (ESI + ). The HPLC method was based on an ACE 3 μm column (3.0 × 50 mm) and a mobile phase (CH3CN/0.1% TFA/H2O). All solvents were HPLC grade and deionized water was monitored at 214 nm. Compounds that did not give satisfactory data were re-analyzed using a method based on a Waters XBridge C18 3.5 μm column (3.0 × 50 mm), 3.5 min gradient mobile phase (CH3CN/10 mM NH4HCO3/H2O). The instrument software was used to integrate the UV response for each peak and provided a list of the peaks and their associated masses. The estimated purity was calculated based on the integral area for the expected mass compared with the areas of all other peaks. The result was manually controlled and if there were deviations from the expected outcome a meticulous investigation of the UV-response and MS was performed.

Cell viability assay. A concentration–response curve of CBK115334 was generated using the Bravo liquid handling system (serial dilution of a 50 mM stock solution was done in 100% DMSO). A total of 150 nl of the serially diluted solutions (positive control 0.67 mM staurosporine and negative control DMSO) were transferred to a white 384-well assay plate (3570, Corning) by means of acoustic dispensing (Echo 550, Labcyte). A Multidrop reagent dispenser (Thermo Scientific) was used to dispense 30 μl of a K562 cell suspension at a density of 33 × 104 cells per ml in supplemented cell culture medium. The cells were cultured at 37 °C in a CellTiter-Glo Luminescent Cell Viability Assay reagent (Promega) using a Multidrop Combi reagent dispenser (Thermo Scientific). The plate was placed on a plate shaker for 15 min prior to detection of the luminescence signal in an Envision plate reader (PerkinElmer). The final highest concentration of CBK115334 in the incubation with cells was 250 μM and the final concentration of the positive control staurosporine was 3 μM. All samples contained 0.5% DMSO. The data were analyzed using microsoft excel and GraphPad Prism 6.

Chemicals and buffers in western blot-based experiments. The cell lysis buffer contained 100 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES, 8
For the cell lysate thermal shift experiments, K562 cells were harvested and washed with Hank’s Balanced Salt Solution. The cells were diluted in lysis buffer supplemented with complete protease inhibitor cocktail. The cell suspensions were freeze-thawed three times using liquid nitrogen and passed through a 27 gauge needle five times. The soluble fraction (lysate) was separated from the cell debris by centrifugation at 20,000 x g for 20 min at 4 °C. The thermal aggregation curve experiments were performed by diluting lysates with lysis buffer supplemented with 200 μM dUMP and divided into two aliquots, with one aliquot being treated with ligand and the other aliquot with vehicle (control). After 10 min incubation at room temperature the respective lysates were diluted into smaller (50 μl) aliquots and heated individually at different temperatures ranging from 40 to 84 °C for 3 min in a Veriti thermal cycler (Applied Biosystems/Life Technologies) followed by cooling for 3 min at room temperature. The heated lysates were centrifuged at 20,000 x g for 20 min at 4 °C in order to separate the soluble fractions from precipitates. The supernatants containing the remaining soluble proteins were transferred to new 0.2 ml microtubes and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blot analysis.

For the in-cell experiments K562 cells were harvested and resuspended with culture medium to a cell density of 5 million cells per ml and seeded into T25 flasks. Cells were treated with either raltitrexed, floxuridine, DI-82 or vehicle (control). After 20 min at 4 °C in a Veriti thermal cycler (Applied Biosystems/Life Technologies) followed by 3 min of cooling at room temperature. The cell lysates were centrifuged at 20,000 x g for 20 min at 4 °C in order to separate the soluble TS-dUMP-CBK115334

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<td>a, b, c (Å)</td>
</tr>
<tr>
<td></td>
<td>108.2, 108.2, 313.9</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>30-3.1 (3.2-3.1)*</td>
</tr>
<tr>
<td>a, β, γ (°)</td>
<td>90, 90, 90</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.9 (99.9)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>11.9 (10.9)</td>
</tr>
</tbody>
</table>

Table 1 | X-ray diffraction data and refinement statistics.

**Expression and purification of human thymidylate synthase.** The gene encoding human TS (NM_001071.2) was subcloned into the pPICZ-Ba vector and expressed in Rosetta BL21-DE3 Escherichia coli (Novagen) in Terrific Broth media supplemented with 30 μg ml⁻¹ of kanamycin and 34 μg ml⁻¹ chloramphenicol. Cells were grown at 37 °C until OD600nm reached about 2.0 and induced with 0.5 mM isopropyl-beta-D-1-thiogalactopyranoside (IPTG) at 18 °C overnight. The cells were harvested by centrifugation at 4,500 x g for 15 min at 15 °C. The cell pellet was resuspended in lysis buffer (100 mM HEPES, 500 mM NaCl, 10 mM imidazole, 10% (v/v) glycerol and 1 mM TCEP at pH 8.0) supplemented with 1:1,000 (v/v) EDTA-free protease inhibitor cocktail (Calbiochem) and 125 U ml⁻¹ of Benzonase (Merck). Cells were lysed by sonication on ice at 70% amplitude, 3 s on/off for 3 min. The lysate was clarified by centrifugation at 47,000 x g for 25 min at 4 °C, and the supernatant was filtered through a 1.3 μm syringe filter to remove cell debris. The cell-free extract was loaded onto a pre-equilibrated HiTrapTM HP column (GE Healthcare) in IMAC wash buffer 1 (20 mM HEPES, 500 mM NaCl, 10 mM imidazole, 10% (v/v) glycerol and 1 mM TCEP at pH 7.5) and subsequently washed with 20 column volumes (CVs) of IMAC wash buffer 1 and 15 CVs of IMAC wash buffer 2 (20 mM HEPES, 500 mM NaCl, 25 mM imidazole, 10% (v/v) glycerol and 1 mM TCEP at pH 7.5). The IMAC wash buffer 2 was eluted with 5 CVs of elution buffer (20 mM HEPES, 500 mM NaCl, 50 mM imidazole, 10% (v/v) glycerol and 1 mM TCEP at pH 7.5) and loaded onto a HiLoad 16/60 Superdex-200 column (GE Healthcare) pre-equilibrated with buffer (20 mM HEPES, 300 mM NaCl, 10% (v/v) glycerol, and 1 mM TCEP at pH 7.5). Based on Nu-PAGE gel results pure protein fractions were pooled and concentrated using a 50 kDa cutoff centrifugal filter concentrator (Sartorius Stadion Biotech). The protein concentration was determined by the absorbance at 280 nm using a Nanodrop spectrophotometer (Thermo Scientific).
the midpoint of the curves ($T_{50}$) was performed using the software package XLfit from IDBS within Microsoft Excel.

**TS enzyme inhibition assay.** Enzymatic activity of recombinant human TS was measured spectrophotometrically at 340 nm by monitoring the absorbance change during the conversion of 5,10-methylenetetrahydrofolate to dihydrofolate using an Infinite M200 spectrometer (Tecan). Measurements were carried out at room temperature and in a buffer of 50 mM Tris at pH 7.5 and 150 mM NaCl. Initial velocities were measured with 250 nM of purified protein, 100 μM 5,10-methylenetetrahydrofolate and 100 μM dUMP in the presence of compounds using the same amount of DMSO as control. Initial rates and activity were analyzed with the software package Prism (GraphPad Software).

**Surface plasmon resonance.** Recombinant human TS protein (25 μg ml $^{-1}$ in a buffer of 10 mM sodium acetate at pH 5.0, 1 mM DUMP and 200 μM methotrexate) were captured on Sensor Chip S-CMS via amine coupling to a level of ~5000 resonance units using Biacore T-200. Raltitrexed was used as a positive control to ensure that the protein remained active after immobilization and during the run. A concentration series (20 nM to 10 μM) of CBK115334 was injected over the prepared surface for 60 s and allowed to dissociate for 60 s with a flow rate of 70 μl min $^{-1}$ at 25 °C. The assay buffer was 20 mM HEPES, 150 mM NaCl, pH 7.5 and 0.005% Tween-20 supplemented with 1% DMSO and 200 μM dUMP. Response data was processed using the BIAevaluation software. Responses were double referenced and solvent-corrected. The data sets were fitted to 1:1 steady-state model for determination of binding constants.

**Decitabine treatment with DCK and DCTD.** Decitabine at a concentration of 1 μM was incubated with 1 mg ml $^{-1}$ of recombinant DCK and 2.5 mM ATP in a buffer of 50 mM Tris at pH 7.5, 150 mM NaCl and 0.5 mM MgCl$_2$ at room temperature for 60 min to generate the corresponding monophosphates. To probe for nucleotide deamination the samples were further treated with 1 mg ml $^{-1}$ of recombinant DCTD in the presence of 1 mM ATP and 10 μM ZnCl$_2$ for 20 min at room temperature. After incubation, the samples were heated at 95 °C for 10 min and centrifuged for 10 min at the highest speed at 4 °C using a benchtop centrifuge. The supernatant was tested without dilution in thermal shift in vitro assays and the TS enzyme inhibition assay. The supernatants from samples without nucleoside were used as the treatment controls.

**Crystallization and structure determination.** TS protein crystallized in sitting drops comprising equal volume of protein (about 24 mg ml $^{-1}$) and reservoir solution at 20 °C. The crystallization condition was composed of 0.1 M sodium cacodylate at pH 6.5 and 15% PEG 4,000. Crystals were soaked with 1 mM compound and 2 mM dUMP in cryo-protectant buffer containing 0.1 M sodium cacodylate at pH 6.5 and 25% PEG 4,000 and 10% DMSO for 15 min, followed by flash frozen in liquid nitrogen. Data collection was performed on beamline MX1 at Australian Synchrotron. X-ray diffraction data was collected at 100 K with a wavelength of 0.9537 Å. It should be noted that CBK115334 is subject to keto-enol isomerization. While the resolution is not sufficient to distinguish between these, the included illustrations are based on the enol form (both forms make interactions with Asn112 and Arg50, but with different donor–acceptor pairs). The structure was solved by molecular replacement using Phaser with the hTS-dUMP-raltrexzide structure (PDB code 1HGY) as the search model. Structure was refined with phenix refine. Ligand structures and restraints files were generated using eLBOW. In the TS-dUMP-CBK115334 complex structure, 90.43% of residues were in favoured regions and 7.92% of residues were in allowed regions. The data collection parameters and refinement statistics are summarized in Table 1. An image of the electron density map of the active site in the co-crystal structure is available in Supplementary Fig. 17.

**Intracellular compound and metabolite concentrations.** Intracellular concentrations of compounds were measured as previously described$^{25,26}$. Briefly, KS62 cells were incubated with the compounds for a predefined time at 37°C in a 5% CO$_2$ atmosphere. After incubation, the cells were centrifuged (300 x g for 5 min) and a medium sample (supernatant) was collected and diluted 1:10 in a 50 mM warfarin solution (internal standard). The cells were washed with phosphate buffered salt solution and lysed with methanol. Methanol was evaporated and the cell samples were reconstituted in 50 mM warfarin. Samples were analyzed with LC–MS/MS with electrospray ionization in negative mode with transitions monitored as listed in Table 2.

**References.**


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Author contributions
H.AL, H.Ax., R.J., D.M.M., T.L. and P.N. conceived the study. H.AL, H.Ax. and T.L. developed the homogenous TS CETSA assay supported by R.J. and D.M.M. and ran the screen. R.J. generated the western blot CETSA data. A.M. and P.A. planned and generated the intracellular concentration data. C.D. generated the in vitro thermal shift and surface plasmon resonance data, and solved the crystal structure of CBK115334 together with A.L, M.H. performed the cheminformatics and metabolic pathway analysis. P.N., P.A. and T.L. lead the project. T.L. and P.N. wrote the first draft of the manuscript. All co-authors contributed to the final draft of the manuscript.

Additional information
Accession codes: The crystal structure of CBK115334 and dUMP in complex with TS has been deposited with the RCSB Protein Data Bank under the accession code 5HS3.

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications


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