Discovery of the first potent and selective inhibitors of the human dCTP pyrophosphatase 1 (dCTPase)

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Supporting Information

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Supplementary Figures

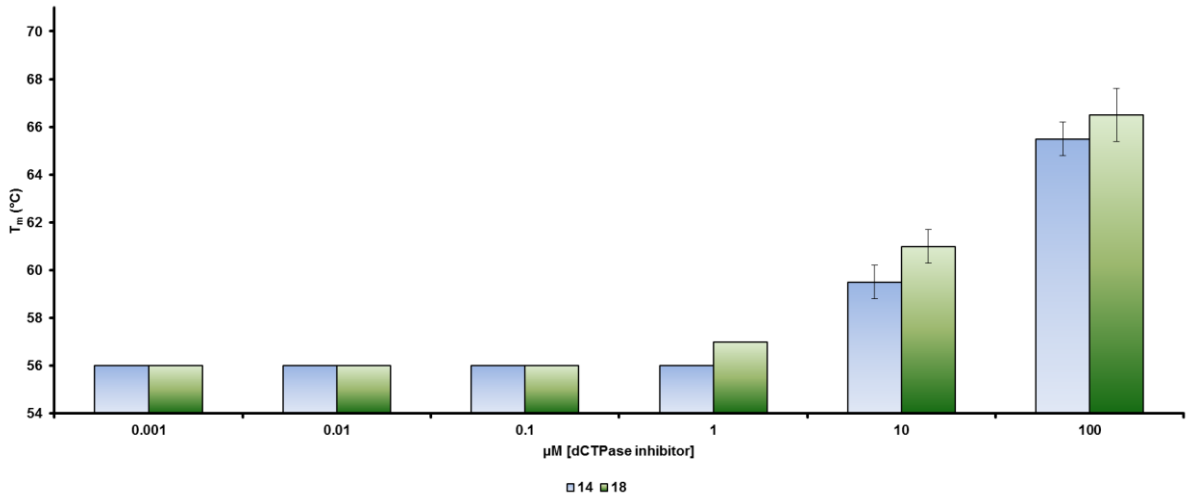


Figure 1. dCTPase inhibitors **14** and **18** stabilize dCTPase. Purified dCTPase was exposed to increasing concentrations of dCTPase inhibitors and the T_m was measured using the Differential Scanning Fluorimetry assay.

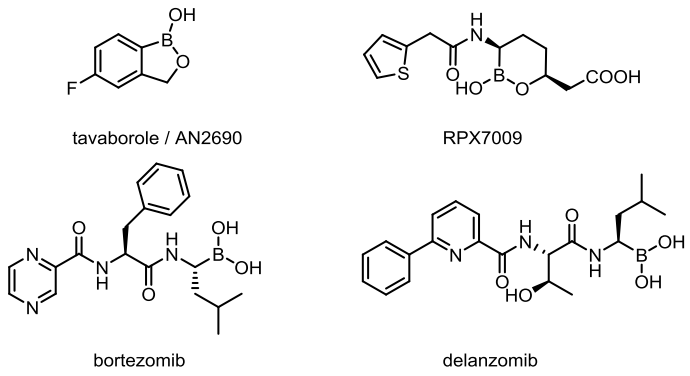


Figure 2. Structures of boron-containing drugs.

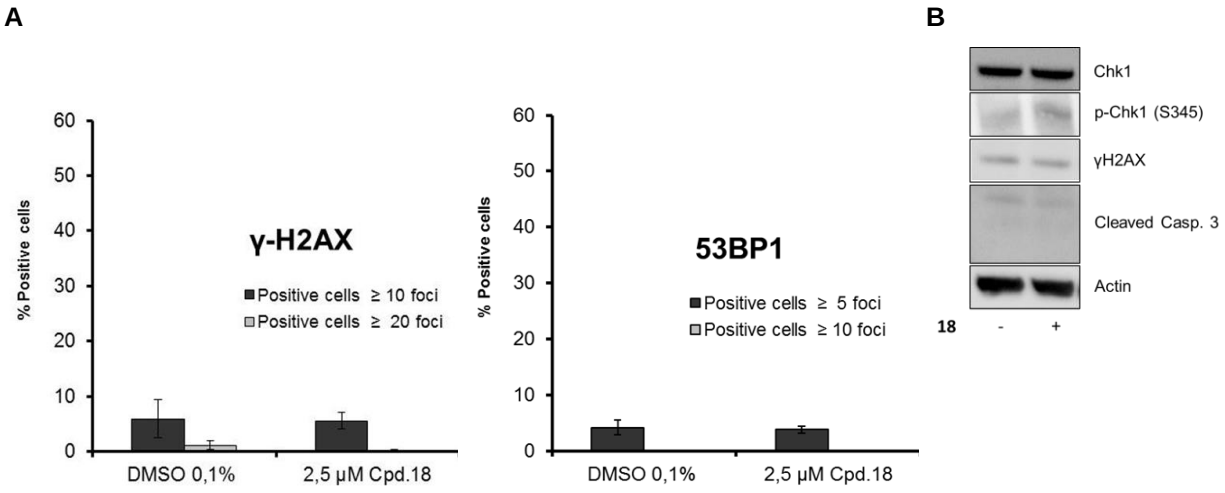


Figure 3. dCTPase inhibitor **18** does not induce DNA damage. A) AML HL60 cells were treated for 24h with low dose **18** and then quantified for the formation of γH2AX and 53BP1 foci using fluorescence microscopy. B) **18** does not induce DNA damage (γH2AX or p-Chk1) or apoptosis (Cleaved caspase 3) by Western blot detection.

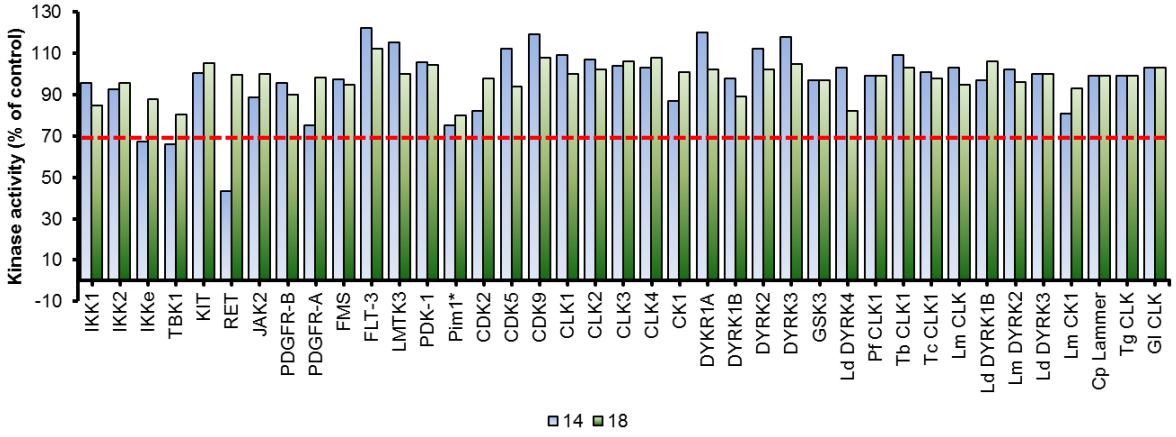


Figure 4. Selectivity profile for compounds **14** and **18**: Kinase enzymes. Tested at 10 µM; red dotted line indicates 30% inhibition level. *Tested at 100 µM.

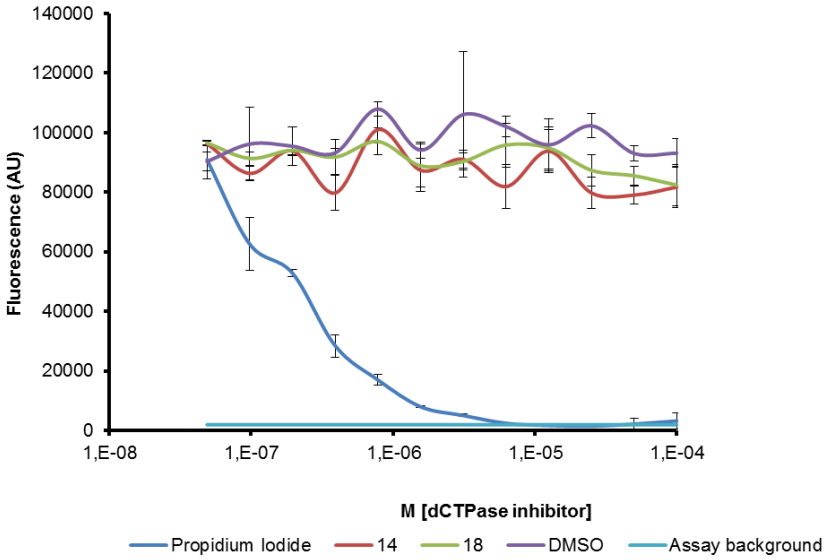


Figure 5. DNA intercalation measured by thiazole orange displacement. The DNA intercalator propidium iodide displaces thiazole orange and leads to a decrease in fluorescence to background levels.

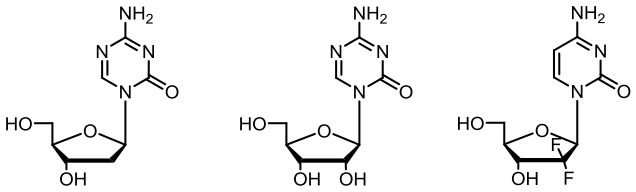


Figure 6. Structures of cytidine analogues.

	14	18
MTH1	>100	6.8
NUDT2	>100	>100
NUDT5	>100	>100
NUDT9	>100	>100
NUDT12	>100	>100
NUDT14	>100	>100
NUDT15	>100	>100
ITPase	>100	>100
dUTPase	>100	>100
dCTPase	0.041	0.046

Table 1. Selectivity profile for compounds **14** and **18** towards NTPases and NUDIX enzymes. Expressed as IC_{50} values (μM).

	14	18
APE1	>100	>100
***E.Coli* Fpg**	>100	>100
NEIL1	>100	>100
MPG	>100	>100
OGG1	>100	>100
SMUG1	>100[a]	>100
UNG2	>100	>100

Table 2. Selectivity profile for compounds **14** and **18** towards selected base excision repair enzymes and DNA intercalation, expressed as IC_{50} values (µM). [a]Full inhibition is not observed, 50% inhibition plateau at 100 µM.

Antibodies

Purified hsdCTPase was applied for polyclonal antibody production in EZBiolabs, USA. dCTPase Ab was purified from rabbit serum on a home-made affinity column with immobilized dCTPase using AminoLinkTM Immobilization Kit (44890, Thermo Scientific). Primary antibodies used: anti-Actin (AC-15, ab6276, Abcam), Anti-phospho-histone H2AX (S139, 05-636, Millipore), anti-53Bp1 (A300-272A, Bethyl Laboratories), cleaved caspase 3 (AB3623, Millipore), anti-Chk1 (#2360, Cell Signaling), anti-phospho Chk1 (S345) (#2341, Cell signalling), goat anti-mouse IgG Alexa Fluor® 488 (A11029, Life technologies), donkey anti-rabbit IgG IgG Alexa Fluor® 555 (A31572, Life technologies), donkey anti-rabbit IRDye 680RD (925-68073, LiCor) and donkey anti-mouse 800CW (925-32212, LiCor).

Purification of human recombinant dCTPase

Human hdCTPase was cloned into a modified pET28 vector (Novagen) with double N-terminal His-tag and TEV protease cleavage site separated from the protein sequence. This construct was transformed into *E. coli* strain BL21(DE3)pLysS, the bacteria was grown in LB medium at 37 °C and dCTPase expression was induced by addition of IPTG to a final concentration of 0.5 mM for 2h. His-tagged dCTPase was purified by immobilized metal ion affinity chromatography (IMAC) using a Ni-Sepharose HisTrap column (GE Healthcare). The His-tag was removed by TEV protease digestion at 4 °C for 24 h followed by passing the digested protein through a HisTrap FF column. Finally, dCTPase was purified using a monoQ column HP, dialyzed against 20 mM HEPES pH 7.5, 300 mM NaCl, 1 mM TCEP, 10% glycerol and kept at -80 °C.

Compound libraries

Screening for inhibitors of human recombinant dCTPase was done at Chemical Biology Consortium Sweden (CBCS) using 10 µM compound concentration in 96-well microtiter plates. The screen campaign was based on a diversity set of small molecule compounds (kindly donated by Biovitrum AB, Sweden). All assay plates contained negative (wells A12-D12) and positive controls (wells E12-H12),

where negative controls represent uninhibited enzyme, corresponding to wells with the equivalent amount of DMSO as the compound containing wells, and positive controls represent the completely inhibited enzyme, corresponding to wells containing 20 mM EDTA (thus chelating all magnesium ions). The compound stock solutions were stored frozen at -20 °C in REMP 96 Storage Tube Racks, where each solution is stored in an individual capped tube containing up to 200 µl. The racks were stored in a REMP Small-Size Store™. To enable the flexibility to screen at multiple locations CBCS has established a routine in which single use daughter plates holding 2 µl of each individual compound solution per well are prepared in multiple copies and then kept at -18 °C in a standard freezer until used (plating service provided by iNovacia AB, Stockholm, Sweden). The compound solutions were placed in columns 1 through to 11 in round-bottom Nunc plates (267245).

Screening assay

The enzymatic assay applied for screening purposes is based on the enzymatic hydrolysis of dCTP by purified human recombinant dCTPase to form dCMP and pyrophosphate. An excess of *E.coli* inorganic pyrophosphatase (PPase) is added to the reaction mixture to hydrolyse pyrophosphate to inorganic phosphate. Inorganic phosphate is measured by monitoring the absorbance at 630 nm after incubation with malachite green reagent, as previously described.[1] dCTPase, PPase and dCTP were diluted in an assay buffer 100 mM Tris-acetate at pH 8.0, 10 mM magnesium acetate, 100 mM potassium chloride, 0.005% Tween-20 and 1 mM dithiothreitol (DTT). The contents of the assay ready plates were diluted 100-fold by assay buffer using a PerkinElmer FlexDrop, followed by transfer of 10 µl of the diluted compound solutions to assay plates using a Cybiwell liquid handling station equipped with a 96-well head. Working solutions of dCTPase and inorganic pyrophosphatase (35 µl) and dCTP (65 µl) were then added to all plates using the FlexDrop. The final conditions in the assay during enzymatic incubation were 25 nM recombinant human dCTPase, 35 µM dCTP and 0.2 U/ml of inorganic pyrophosphatase in a total assay volume of 100 µl in a transparent 96-well plate (Nunc 269620). Following incubation of enzymes and substrate at room temperature for 1h, the reaction was terminated and the signal developed by the addition of 25 µl of the malachite green reagent using a MultiDrop (Thermo Scientific). The plates were analysed in a microplate reader using a filter at 630 nm (Victor 2, PerkinElmer). Reported IC_{50} values are the average of two experimental determinations, unless stated otherwise.

Jump Dilution assay

Performed according to a modified procedure from Copeland et al.[2] 500nM inhibitor (10 x IC_{50} concentration) was pre incubated for 1 hour with 3.5µM dCTPase with His tag (100 x enzyme concentration) followed by a 100-fold dilution with dCTP substrate solution to a final concentration of 5 nM compound, 35 nM enzyme and 35µM dCTP substrate. The assay buffer consisted of 100mM Tris-Acetate (pH 8), 100 mM potassium chloride, 10 mM magnesium acetate, 1mM dithiothreitol and 0.005% Tween20. The enzymatic reaction was incubated at different time points and terminated by the addition of Malachite Green reagent followed by an absorbance measurement at 630nm on a

SpectraMax Plate reader after an additional 10 and 15 min incubation. The positive control contained both PPase and dCTPase (0% inhibition) and the negative control contained only PPase (100% inhibition).

Differential Scanning Fluorimetry assay

Differential Scanning Fluorimetry (DSF) was used to identify compounds stabilizing the purified recombinant human dCTPase enzyme. The degree of protein unfolding as a function of temperature is detected by an increase in fluorescence of the Sypro Orange dye upon binding to the hydrophobic parts of the unfolding protein. Compounds that bind to and stabilize the enzyme raise the protein melting point (T_m) leading to temperature shift of the increase in fluorescence upon protein unfolding. The assay buffer in which dCTPase, inhibitors and dye were diluted consisted of 100 mM Tris-acetate pH 8.0, 100 mM KCl, 0.005% Tween 20 and 1 mM DTT. The assay concentrations used were 0.001-100 µM inhibitor, 20 µM recombinant human dCTPase and Sypro Orange 5X; 1% final concentration of DMSO. The assay volumes were 20 µl in 96-well Q-PCR plates. A BioRad Q-PCR instrument was used to ramp the temperature from 20-100 °C at 1 °C/min and analysing fluorescence intensity at each step. The Bio-Rad software was used to calculate the T_m (negative) of each well of the plate.

NTPases / NUDIX hydrolases selectivity assays

Performed according to Gad *et al*.[3]

Selectivity assay for DNA repair enzymes

DNA repair enzyme activity was measured using an adapted molecular beacon protocol from Svilar et al.[4] The assay contains a duplex containing a FAM fluorophore on the 5' end of one strand oligonucleotide (5'-(FAM)CTGCCAXCACTGCGTCGACCTG-3') and a dabcyl quencher at the 3' end of the complementary strand (5'-CAGGTCGACGCAGTGYTGGCAG(Dab)-3'. X= modified base, Y: base paired with damaged base when annealed to the FAM-containing strand). Sequential DNA glycosylase and/or AP endonuclease activities leads to the generation of a strand break and dissociation of the FAM-containing strand which generates the signal. Purified DNA glycosylases and AP endonuclease 1 were incubated in reaction buffer containing 25 mM Tris-HCl pH 8.0, 15 mM NaCl, 2 mM $MgCl_2$, 0.0025% Tween20 and 0.5 mM DTT in black 384-well plates under the following conditions: APE endonuclease 1 (APE1) assay: X=AP-site, Y=A, 20 nM DNA, 0.2 nM APE1, *E.coli* formamidopyrimidine [fapy]-DNA glycosylase (Fpg) assay: X=AP-site, Y=C, 15 nM DNA, 0.05 U Fpg per reaction. Endonuclease 8-like 1 (NEIL1) assay: X=thymine glycol, Y=A, 10 nM DNA, 5 nM NEIL1, 2 nM APE1. Methylpurine-DNA glycosylase (MPG) assay: X=inosine, Y=T, 0.3 U MPG per reaction, 2 nM APE1. 8-oxoguanine-DNA glycosylase (OGG1) assay: X=8oxo, Y=C, 10 nM DNA, 0.8 nM OGG1, 2 nM APE1. Single-strand selective monofunctional uracil-DNA glycosylase 1 (SMUG1) assay: X=uracil, Y=guanine, 250 nM DNA (of which 90% was unlabelled), 0.25 U SMUG1 per reaction and 2 nM APE1. Uracil-DNA glycosylase 2 (UNG2) assay: X=uracil, Y=A, 500 nM DNA (of which 95% was

unlabelled), 0.075 nM UNG2 and 2 nM APE1. None of the reactions yielded any signal on undamaged control duplex oligos (X=G,Y=C) under these conditions (not shown. The reaction buffer was 25 mM tris-HCl pH 8.0, 15 mM NaCl, 2 mM $MgCl_2$, 0.0025% tween20 and 0.5 mM DTT and was performed in black 384-well plates. The signal was read in a Hidex sense plate reader using the 485(10) filter for excitation and the 535(20) filter for emission. Fpg, MPG and SMUG1 were purchased from New England Biolabs while the rest of the enzymes were expressed in bacteria with histidine-tag and purified essentially as described for dCTPase.

Kinase selectivity assays

For KIT, RET, JAK2, PDGFR-B, PDGFR-A, FMS, FLT-3, IKK2, IKK1, TBK1, IKKe, LMTK3, PDK-1:

Kinase assays. Kinase activity was measured using a LANCE Ultra time-resolved fluorescence resonance energy transfer (TR-FRET) assay and purified recombinant kinases (all from Carna Biosciences, except PDK-1 which was from SignalChem). Kinase assays were carried out in 5 µl kinase buffer (50 mM Hepes pH 7.4, 10 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, and 0.01% Tween-20) with an ATP concentration about two-fold the *Km* for each enzyme. The conditions for each kinase were as follows (reaction time for each kinase indicated in parenthesis): 4 nM IKKα (90 min) and 1 nM IKKβ (60 min) with 2 µM ATP and Ulight-IκBα (Perkin Elmer); 4 nM IKKε/TBK1 (90 min) with 10 µM ATP and Ulight-rpS6 peptide(Perkin Elmer); 0.3 nM JAK2 (60 min) with Ulight-JAK1 (Perkin Elmer) and 10 µM ATP; 0.25 nM ABL (90 min) with 2 µM ATP and Ulight-pGT (Perkin Elmer); 0.1 nM FLT3 (90 min) with 25 µM ATP and Ulight-pGT; 0.3 nM KIT (30 min), 0.4 nM FMS (60 min), 1 nM PDGFRα and 0.5 nM PDGFRβ all with 10 µM ATP and Ulight-pGT; 0.1 nM RET (30 min) with Ulight-pGT and 0.25 µM ATP. PDK1 was incubated with 125 nM biotin-PDKTide (AnaSpec) and 0.5 µM ATP for 30 min. All kinases were incubated at room temperature for the indicated periods of time in the presence of solvent (1% DMSO) or 10 µM TH620/TH859. Kinase reactions with IKKs were stopped by adding 5 µl of 20 mM EDTA in LANCE detection buffer containing 2 nM of the corresponding Europium-labelled antibody (Eu-pIκBα or Eu-prpS6, Perkin Elmer). PDK1 reaction was stopped with 20 mM EDTA/LANCE containing 2 nM Eu-labelled phospho-Thr antibody (Perkin Elmer) and 25 nM Sa-XL665 (CisBio). All Tyrosine kinases were stopped with Eu-PT66 antibody (CisBio) in HTRF detection buffer (CisBio). Following a 2 h incubation at RT, the TR-FRET signals at 620 and 665 nm were measured in a CLARIOstar (BMGLabtech) multilabel reader and the 665/620 ratios were used to calculate the percentage of kinase activity remaining in the presence of compound with respect to solvent controls.

For Pim1:

Active recombinant GST-tagged M_1-K_{313} Pim1 was purchased from ProQinase (Germany). Assay conditions: Pim1 reaction were performed in 60 mM HEPES/NaOH pH 7.5, 0,0013% NP40, 10% DMSO, 3 mM $MnCl_2$/$MgCl_2$, 50 µM ATP/[γ-33P]ATP, 50 µM peptide substrate (R11-SGRARTSSFAEPGGK), 20 ng active enzyme. The reaction was performed in 10 ml at 30 °C for 10 min. Reaction was stopped by adding 5µl of phosphoric acid 0.8%. Aliquots (10 µL) were then transferred into a P30 Filtermat (PerkinElmer), washed five times with 75 mm phosphoric acid and

once with acetone for 5 min. The filter was dried and transferred to a sealable plastic bag, and scintillation cocktail (4 mL) was added. Spotted reaction was read in a scintillation counter (Trilux, Perkinelmer). To avoid adsorption of enzyme and peptide substrate, low binding plasticware (Eppendorf, LoBind) was used.

For CDK2, CDK5, CDK9, CLK1, CLK2, CLK3, CLK4, CK1, DYKR1A, DYRK1B, DYRK2, DYRK3, GSK3, Ld DYRK4, Pf CLK1, Tb CLK1, Tc CLK1, Lm CLK, Ld DYRK1B, Lm DYRK2, Ld DYRK3, Lm CK1, Cp Lammer, Tg CLK, Gl CLK:

Performed according to Tahtouh *et al*.[5]

DNA intercalation assay

DNA intercalation was measured in a thiazole orange (ThO) displacement assay performed according to Tse *et al*.[6] ThO is a weak intercalating compound that displays a several houndred-fold increase in fluorescence when it binds DNA. Briefly, 50 nM of a duplex oligo substrate DNA repair assays described above is incubated in black 384-well plates with 500 nM ThO in the presence of compound 14 and 18 with DMSO as control under the same buffer conditions as for the DNA repair assays. Propidium iodide, a well-known and frequently used intercalator, was used as positive control. Fluorescence was read after incubation for 5 minutes at room temperature in a Hidex sense plate reader, using the 485(10) filter for excitation and the 535(20) filter for emission.

Photo-stability measurements

A 10 mM solution DMSO stored in a 2 mL Eppendorf tube at room temperature under normal laboratory light conditions was periodically monitored by LC-MS over a period of 5 months. 5 µL samples were diluted in 200 µL of MeOH and analyzed using LC-MS Method B1090A (see Chemistry General Information) at 305, 254 and 220 nM wavelengths. No UV-active peaks or mass signals appeared during the monitored period.

Genotoxicity assay - Immunofluorescence

For 53BP1 and γ-H2AX quantification, HL60 cells were seeded in a T25 cell culture flasks ($8x10^5$ cells per flask) and treated with 2.5 µM **18** for 24h. Immunofluorescence staining was performed as described by Orta *et al*.[7] Images were acquired in a Zeiss LSM-780 confocal microscope with the 63x oil objective. A total of 150 cells /slide were counted and cells with 5 or more co-localized foci of 53BP1 and γ-H2AX were considered as positive cells.

Cell Culture

HL60 cells were grown in RPMI-Glutamax (Lifetechnologies, cat. Nr. 61870-010), containing 10% fetal bovine serum (FBS), penicillin (50 U/ml) and streptomycin (50 µg/ml). Cells were maintained at 37ºC in a 5% CO_2 atmosphere.

Flow Cytometry

All experiments were performed on a Navios flow cytometer from Beckman Coulter. For generation of DNA histograms and scoring of (SubG1) dead cells, cells were harvested at the indicated time point, spun down, washed once in cold PBS and then resuspended in propidium iodide buffer (20 mM Tris pH 8.0, 50 µg/ml PI, 100 mM NaCl, 0.1% NP40, 20 µg/ml RNAse). After 1h incubation in the dark (4 °C) the samples were analysed in the FL3 channel on a linear scale for DNA histograms and in the FL2 channel on a logarithmic scale for subG1 content (one log scale below the G1 population was scored as dead cells).

Cellular Thermal Shift (CETSA)

Performed according to Molina *et al*.,[8] with the exception of use of Complete protease inhibitor cocktail (Roche) in the lysis buffer.

Viability Assays and Combination Index Analysis

Resazurin viability assay was performed according to Gad *et al*.[3]

Combination Index analysis was performed using Compusyn according to Chou.[9]

ADME

Performed according to Gad *et al*.,[3] except for the Cytochrome P450 (CYP) inhibition assay. A test compound (0-100 µM) and specific probe substrates were pre-incubated with human liver microsomes for 10 minutes, before the reactions were initiated by the addition of NADPH to give a final incubation volume of 100 µL. After different incubation times (enzyme specific), the reactions were terminated by the addition of ice-cold acetonitrile (100 µL) containing internal standard (warfarin, final concentration of 50 nM). All incubations were performed in duplicate.

For GSH adduct detection, compounds **18** and **30** at the final concentration of 10 umol/L were incubated in the presence of human liver microsomes (HLM, final concentration 1 ug/ul) over 30 and 60 min. The incubations were performed in 100 mM KPO4 buffer, pH 7.4 and in the presence of 5 mM glutathione (GSH). The incubations were started by adding of NADPH at the final concentration of 1 mM. After termination with ice-cold acetonitrile the samples were centrifuged 20 min at 3500 rpm, at 4oC and supernatant was transferred to a new vial. The samples were evaporated and rest was dissolved in acetonitrile/water (50:50, v/v).

The samples were analyzed using UPLC-Q-Trap mass spectrometer (from AB Sciex) operating in neutral lost and ion precursor modes. No indication of glutathione adducts for the tested compounds were detected.

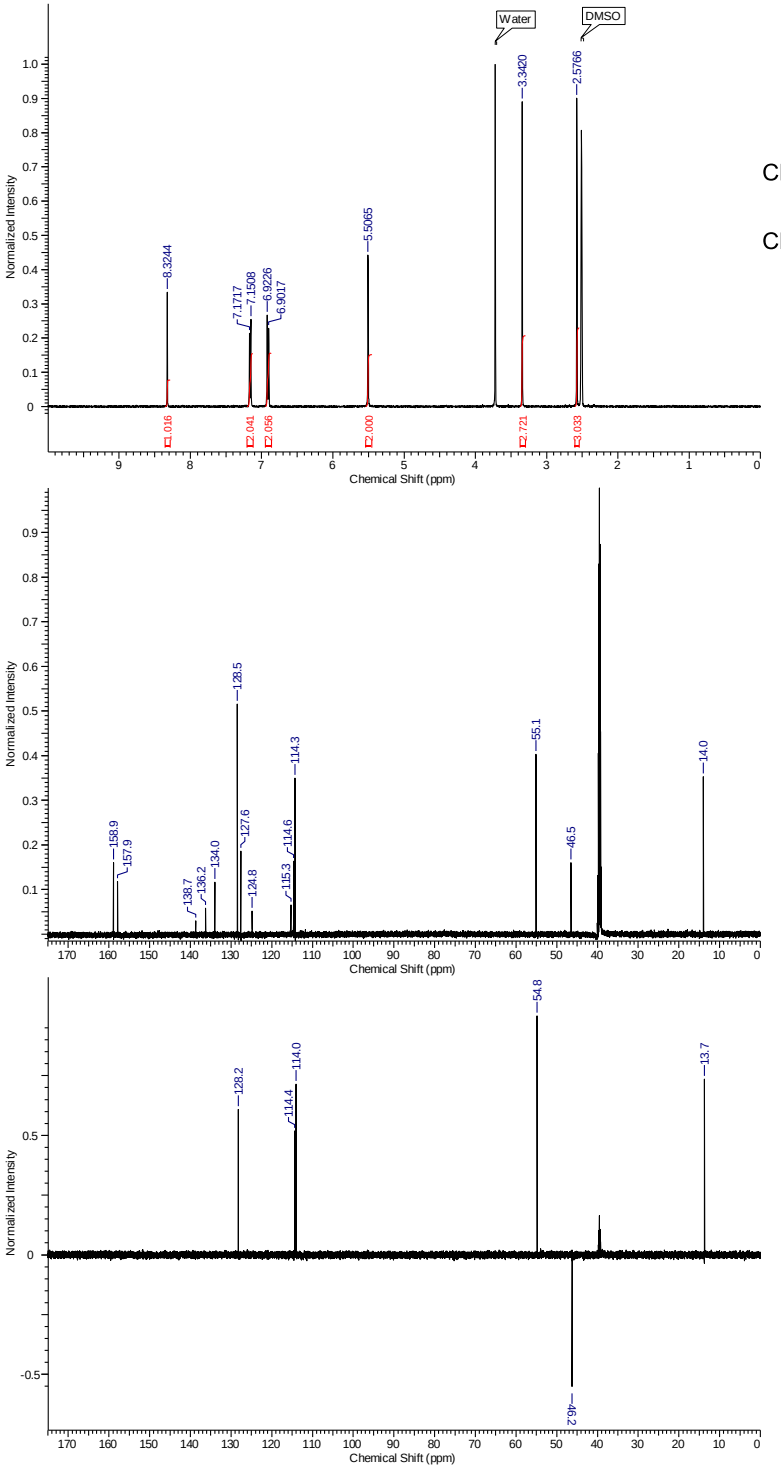
Following probe substrates were used for measurement of activity of respective cytochrome P450 enzymes: CYP1A2–Phenacetin, CYP2C9-Diclofenac, CYP2C19-*S*-Mephenytoin, CYP2D6-Bufuralol and CYP3A4-Midazolam and Testostrone.

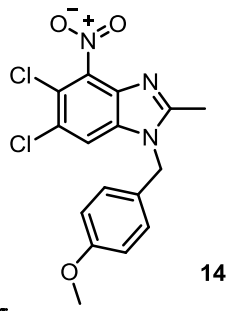
Different control incubations were included in the assays. As negative controls (single incubations), incubations were performed without NADPH (volume adjusted with phosphate buffer), and without microsomes (only phosphate buffer). In order to verify that the system responds proper to inhibition, the compounds with known inhibitory potency towards each CYP isoform were used as positive controls. The terminated incubations were kept on ice for about 30 minutes before they were centrifuged at ca 3000 rpm for 15 min. The samples were analysed by LC/MS/MS. Standard curves were prepared for the substrate metabolites in 50 mM phosphate buffer, pH 7.4, in appropriate ranges (100 µL total volume).

Data analysis and statistics

All statistical significant difference was calculated in Excel or GraphPad by using the student t-test

Copies of NMR spectra for relevant compounds (14,18 and 30)





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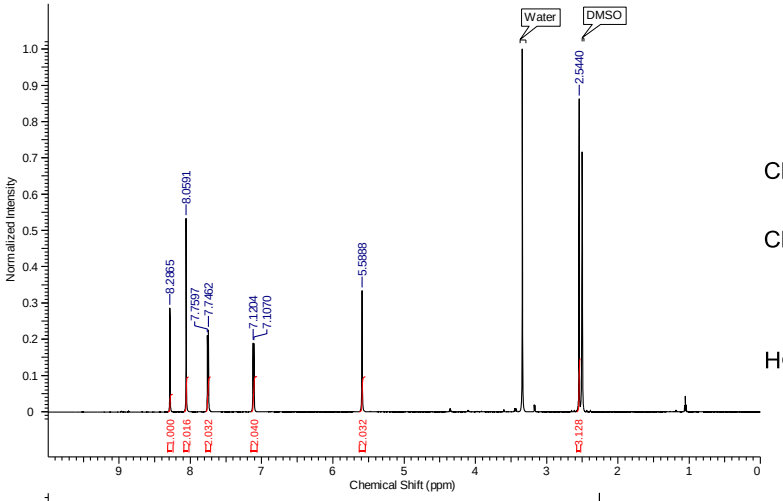
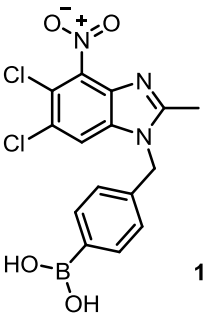
^{1}H and ^{13}C NMR spectra for compound **14**





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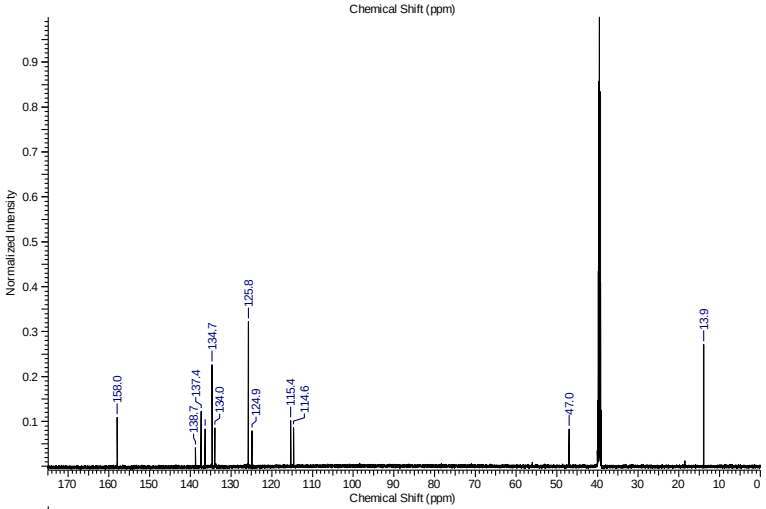
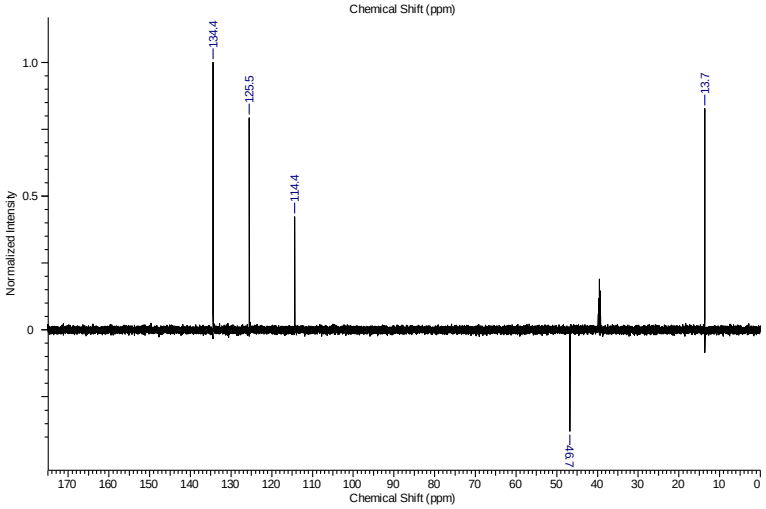
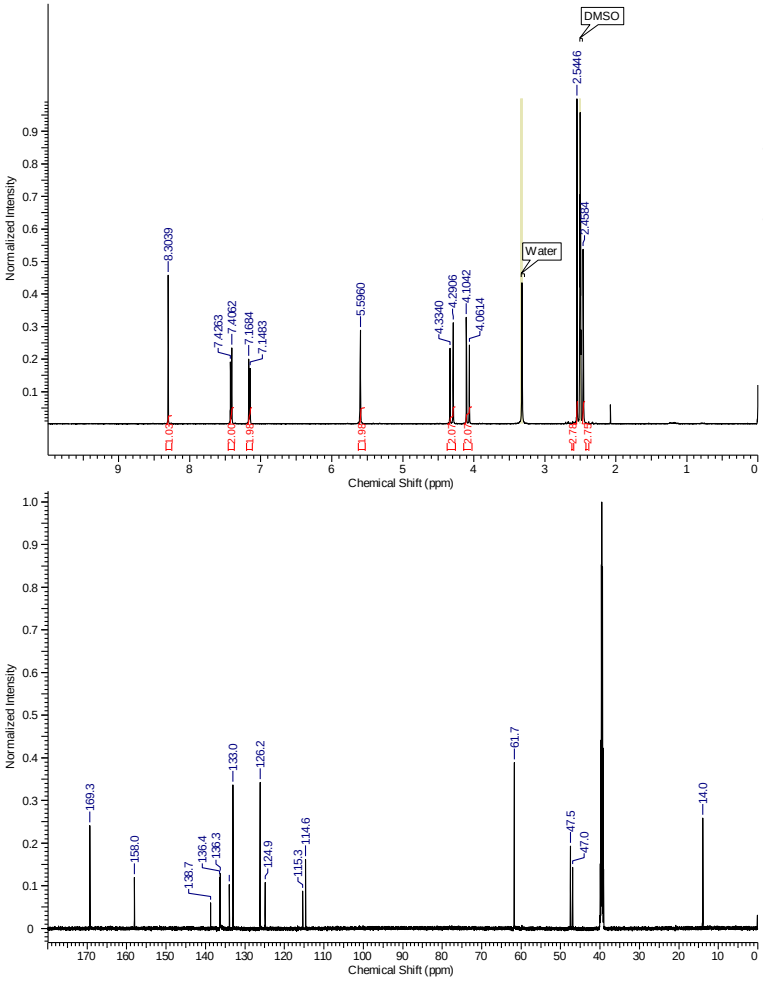
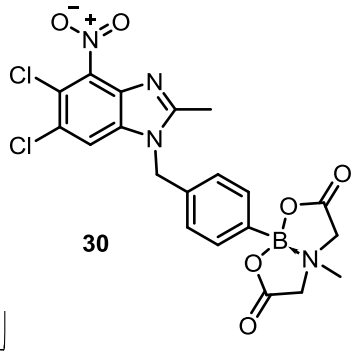




^{1}H and ^{13}C NMR spectra for compound **18**





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^{1}H and ^{13}C NMR spectra for compound **30**

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