

## Validation and development of MTH1 inhibitors for treatment of cancer

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**Background:** Previously, we showed cancer cells rely on the MTH1 protein to prevent incorporation of otherwise deadly oxidised nucleotides into DNA and we developed MTH1 inhibitors which selectively kill cancer cells. Recently, several new and potent inhibitors of MTH1 were demonstrated to be non-toxic to cancer cells, challenging the utility of MTH1 inhibition as a target for cancer treatment.

**Material and methods:** Human cancer cell lines were exposed *in vitro* to MTH1 inhibitors or depleted of MTH1 by siRNA or shRNA. 8-oxodG was measured by immunostaining and modified comet assay. Thermal Proteome profiling, proteomics, cellular thermal shift assays, kinase and CEREP panel were used for target engagement, mode of action and selectivity investigations of MTH1 inhibitors. Effect of MTH1 inhibition on tumour growth was explored in BRAF V600E-mutated malignant melanoma patient derived xenograft and human colon cancer SW480 and HCT116 xenograft models.

**Results:** Here, we demonstrate that recently described MTH1 inhibitors, which fail to kill cancer cells, also fail to introduce the toxic oxidized nucleotides into DNA. We also describe a new MTH1 inhibitor TH1579, (Karonudib), an analogue of TH588, which is a potent, selective MTH1 inhibitor with good oral availability and demonstrates excellent pharmacokinetic and anti-cancer properties *in vivo*.

**Conclusion:** We demonstrate that in order to kill cancer cells MTH1 inhibitors must also introduce oxidized nucleotides into DNA. Furthermore, we describe TH1579 as a best-in-class MTH1 inhibitor, which we expect to be useful in order to further validate the MTH1 inhibitor concept.

**Key words:** MTH1, reactive oxygen species, cancer, small molecule inhibitors, DNA damage

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## introduction

Oxygen metabolism is central to vertebrates and other life forms and reactive oxygen species (ROS) have both beneficial and deleterious functions in cells. There is overwhelming evidence that many diseases are associated with loss of balanced redox homeostasis and/or ROS, e.g. cancer, cardiovascular disease, hypertension, inflammatory diseases (e.g. atherosclerosis, rheumatoid arthritis), ischemia-reperfusion injury, diabetes mellitus, neurodegenerative diseases, and ageing [1]. Recently, we and others described that the normally non-essential MTH1 enzyme is required for survival of cancer cells independent of tissue of origin [2–6]. Dysfunctional redox regulation and ROS in cancer cells predominantly damage nucleobases in the free deoxynucleoside triphosphate (dNTP) pool [7] and the role of the MTH1 protein is to prevent these oxidized dNTPs, e.g. 8-oxodGTP or 2-OH-dATP, from being incorporated into DNA and killing cells. The MTH1 protein is non-essential in non-transformed cells and MTH1 knockout mice live and grow old [3, 8]. We have developed small molecule MTH1 inhibitors that are selectively toxic to cancer cells while sparing normal, healthy cells, which may represent a paradigm shift in the treatment of cancer [3]. Three recent papers question the validity of MTH1 as a target and described potent MTH1 inhibitors that failed to kill cancer cells [9–11], whilst presumably targeting the MTH1 protein in cells.

Here, we evaluate some of these inhibitors and observe that these also fail to introduce the toxic 8-oxodG lesion into DNA. Furthermore, we describe TH1579 (Karonudib) as a potent MTH1 inhibitor with excellent pharmacokinetic and pharmacodynamic properties, which supports the clinical potential of an MTH1 inhibitor.

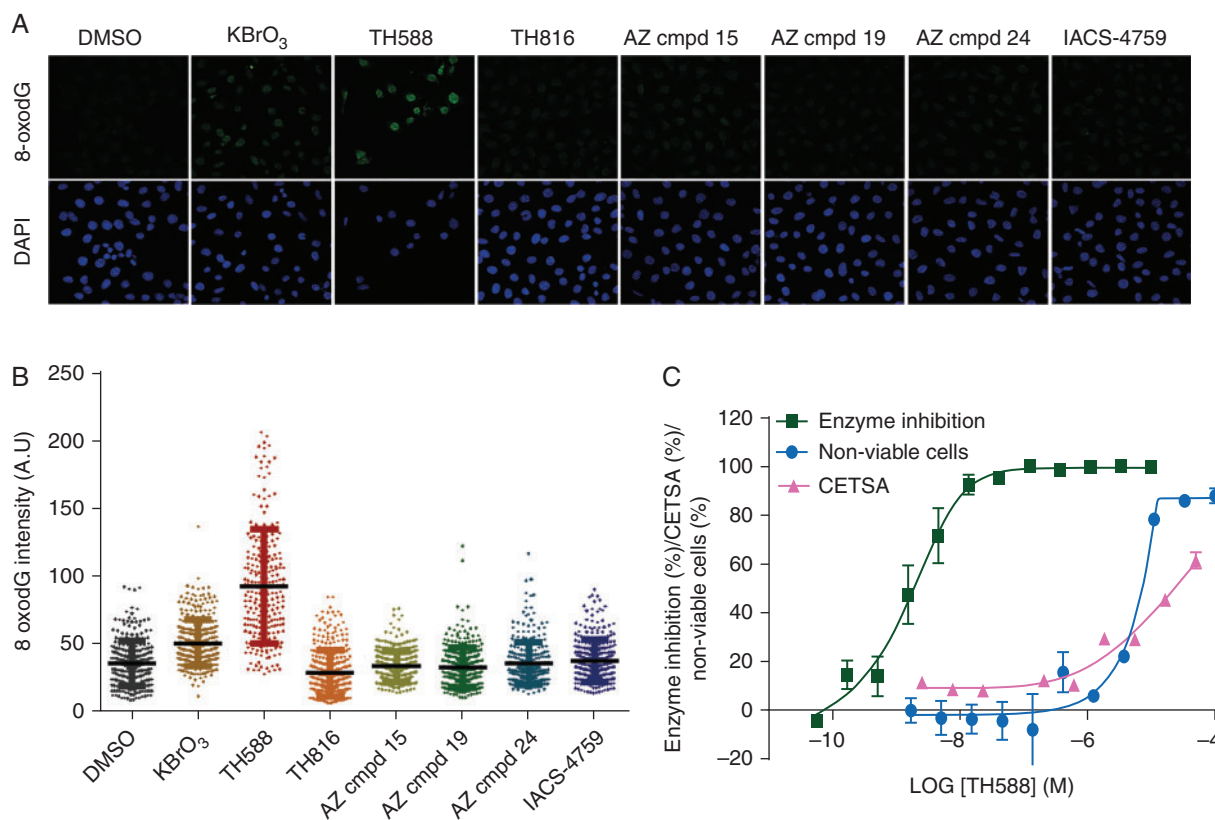
## material and methods

### drugs, assay conditions and statistical analysis

All compounds were synthesized in house except AZ compounds 15, 19 and 24 [10] and IACS-4759 [9] that were provided by Astra Zeneca and MD Anderson Cancer Center.

All cells were cultured at 37°C in 5% CO<sub>2</sub> (10% FBS). Cell viability was measured with Resazurin assay and in clonogenic outgrowth assays, where cells were grown out to colonies for 8–10 days, prior to staining and colonies with ≥50 cells were counted as positive. To determine the effect of MTH1 siRNA on viability 1000 cells were seeded onto the mixture of interferin and Non target siRNA control or MTH1 siRNA (10 nM) in 96 well plate, 24 h later media was replaced with fresh media and incubated for another 5 days.

For statistical analysis one-way and two-way ANOVA analysis was performed in GraphPad Prism software. All other



**Figure 1.** MTH1 inhibitors not increasing 8-oxodG in DNA do not cause cell death. (A) Representative confocal images of 8-oxodG staining in NTUB1/P cancer cells following treatment with KBrO<sub>3</sub> and various MTH1 inhibitors. (B) Quantification of 8-oxodG intensity following 24 h treatment with TH588 (10 μM), AZ compound 15 (10 μM), AZ compound 19 (10 μM), AZ compound 24 (10 μM), IACS-4759 (10 μM) and TH816 (10 μM) and 30 min treatment with KBrO<sub>3</sub> (50 mM). Nuclear intensity was measured using cell profiler ( $n = 2$ ). (C) Enzymatic inhibition of TH588 (square label), effect of TH588 to reduce BJ-hTERT-Ras-Sv40T cell viability using resazurin assay (circle) and target engagement of TH588 in BJ-hTERT-Ras-Sv40T cells using CETSA (triangle). Data shown as % of controls, mean ± s.d. of at least two independent experiments performed in duplicate.

experiments are described in detail in supported materials and methods.

## results

### MTH1 inhibitors failing to kill cancer cells fail to introduce 8-oxoG into DNA

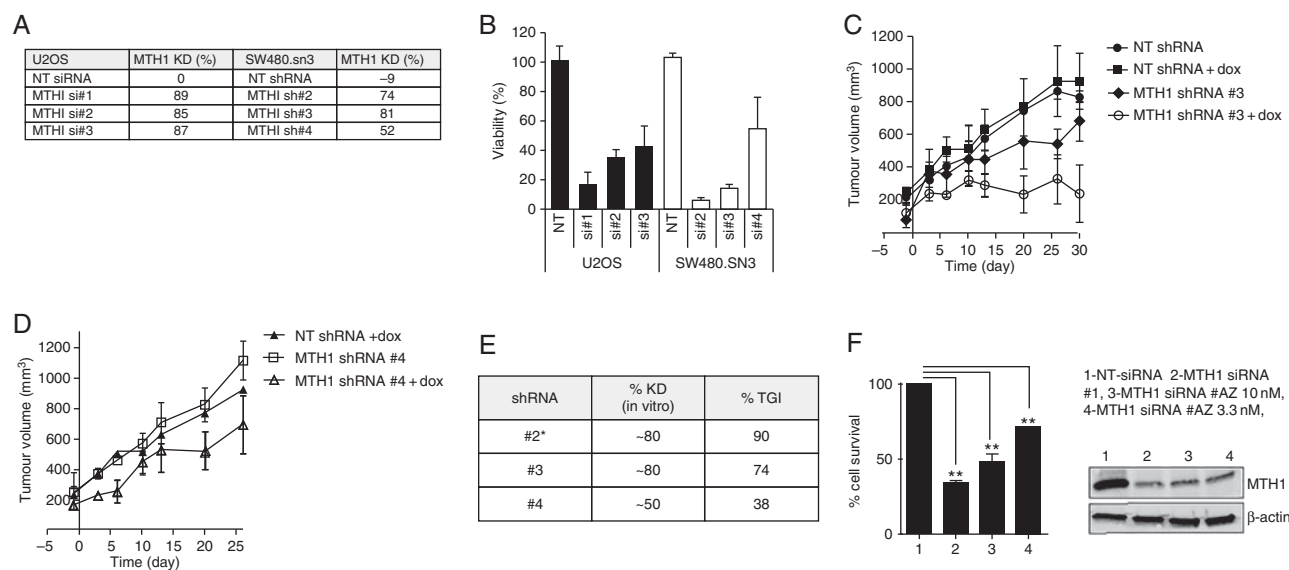
We hypothesized the reason for lack of cellular effect by the recently reported MTH1 inhibitors [9–11] could be that they failed to introduce the toxic lesion, 8-oxoG into DNA. Indeed neither the AZ nor IACS compounds introduce 8-oxoG into DNA as visualized by immunofluorescence (Figure 1A and B). Similarly, TH816, an analogue of TH588, with a biochemical IC<sub>50</sub> of 1 nM, that only induces cancer cell toxicity at high μM concentrations (supplementary Figure S1, available at *Annals of Oncology* online), does not cause increased 8-oxoG levels in DNA at concentrations below 10 μM (Figure 1A and B), which is consistent with lack of target engagement in cells at lower concentrations (supplementary Figure S1, available at *Annals of Oncology* online). TH588 elevates 8-oxodG in DNA (Figure 1A and B) and has a good correlation between target engagement within living cancer cells and reduced cancer cell viability (Figure 1C). There is a possibility that TH588, but not the IACS and AZ compounds, inhibits an MTH1-independent 8-oxodGTPase activity in the cell, resulting in the increased levels of 8-oxodG in DNA. MTH2 and MTH3 have been described as having 8-oxodGTPase activity *in vitro* [12, 13], however we

recently demonstrated that neither of the two proteins have physiological 8-oxodGTPase activities [14]. TH588 does not inhibit MTH2 or MTH3 enzymatic activities [3] nor does our more optimized compound TH1579 (see below). Using bioinformatics approaches (supplementary Figure S2, available at *Annals of Oncology* online) we wanted to identify possible sequences encoding novel 8-oxodGTPases, but besides MTH1 and other NUDIX family members, only four hits that were deemed irrelevant were identified (PDB codes: 3LVW, 2OYL, 3BHD and 1DJ9).

### MTH1 siRNA knockdown reduces viability

We have used three different siRNA sequences and four shRNA sequences (one overlapping with siRNA) to investigate the role of MTH1 in cancer cells (supplementary Figure S3, available at *Annals of Oncology* online). Whilst shRNA #1 had unsuccessful knockdown, five sequences show >70% knockdown and one sequence shows ~50% knockdown (Figure 2A). A significant reduction of cancer cell viability and survival was observed with siRNA #1,2,3 and shRNA #2,3,4 (Figure 2B). Furthermore, when MTH1 was suppressed in the SW480 MTH1 shRNA #2,3,4 mice xenograft, all three shRNAs reduced tumour growth corresponding to the level of MTH1 knockdown (Figure 2C–E and reference [3]).

Recently Kettle et al. [10] reported one MTH1 siRNA that does not influence cancer cell survival. In our hands, we observe



**Figure 2.** MTH1 suppression reduces cancer cell survival and viability. (A) Level of knockdown of MTH1 protein following various siRNA and shRNA measured using western blot. The siRNA/shRNA sequences are provided in supplementary Figure S3, available at *Annals of Oncology* online. Values shown as % of NT RNA control for MTH1 siRNA values and as % of uninduced samples (no doxocyclin) for MTH1 shRNA. Data shown from one representative experiment and  $\geq 3$  independent experiments have been performed. (B) Effect on U2OS and SW480 cells survival following various siRNA/shRNA knockdown. Values shown as mean  $\pm$  s.d. of two independent experiments. (C and D) Human colon cancer SW480 cells were transfected with a doxocyclin inducible shRNA# 3 (C), shRNA#4 (D) or non-targeting (NT) control and s.c. implanted into the flank of female SCID mice. When tumour reached approximately 200 mm<sup>3</sup> (Day 0), doxocycline was introduced into the drinking water resulting in suppression of MTH1 levels in the tumour as indicated in the figure. Values are shown as mean  $\pm$  s.e.m. of  $n = 5$ /group. (E) Table showing *in vitro* levels of MTH1 after doxocyclin induced shRNA#2,3,4 knockdown compared to *in vivo* effect on tumour growth as measured as % tumour growth inhibition (TG) where 0% is no inhibition compared to control group. (TG(%) = (TV(control) - TV(treatment))/TV(control)  $\times$  100, where TV is tumour volume the last day the first control animal reached TV of 1000 mm<sup>3</sup>. Results with shRNA#2 has previously been reported in Gad et al. [3]. (F) Effect on NTUB1/P cancer cell viability following MTH1 knockdown using AZ siRNA (sequence published in [10]). Data shown as mean  $\pm$  s.e.m. of at least 2 independent experiments.

decreased cell survival when using the same siRNA MTH1 sequence (Figure 2F).

### MTH1 inhibitor shows different mode of action compared to anti-microtubule agent

Kawamura et al. [11], recently showed that TH588 and TH287 inhibit beta-tubulin *in vitro* at concentrations above 30  $\mu\text{M}$  and show similar proteomic profile as anti-microtubule agents Nocodazole and Paclitaxel. Here we treated HCT116 cells with TH588, Paclitaxel, 5-Fluorouracil, or Camptothecin for 48 and 72 h and performed a proteomic study identifying 3982 proteins. Unsupervised principal component analysis (PCA) on the whole dataset demonstrated that the results clustered reasonably well (data not shown). Supervised OPLS analysis of the whole dataset revealed the mode of death induced by TH588 was unique from all other molecules used in this study, including Paclitaxel, as well as starvation/senescence (Figure 3A). Furthermore, the effect on tubulin proteins levels following TH588 treatment differed from Paclitaxel treatment (Figure 3B). In addition, MTH1 inhibitors induce 53BP1 foci that can be rescued by MutT expression (supplementary Figure S4A and B, available at *Annals of Oncology* online), while most anti-microtubule agents tested did not induce 53BP1 foci (supplementary Figure S4A, available at *Annals of Oncology* online). Taken together the data supports different mechanisms of action of these two classes of drugs.

### TH1579 is a potent MTH1 inhibitor causing accumulation of 8-oxoG into DNA, in an MTH1-dependent manner

While further refining our ideas on the role of MTH1 inhibition in TH588-mediated cell death, we optimized TH588 and generated a more potent and orally available MTH1 inhibitor TH1579 (Karonudib) [15].

TH1579 inhibits MTH1 activity with an  $\text{IC}_{50}$  value of nM and effectively introduces 8-oxoG into cells. This effect can be fully reversed with the antioxidant N-acetylcysteine (NAC) or overexpression of human MTH1 or the bacterial MutT enzyme (Figure 4A–D). TH1579 kills SV40 large T and Ras transformed BJ cells (BJ-hTERT-Ras-SV40T) at nM concentrations, and is less toxic to the non-transformed counterpart, BJ-hTERT cells (Figure 4E). TH1579 stabilizes the MTH1 enzyme in cells at approximately the same concentration it exerts its cellular toxicity (Figure 4F). TH1579 triggered the same p53 dependent DNA damage response as previously demonstrated with TH588 (Figure 4G and H).

### selectivity profile of TH1579

TH1579 was demonstrated to be a highly selective MTH1 inhibitor when assayed against a panel of NUDIX hydrolase and (d)NTPase enzymes (Figure 5A). Against a panel of 87 other purified proteins, TH1579 showed good selectivity (supplementary Figure S5A, available at *Annals of Oncology* online), and no cross-reactivity against a panel of 45 kinases (supplementary Figure S5B, available at *Annals of Oncology* online), 6 base excision repair proteins (supplementary Figure S5C, available at *Annals of Oncology* online) and did not intercalate with DNA

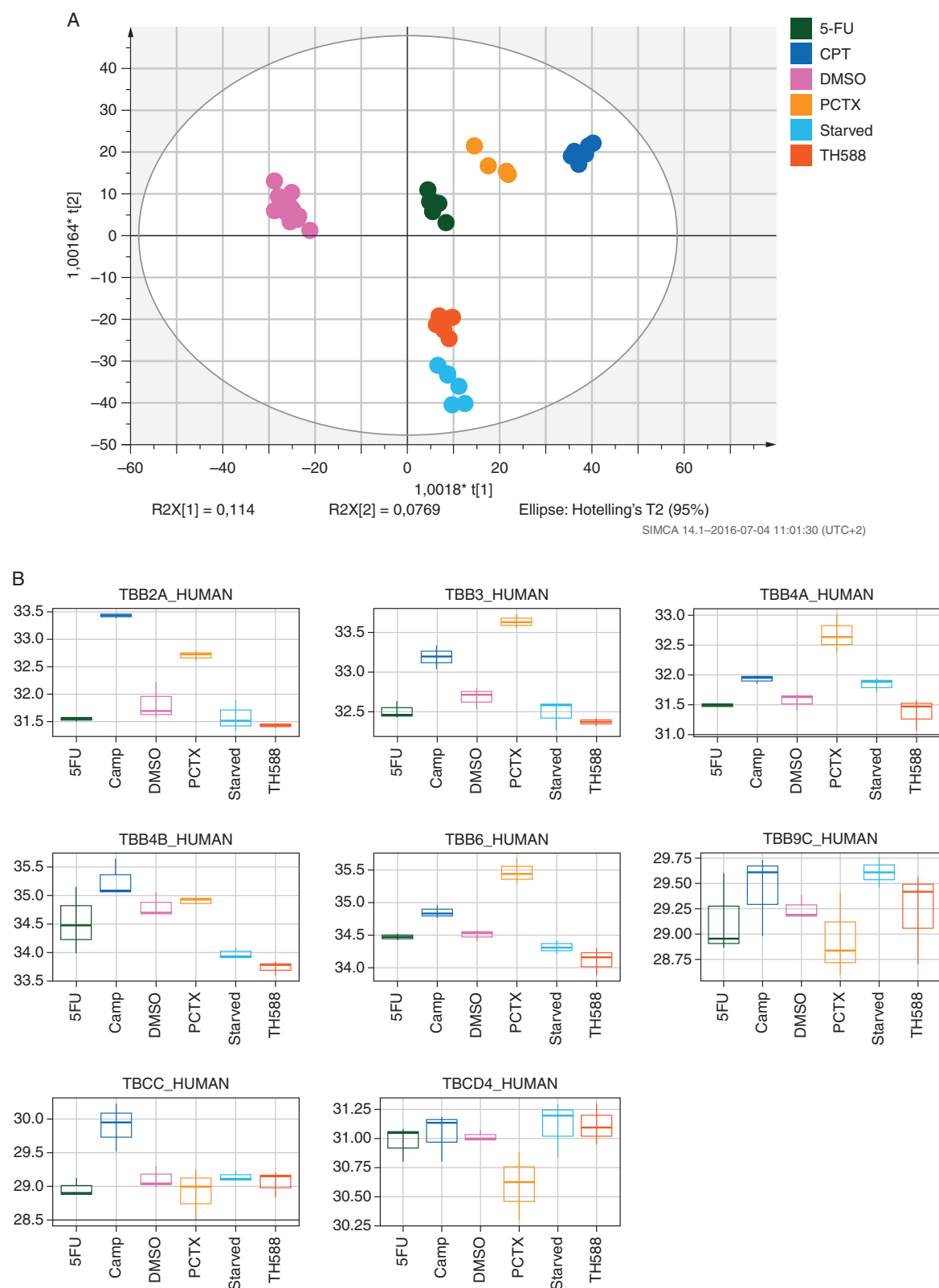
(supplementary Figure S5D, available at *Annals of Oncology* online) at the concentrations tested.

The selectivity data we generate here are relatively limited and therefore we performed a proteome wide Cellular Thermal Shift Assay coupled to mass spectrometry (CETSA-MS) study by treating BJ-hTERT-Ras-SV40T cells with 20  $\mu\text{M}$  of TH1579 (Figure 5B). 9301 proteins (based on 113126 peptides) were detected and out of these, 6405 complete melting curves were obtained. MTH1 was by far the most prominent protein engaged by TH1579 (Figure 5C) showing a thermal shift of approximately 12°C (Figure 5D), an effect that was confirmed in both living cells and cell lysate using western blot (Figure 5E and F). A curated list of the proteins showing significant shifts can be seen in supplementary Table S1A, available at *Annals of Oncology* online. Some of these hits were not confirmed by using western blot and some were only confirmed in living cells but not in cell lysate (supplementary Figures S6A–H, available at *Annals of Oncology* online), suggesting that it acts downstream of MTH1 inhibition. Interestingly, in view of the paper by Kawamura et al. [11], none of the tubulin proteins identified in our experiments were detected as significant targets after TH1579 treatment of living intact cells (supplementary Table S1B, available at *Annals of Oncology* online).

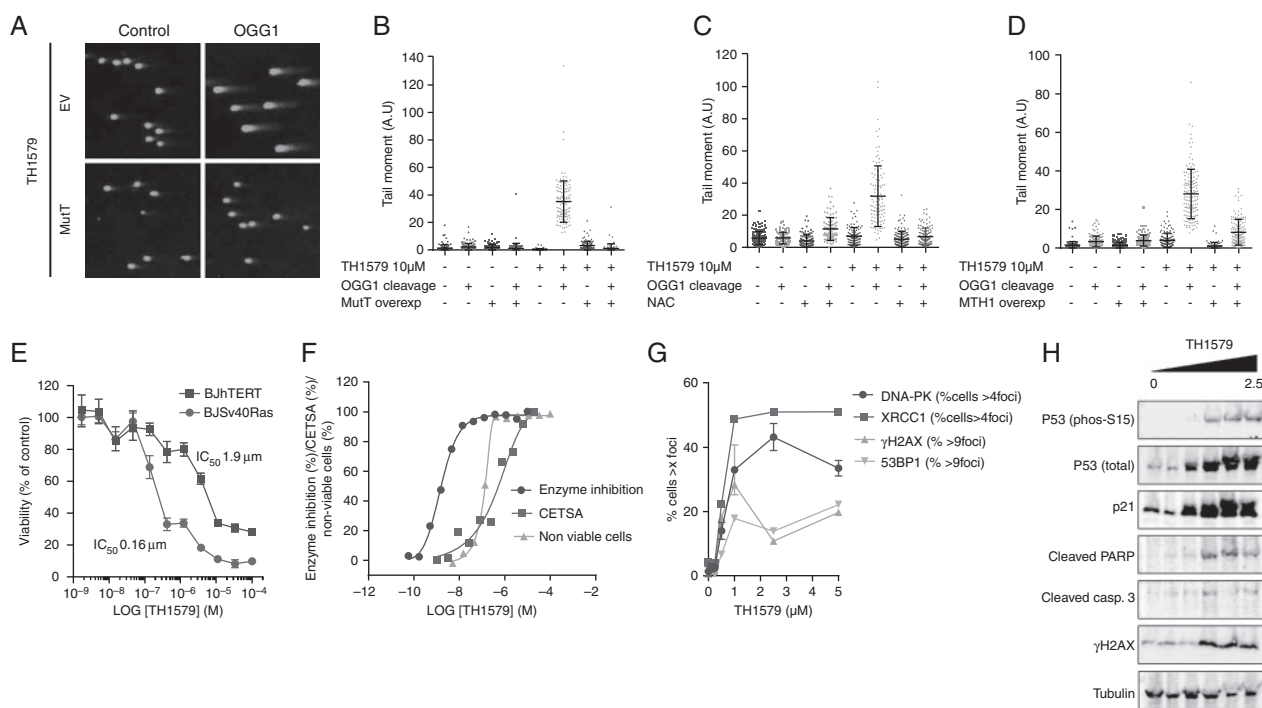
### TH1579 is effective in preclinical xenograft models

TH1579 shows good bioavailability (>60%), half-life of ~4 h, a volume of distribution ( $V_d$ ) of ~3 L/kg and a  $C_{\text{max}}$  of ~7  $\mu\text{M}$  following a 50 mg/kg dose given by oral gavage in NOD-SCID mice (supplementary Table S2, available at *Annals of Oncology* online). In a human colon cancer SW480 xenograft mice model, TH1579 (30 mg/kg b.i.d., p.o., daily dose) significantly reduced the tumour growth, while once daily TH1579 and 5-FU (30 mg/kg 3 times/week, i.p.) did not (Figure 6A). No significant change in *MTH1*, *MTH2*, *MutYH*, *NUDT5* or *OGG1* mRNA levels were detected after TH1579 treatment (supplementary Figure S7A, available at *Annals of Oncology* online). TH1579 (30 mg/kg b.i.d. and q.d. daily dose) was well tolerated, while 5-FU treatment reduced body weight (Figure 6B). Next, a dose response study was performed using 30, 60 and 90 mg/kg b.i.d. daily treatment of TH1579 in the same disease model. Two animals in the 90 mg/kg daily dose group was euthanized at Day 19, due to a significant body weight drop, diarrhoea, piloerection and reduced locomotion. The treatment of all other animals in the 90 mg/kg group and in the 60 mg/kg group was stopped on Day 21 and Day 25, respectively, due to body weight reduction. The adverse events observed at the higher doses were reversible and the animals regained their body weight to control levels 1 week after end of treatment. The low dose (30 mg/kg b.i.d.) treatment group was treated for 30 days, with no significant effect on body weight or behaviour (Figure 6C). Interestingly, in the 90 mg/kg b.i.d. treatment group the tumours did not start to regrow until additional 30 days after finalizing the treatment (Figure 6C). In a BRAF V600E-mutated malignant melanoma patient-derived xenograft model previously described [3, 16], TH1579 (45 mg/kg b.i.d., p.o., 5 days a week) significantly hindered tumour growth (Figure 6D).

Various treatment regimen schedules were investigated resulting in a three time per week treatment schedule showing similar



**Figure 3.** Effect of death pathways on the proteome of HCT116 cells treated for 72 h by TH588 (5  $\mu$ M), Paclitaxel (PCTX, 100 nM), 5-Fluorouracil (5-FU, 50  $\mu$ M), Camptothecin (CPT, 75  $\mu$ M) and starvation compared to DMSO-treated control. (A) Supervised principal component analysis OPLS-DA of the protein abundancies. The first component ( $x$ -axis) separates treated versus untreated cells, while the second component ( $y$ -axis) demonstrates that the effect of TH588 is closer to starvation than to that of common anticancer drugs 5-FU, CPT and PCTX. (B) Relative protein expression levels for selected proteins.



**Figure 4.** MTH1 inhibitor TH1579 induces 8-oxodG, DNA damage and reduces cancer cell viability. (A and B) Modified comet assay of U2OS cells transfected with MutT and empty vector (EV) following 10 μM of TH1579 for 24 h. Quantifications (B) and representative images (A) of comets treated without (Control) and with OGG1 to identify 8-oxodG. (C and D) 8-oxodG analysis using the modified comet assay in U2OS cells (C) or SW480 cells with MTH1 overexpression (D). Cells were treated with 10 μM TH1579 for 24 h. In (C), cells were pre-treated with 5 mM NAC for 4 h before washout and treatment with TH1579. (E) BJ hTERT and BJ-hTERT-Ras-Sv40T cells were treated for 24 h with a range of concentrations of TH1579. TH1579 was washed out and following additional 48 h culture, viability was measured using resazurin. Data shown as mean ± s.e.m. of four independent experiments. (F) The enzymatic inhibitory effect following TH1579 treatment (circle), the effect of TH1579 on BJ-hTERT-Ras-Sv40T cell viability following 72 h treatment (triangle) and target engagement in BJ-hTERT-Ras-Sv40T cells following 60 min treatment using CETSA (square). Data presented as mean % of control of at least two independent experiments. (G) DNA damage foci in U2OS cells treated with TH1579. Cells were seeded in 96-well plates and treated with compound for 72 h before fixation. Samples were immunostained with indicated primary antibody marker together with fluorescent conjugated secondary antibodies. Images were acquired in an ImageXpress instrument and foci were quantified in Cell Profiler software. (H) Western blot analysis of HCT116 cells treated with increasing concentrations of TH1579 (0, 0.1, 0.2, 0.5, 1.0 and 2.5 μM).

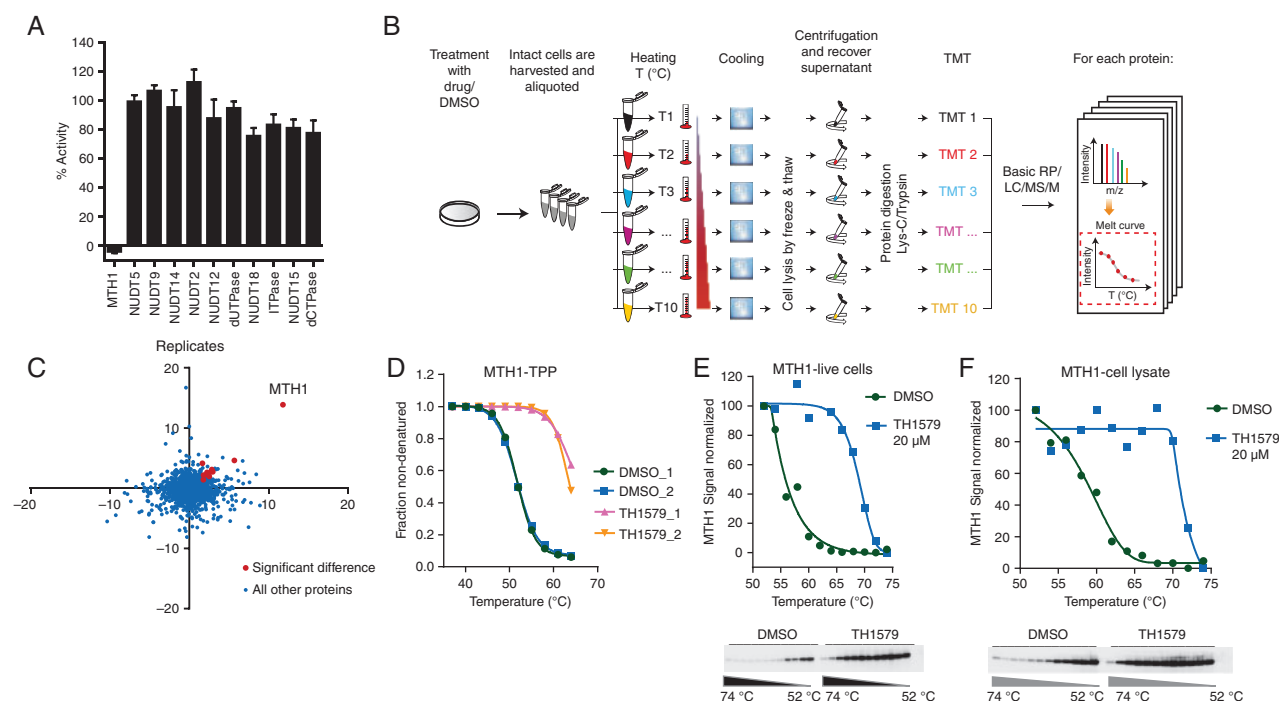
efficacy as daily exposure and with significantly improved tolerability (data not shown). A TH1579 dose dependent reduction in tumour growth (supplementary Figure S7B–G, available at *Annals of Oncology* online) and MTH1 target engagement inside the tumour (Figure 6E) were observed in HCT116 xenograft mouse model. The level of 8-oxodG (Figure 6F, supplementary Figures S7H and S8A–C, available at *Annals of Oncology* online) was significantly and dose dependently increased in HCT116 tumours from TH1579 treated animals. In addition, DNA damage and apoptosis markers (Figure 6G–I and K, supplementary Figure S8E and D, available at *Annals of Oncology* online) were elevated and cell proliferation marker reduced (Figure 6J) following TH1579 treatment. These data support the *in vitro* findings that the MTH1 inhibitor TH1579 induces elevated 8-oxodG levels, DNA damage signalling and reduces tumour growth.

## discussion

Three recent reports described potent MTH1 inhibitors that engaged the MTH1 enzyme in cells but did not kill cells [9–11]. Here, we show that the lack of toxicity is correlated with an inability to increase oxidized lesions in DNA, which is the suggested toxic lesion and thus compounds that fail to generate the

toxic lesion in DNA do not kill cells. One question is if the 8-oxodG lesion in DNA found after TH588 or TH1579 treatment is a consequence of inhibition of MTH1 in cells? Here, we provide evidence that overexpression of MTH1, bacterial MutT or using the antioxidant NAC, can prevent 8-oxodG incorporation into DNA, providing evidence that incorporation of 8-oxodG into DNA following TH1579 treatment is a consequence of abrogated MTH1-dependent 8-oxodGTPase activity. Another relevant question is if 8-oxodG incorporation into DNA is toxic at all? Recently, we demonstrated that microinjection of 8-oxodGTP or 2-OH-dATP is only toxic to zebrafish embryos in the presence of the MTH1 inhibitor TH588 [17]. Hence, the fish embryos are protected from toxic oxidized nucleotides being incorporated into DNA using a process that is targeted by the TH588 compound. MTH1 is the only enzyme described to carry out this function in cells to date and our bioinformatics searches failed to identify any protein with similar activity.

The Kettle study [10] reported a SW480 MTH1<sup>-/-</sup> clone 3, while the other 6 MTH1<sup>-/-</sup> clones described to us aggregated with a morphology different from wild type cells (personal communication). Indeed, we do expect some MTH1<sup>-/-</sup> cancer cells to survive, since in our hands siRNA targeting does not work in all cell types [3] and this enzyme is unlikely to be



**Figure 5.** Selectivity of TH1579. (A) Selectivity of TH1579 (100µM) compared to other nudix proteins. Values shown as mean  $\pm$  s.d. of a representative experiment with each datapoint performed in triplicate. The experiment was repeated once with similar results. (B) Experimental design of the thermal proteome profiling (TPP) based on CETSA. (C) Scatter plot of  $T_m$  shifts calculated from the two biological replicates of TH1579 versus DMSO controls in live cells. Red circles represent significant  $T_m$  shifts that fulfilled the criterias, blue circles show all remaining proteins. MTH1 is highlighted on the graph with a  $T_m$  shift of 12°C. (D,E,F) Melting curves for MTH1 (D) generated from LC/MS/MS, (E) from western blot (shown below graph) in live cells and (F) from western blot (shown below graph) with cell lysate. For experiments shown in (B)–(F) 20 µM TH1579 was added to BJ-hTERT-Ras-Sv40T cell lysate or live cells for 30 min followed by CETSA.

required for all cancer cell survival. Moreover, resistance mechanisms are likely to emerge, if not by genetic then by phenotypic changes. Also, effects of chemical inhibition and gene loss do not always correlate when it comes to cancer drugs. In fact, gene loss is often a resistance mechanism for many anti-cancer treatments targeting DNA repair [18].

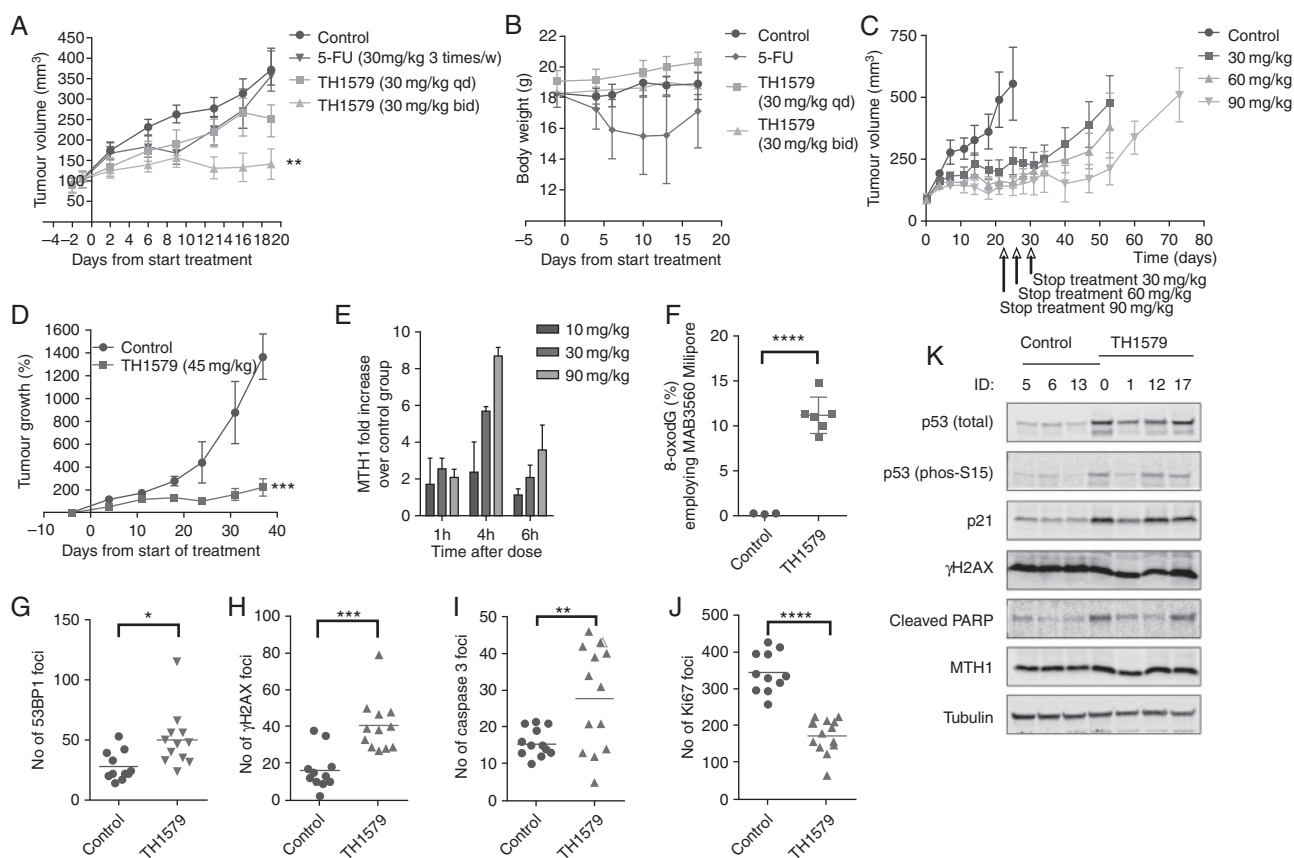
Our current perception is that MTH1 inhibitors TH588 and TH1579 are much more effective in cell killing than MTH1 protein loss, which we speculate can be explained by slow depletion of MTH1 protein levels allowing time for adaptation.

Here, we cannot give an explicit biochemical explanation to why the potent MTH1 inhibitors previously described by Kettle and Petrocchi [9, 10] do not result in incorporation of 8-oxodG into DNA nor why they do not kill cancer cells. There are four different isoforms of MTH1, which can be differentially inhibited by various MTH1 inhibitors (data not shown) and there are further emerging post-translational modifications on MTH1 that may affect the efficiency of MTH1 inhibition. Clearly, more in depth understanding of the complex MTH1 biology is required to answer these questions. Another possibility is that the TH588 and TH1579 compounds have relevant off-target effects, which work together with MTH1 inhibition to provide the cell killing effects. Here, we present our extensive efforts trying to identify putative off-target effects of our compounds. The use of cellular thermal shift assay (CETSA) combined with proteomics was recently described as a method to investigate how drug candidates can permeate and bind to targets within the cell [19, 20]. Using proteome-wide CETSA we observe MTH1 as the

clear top-ranked hit. The overall conclusion is that our compounds are highly selective in binding and inhibiting MTH1, although off-target effects cannot be excluded. Kettle and co-workers performed a kinase screen (267 kinases, Millipore) using TH588, which also did not generate any putative off-targets (personal communication).

Recently, TH588 was reported to affect tubulin polymerization *in vitro* [11]. Using CETSA proteomics, we find no evidence of target engagement of the tubulin proteins detected and identified. Furthermore, in additional proteomics studies we show distinct death pathways between TH588 and the anti-microtubule agent Paclitaxel. However, this does not exclude any putative tubulin effects of MTH1 inhibitors.

The MTH1 biology is highly reminiscent of the complexities in PARP biology uncovered thus far. By analogy, in the PARP-BRCA story, not all PARP inhibitors nor siRNA approaches recapitulate the effects we observed with certain small molecule PARP inhibitors [21]. This underscores the difficulties in using RNAi when validating novel targets in DNA repair. Furthermore, some PARP inhibitors poorly kill BRCA defective cells in spite of being low nM inhibitors and the underlying reason, discovered much later, is that PARP trapping correlated with killing BRCA mutated cells, and not inhibition of PARP1 [22]. In spite of intense research on PARPs over the last decade, a complete biochemical understanding of the PARP trapping mechanism is still missing. This has however not stopped a PARP inhibitor being approved by the FDA and EMA for treatment of BRCA mutated ovarian cancers.



**Figure 6.** MTH1 inhibitor TH1579 significantly hinders tumour growth and induces 8-oxodG, DNA damage and apoptosis *in vivo*. (A) Effect of TH1579 (oral gavage, daily dosing as labelled in graph) or 5-FU (i.p., three times per week as labelled in graph) in human colon SW480 tumour xenograft model. TH1579 was administered via oral gavage and 5-FU via the i.p. route. Control animals obtained same vehicle as TH1579. Data shown as mean  $\pm$  s.e.m.,  $n = 6$ /group.  $^{**}P < 0.01$ . 2-way ANOVA, followed by Bonferroni's multiple comparison test. (B) Effect on body weight of TH1579, 5-FU and vehicle treatment in SW480 xenograft model. Values are shown as mean  $\pm$  s.e.m. and treatment as indicated. (C) Dose response in human colon SW480 xenograft model. Values are shown as mean  $\pm$  s.e.m., with  $n = 5-7$ /group. (D) Effect of TH1579 (45 mg/kg b.i.d. daily) treatment in BRAF V600E-mutated malignant melanoma patient derived xenograft versus vehicle treated animals. The patient whose tumour was injected into mice exhibited resistance to carboplatin/dacarbazine/vemurafenib.  $^{**}P < 0.01$ . 2-way ANOVA, followed by Bonferroni's multiple comparison test. (E) MTH1 target engagement by TH1579 at doses indicated in HCT116 xenograft tumours at 1, 4 or 6 h after p.o. administration. MTH1 target engagement was measured using CETSA. Values show mean  $\pm$  s.d. of two individuals at each timepoint. (F) 8-oxodG level in HCT116 xenograft tumours 1 h after 90 mg/kg p.o., b.i.d. TH1579 treatment for 1-3 days. Values shown as individual data from 6 TH1579 treated animals and 3 vehicle treated animals.  $^{****}P < 0.0001$  Student *t*-test. (G) 53BP1 foci measured in tumours from HCT116 xenograft treated with 90 mg/kg TH1579 p.o., b.i.d. for 4 days.  $^{*}P < 0.05$ , Student's *t*-test. (H)  $\gamma$ H2AX foci measured in tumours from HCT116 xenograft treated with 90 mg/kg TH1579 p.o., b.i.d. for 3 days.  $^{***}P < 0.001$  Student *t*-test. (I) Cleaved caspase measured in tumours from HCT116 xenograft mice treated with 90 mg/kg TH1579 p.o., b.i.d. for 3 days.  $^{**}P < 0.01$ , Student's *t*-test. (J) Ki67 foci measured in tumours from HCT116 xenograft mice treated with 90 mg/kg TH1579 p.o., b.i.d. for 3 days,  $^{****}P < 0.0001$ , Student's *t*-test. (K) Western blot showing level of p53 (total and phospho-S15), p21, cleaved PARP,  $\gamma$ -H2AX, MTH1 and alpha-tubulin (as loading control) measured in tumours from HCT116 xenograft treated with 90 mg/kg TH1579 p.o., b.i.d. for 3 days (four individuals treated with drug, three vehicle treated individuals).

Here, we describe an improved MTH1 inhibitor TH1579, which has favourable pharmacokinetic and dynamic properties and potently inhibits tumour growth in chemotherapy resistant patient derived malignant melanoma and human colon cancer mouse xenograft models. We believe MTH1 inhibitors may become an important therapeutic option in the future treatment of cancer, a hypothesis that we hope soon is to be tested in phase I clinical trials.

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## disclosure

A patent has been filed with data generated in this manuscript where T.K., M.S. and T.H. are listed as inventors.

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# Short, full-dose adjuvant chemotherapy (CT) in high-risk adult soft tissue sarcomas (STS): long-term follow-up of a randomized clinical trial from the Italian Sarcoma Group and the Spanish Sarcoma Group

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