

35 **ABSTRACT**

36 **Background:** Osteosarcoma (OS) is the most common primary malignant bone tumour.
37 Unfortunately, no new treatments are approved and over the last 30 years the survival rate
38 remains only 30% at 5 years for poor responders justifying an urgent need of new therapies.
39 The MutT Homolog 1 (MTH1) enzyme prevents incorporation of oxidized nucleotides into
40 DNA and recently developed MTH1 inhibitors may offer therapeutic potential as MTH1 is
41 overexpressed in various cancers.

42 **Methods:** The aim of this study was to evaluate the therapeutic benefits of targeting MTH1
43 with two chemical inhibitors, TH588 and TH1579 on human osteosarcoma cells. Preclinical
44 efficacy of TH1579 was assessed in human osteosarcoma xenograft model on tumour
45 growth and development of pulmonary metastases.

46 **Findings:** MTH1 is overexpressed in OS patients and tumour cell lines, compared to
47 mesenchymal stem cells. *In vitro*, chemical inhibition of MTH1 by TH588 and TH1579
48 decreases OS cells viability, impairs their cell cycle and increases apoptosis in OS cells.
49 TH1579 was confirmed to bind MTH1 by CETSA in OS model. Moreover, 90mg/kg of
50 TH1579 reduces *in vivo* tumour growth by 80.5% compared to non-treated group at day 48.
51 This result was associated with the increase in 8-oxo-dG integration into tumour cells DNA
52 and the increase of apoptosis. Additionally, TH1579 also reduces the number of pulmonary
53 metastases.

54 **Interpretation:** All these results strongly provide a pre-clinical proof-of-principle that TH1579
55 could be a therapeutic option for patients with osteosarcoma.

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57 Cancers Santé.

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60 **Key words:** Osteosarcoma, bone tumours, MTH1, ROS, DNA damage

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62

63 **Research in Context section**

64

65 *Evidence before study*

66 Osteosarcoma is the most prevalent primary malignant bone tumour with poor prognosis
67 mainly affecting children and young adults. Since current therapies did not change in the last
68 30 years, there is a real need of new therapeutic strategies. Targeting MTH1 using TH1579
69 has shown to inhibit tumour growth in preclinical studies in various cancers.

70

71 *Added value of this study*

72 Using in vitro experiments and mouse models of osteosarcoma, we report that TH1579,
73 MTH1 inhibitor, inhibits cell growth and induces apoptosis in osteosarcoma cells and, delays
74 tumor growth and blocks the development of pulmonary metastases in preclinical model of
75 osteosarcoma.

76

77 *Implications of all the available evidence*

78 In this study, we provide the new insight in the therapeutic efficacy of TH1579 on tumour
79 growth and metastases development in preclinical model of osteosarcoma suggesting that
80 TH1579 could be a therapeutic option in treatment of patient with osteosarcoma.

81 **Introduction**

82

83 Osteosarcoma (OS) is the most common primary malignant bone tumour. It
84 mainly affects children and young adults, with an incidence peak at around 18 years
85 old, and is mostly localized in long bones (1). OS involves deregulation of the
86 equilibrium between bone formation and bone resorption, which leads to ectopic
87 bone formation and osteolysis. Current strategies combine surgical tumour excision
88 and chemotherapy (2, 3). Unfortunately, 5 years survival rate drops from 75% to 25%
89 for bad responders (4, 5), hence the urgent necessity to develop new therapeutic
90 strategies. In this context, DNA damaging compounds are particularly interesting
91 candidates. Indeed, their antitumour effects have been known for over half a century
92 (6, 7). In normal cells, DNA damages lead to cell cycle arrest in order to prevent
93 transmission of genetic alterations during mitosis. Nevertheless, tumour cells have
94 high tolerance to DNA damage, allowing greater proliferation rates (8). However this
95 fast proliferation also increases the chances of cell death through the transmission of
96 fatal DNA damages to daughter cells. Therefore, several anticancer strategies are
97 based on the induction of DNA damages to tumour cells. For example in OS cells,
98 DNA damaging agents such as cisplatin, doxorubicin and methotrexate have been
99 used in clinic for decades to treat the disease (1, 8, 9).

100 Recently, there has been a growing interest for antitumour capabilities of the
101 reactive oxygen species (ROS), notably through their ability to induce DNA damage
102 (10). ROS can indeed interact with the pool of free nucleotides, creating oxidized
103 nucleotides. These oxonucleotides can be integrated into the DNA double helix by a
104 DNA polymerase, during replication, and disturb cellular processes (11). Base
105 Excision Repair (BER) mechanism then lead to excision of oxonucleotides from DNA

106 by glycosylases such as the 8-oxoguanineglycosylase 1 (OGG1). However, this
107 repair mechanism can be overwhelmed, leading to cell death. The MutT Homolog 1
108 (MTH1) or Nudix-Type 1 (NUDT1) protein is part of the nudix family of hydrolases.
109 MTH1 acts as a nucleotide pool sanitation enzyme by hydrolyzing oxonucleotides
110 triphosphates such as 8-oxo-dGTP, 2-OH-dATP or 8-oxo-dATP, into their
111 monophosphates counterparts. Its activity prevents the integration of oxidized
112 nucleotides into DNA, since monophosphate nucleotides cannot be used by the DNA
113 polymerase (12, 13). Thus, MTH1 protects cells against ROS effects on DNA
114 integrity. Therefore, targeting MTH1 could imply impairing the genome integrity, *i.e.*
115 through the targeting of DNA building blocks, nucleotides. Free nucleotides are also
116 more likely to be oxidized by ROS than DNA macromolecules (12). Furthermore,
117 ROS are by-products of cell metabolism (14), and it has been shown that tumour
118 cells produce high quantity of ROS compared to normal cells, due to their fast
119 metabolism, their environment and mutations (15-17). Consequently, tumour cells
120 could strongly depend on MTH1 activity to protect their genome integrity, and thus
121 increase its expression compared to normal cells. MTH1 has indeed been found to
122 be overexpressed in several types of cancer such as breast and colorectal cancer
123 (18, 19), while MTH1-deficient mice developed spontaneous tumours 18 months after
124 birth (20). Since 2014, several studies have tested the effects of MTH1 inhibition in
125 different cancer types including osteosarcoma, melanoma, breast, cervical or
126 colorectal cancer. MTH1 inhibition reduced cell viability and colony formation,
127 increased oxidized nucleotides integration in DNA, and induced DNA damage (21-
128 24). *In vivo*, MTH1 inhibition decreased tumour growth and increased survival in
129 mouse xenograft models (21-23), leading to an increased interest in MTH1 as
130 therapeutic target in cancer (21, 22). But more recently, these results were

131 challenged by questioning the role of MTH1 in tumour biology, as well as the benefits
132 of targeting this protein in cancer (25-28).

133 In this study we investigated the therapeutic efficacy of TH588 (first generation
134 of MTH1 inhibitor) and TH1579 (optimized analogue of TH588), in OS. TH1579
135 (Karonudib) is currently in a phase I clinical trial. Using a panel of human OS cell
136 lines and a xenograft model, we evaluated the effects of TH588 and TH1579 on
137 several tumour processes such as proliferation, tumour growth or metastatic
138 dissemination.

139

140 **Materials and Methods**

141 **Tumour cell lines**

142 Human osteosarcoma cancer cell lines HOS-MNNG, KHOS, MG63, U2OS, SJSA-1,
143 G292, Cal72 and 143B were purchased from the ATCC and maintained in DMEM
144 (Invitrogen-Life Technologies, Inc.) supplemented with 10% FBS and 2 mmol/L L-
145 glutamine. All cell lines were cultured in a humidified 5% CO₂/air atmosphere at 37°
146 C.

147 **Tissue specimens and mesenchymal stem cells**

148 Patient tumour biopsy specimens were collected at Nantes University Hospital
149 (Nantes, France). Samples were obtained following patient informed consent, and
150 after ethical approval by the Nantes University Hospital Ethics Committee.

151 Human mesenchymal stem cells (hMSCs) from healthy donors and osteoblasts
152 derived from hMSCs on osteogenic differentiation are used as controls. Briefly,
153 hMSCs are cultured in DMEM supplemented with vitamin D3 and dexamethasone.
154 Then ascorbic acid and β -glycerophosphate was added to osteogenic medium.
155 Before their use, the cells induced to osteogenic differentiation were tested for
156 mineralization using alizarin red staining as described previously (29).

157

158

159 **Therapeutic agent**

160 MTH1 inhibitors, TH588 and TH1579 (21, 22) was provided by the Karolinska
161 Institutet and used for *in vitro* and *in vivo* studies. For the *in vitro* studies, the drugs
162 were dissolved in dimethyl sulfoxide (DMSO) at 10 mmol/L stock solution and stored

163 at -20°C . For *in vivo* studies, TH1579 was dissolved in an acetate plus cyclodextrin
164 buffer at the indicated concentrations.

165

166 **Cell proliferation assay and clonogenic assay**

167 OS cells were seeded in 96 wells plates. 24 h after seeding, OS cells were treated
168 with the drugs at the indicated doses (from 78nmol/L to 20 $\mu\text{mol/L}$). After 72 h of
169 treatment, the cells were fixed with 1% glutaraldehyde under agitation for 5 min,
170 washed with water then stained with 0.1% crystal violet for 5 min. After drying, the
171 dye was diluted in Sorensen's buffer (5 min in trisodium citrate, HCl 0,1N and
172 ethanol) and the absorbance was measured at 570 nm using a spectrophotometer
173 (Perkin Elmer Wallac 1420 Victor²). The proliferation rate is represented as a
174 percentage of untreated cells.

175 Regarding the clonogenic assay, cells were treated for 48 h with 2 or 8 $\mu\text{mol/L}$ of
176 TH588 or TH1579. The cells were then trypsinated and plated at 1000 cells/well in 6
177 wells plate. 10 days later, the cells were fixed and stained with crystal violet as
178 described above. The colonies were then counted.

179 **Transfection and transduction**

180 OS cells were transfected with 30nmol/L of siRNA (sc-62647, sc-37007; Santa Cruz
181 Biotechnology) using Lipofectamine RNAimax (thermofisher scientific) following the
182 provider's recommendation. For the transduction, the shRNA (MOI20) targeting
183 MTH1.pLKO.1shMTH1-1 and shMTH1-2 was a gift of Bob Weinberg (Addgene
184 plasmids # 21297 and # 21298)(30). The proliferation assay was performed as

185 described above (without treatment), on cells trypsinated and re-seeded after 48 h of
186 transfection or 72 h of transduction.

187

188 **Apoptosis assay**

189 Caspase activity was measured in protein extract using the Apo-ONE caspase 3/7 kit
190 from Promega. The cells were treated for 48 h and the assay was performed
191 according to the supplier's recommendation. The fluorescence was measured at
192 521nm on the Tristar LB 941 (Berthold Technologies). The enzymatic activity was
193 normalized by the protein quantity.

194

195 **Cell-cycle analysis**

196 Osteosarcoma cell were treated at indicated concentrations of TH588 or TH1579 for
197 48 h, trypsinized, washed twice, in cold PBS (5 min), and fixed with 70% ethanol at
198 4°C for 30 min. After washing, cells were incubated for 10 min in 4° C phospho-
199 citrate buffer, washed and incubated in PBS containing 100 µg/mL ribonuclease A,
200 for 30 min at 37° C. Cells were then incubated in 50µg/mL propidium iodide for 20
201 min at 4° C. Cell-cycle distribution was analyzed by flow cytometry (Beckman Coulter
202 Epics Elite, Beckman Coulter), based on 2N and 4N DNA content.

203

204 **Comet assay**

205 OS cells were treated with TH588 or TH1579 for 24 h, then trypsinated, included in
206 low melting agarose (37°C) and put on Superfrost plus slides (ThermoFisher

207 scientific). The cells were then lysated overnight at 4° C, in a DMSO, NaOH, triton
208 and Tris-HCl containing buffer. The cells were then washed in a digestion buffer
209 (Hepes, KCl, 500mmol/L EDTA and BSA in water; 3 x 20 min) and treated with 2µG
210 OGG1 (diluted in the digestion buffer) when indicated, for 45 min at 37° C. After 30
211 min in a denaturation buffer (EDTA, 10N NaOH in water), an electrophoresis was
212 performed at 300mA, for 30 min in the denaturation buffer. The slides are then
213 dipped in a pH 7.5 neutralization buffer for 45 min (Tris and HCL in water). DNA
214 breaks are detected using the Thermofisher Sybr Gold dye and quantified using FIJI
215 and the Open Comet plugin.

216

217 **8-oxo-dG assay**

218 It is well known that Avidin binds with high specificity to 8-oxo-dG and used to
219 evaluate the incorporation of 8-oxo-dG into DNA (31). Tumor cells were treated with
220 TH1579 at indicated concentrations for 24 h in slide 8 wells (Ibidi). Then, Cells were
221 fixed in methanol at -20° C for 20 min following by incubation in TBS with 0.1% Triton
222 X-100 for 15 min. Cells were incubated in blocking solution (15% FBS, 0.1% Triton X-
223 100 in TBS) for 2 h at room temperature, and thereafter incubated with 10 µg/mL
224 Alexa488-conjugated Avidin (Invitrogen) in blocking solution for 1 h at 37° C. Then,
225 the cells were washed in TBS 0.1%Triton X-100 for 2 x 5 min at room temperature,
226 following by a quick rinse in distilled water. DNA was counterstained with DAPI for 15
227 min in dark at room temperature. The cells were rinsed in TBS and Ibidi mounting
228 Medium were added in the wells of the slides. Images were acquired with the
229 Olympus IX73 inverted microscope.

230

231 **Quantitative reverse transcription PCR**

232 Total RNA was extracted using the Macherey-Nagel Nucleospin RNA. Following a
233 reverse transcription on 1 µg of total RNA, the cDNA were diluted to 10 ng/µL. A
234 Real-time monitoring of PCR amplification of cDNA was performed using DNA
235 primers (Supplementary Table I) on CFX96 Detector System (Bio-Rad) with SYBR
236 PCR Master Mix (Bio-Rad). Target gene expression was normalized to GAPDH level
237 in respective samples as an internal standard, and the comparative cycle threshold
238 (Ct) method was used to calculate relative quantification of target mRNAs. Each
239 assay was performed in triplicate.

240

241 **Western blot**

242 Samples containing equal amounts of protein from lysates of cultured osteosarcoma
243 cells underwent electrophoresis on SDS-polyacrylamide gel and were transferred to
244 PVDF membranes blocked in odyssey blocking buffer (LI-COR). Blots were probed
245 overnight at 4° C with primary antibodies (Supplementary Table II). After incubation,
246 the membranes were washed in PBS containing 0.05% Tween. Membranes were
247 then probed with a fluorescent antibody (Li-COR IRDye) diluted in the odyssey
248 blocking buffer. Antibody binding was visualized using the LI-COR odyssey Fc

249 **Immunofluorescence**

250 Cells were cultured in an imaging chamber (Ibidi) and treated as indicated in the
251 figure. As control, cells were irradiated at 15Gy, delivered at a rate of 1,67 Gy/min
252 (Faxitron CP160 irradiator, Faxitron X-ray Corporation, IL, USA). Then, cells were
253 fixed with 4% paraformaldehyde for 20 min at room temperature, then washed

254 (washing buffer: PBS-Tween20 0.05%) and permeabilized 10 min at room
255 temperature with PBS-0.1% triton X100. Cells were then blocked 10 min with goat
256 serum (1/200) in washing buffer, then incubated overnight, at 4° C, with primary
257 antibody (Cell signaling Technology γ H2AX antibody). Afterward, cells were washed
258 and incubated with alexa Fluor 488 antibody (60 min at room temperature), then with
259 DAPI.

260

261 **Transwell motility and invasion**

262 Cell migration and invasion assays were performed in Transwell chamber (8- μ m pore
263 size; Falcon). For cell invasion experiments, inserts were coated with 100 μ L
264 basement membrane Matrigel (3 μ g/ml; Becton Dickinson) for 90 min at 37° C. HOS-
265 MNNG cells (1×10^5 cells/mL) were resuspended in DMEM containing 0.1% FBS, and
266 300 μ L of this cell suspension was loaded into each insert (top chamber). DMEM
267 10% FBS was placed in the bottom chamber (750 μ L/well). The plates were
268 incubated for 6 h at 37° C for migration and 24 h for invasion. Then, the inserts were
269 carefully collected, the non-migrating cells were removed, and the migrating cells on
270 the under-surface of the inserts were fixed with 1% glutaraldehyde under agitation for
271 5 min, washed with water, and then stained with 0.1% crystal violet for 5 min. The
272 cells were then counted by bright-field microscopy.

273

274 **Animal treatment**

275 Female athymic nude mice were injected intramuscularly close to the tibia with
276 1.5×10^6 HOS-MNNG cells. Once their tumours were palpable, mice were randomly

277 assigned to vehicle, TH1579 45 mg/kg or TH1579 90 mg/kg groups and treated orally
278 b.i.d daily three times per week until day 48. Each experimental group consisted of 8
279 mice. Tumour volume was measured three times per week. Tumour volume was
280 calculated by the formula: length × width × depth × 0.5432. Data points were
281 expressed as average tumour volume ± SEM. Tumours and tibia were harvested for
282 further analyses [micro computed tomography (microCT), histology]. Animal care and
283 experimental protocols were approved by the French Ministry of Research and were
284 done in accordance with the institutional guidelines of the French Ethical Committee
285 protocol agreement number 1280.01 and under the supervision of authorized
286 investigators.

287

288 **MicroCT analysis**

289 Tibias were scanned using high-resolution microcomputed tomography (Skyscan
290 1076) at 50 kV and 200 mA using a 0.5 mm aluminium filter and a detection pixel size
291 of 9 µm. Images were captured every 0.8° through 180° rotation, and analyzed using
292 Skyscan software. After scanning, 3D microstructural image data was reconstructed
293 using the Skyscan NRecon software. Bone parameters were calculated using the
294 Skyscan CT Analyzer (CTAn) software. Using this software, the volume of interest
295 was defined as cortical remodeling bone. Binarized images were performed: an
296 upper threshold of 255 and a lower threshold of 55 was used to delineate each pixel
297 as “bone” or “non-bone” Morphological traits of the mid-diaphyseal cortical shell were
298 performed at a site starting 200 slices from the peronea insertion and then extending
299 from this position for 700 slices (each slice = 9 µm). Then, total cross sectional area
300 (Tt.Ar), cross sectional cortical area (Ct.Ar), cortical bone fraction (Ct.Ar/Tt.Ar),

301 cortical thickness (Ct.Th), and cross sectional moment of inertia (polar) (CSMI) was
302 measured in 2D.

303 **Immunohistochemistry**

304 IHC was performed on formalin-fixed, paraffin-embedded 3 µm sections of tumour
305 samples. Immunostaining was conducted using the primary antibodies cleaved
306 caspase-3 (1/400; Cell Signaling Technology), CD146 (1/200; Abcam), gH2AX
307 (1/750; Millipore), 8-oxo-dG (1/750; Millipore) and Sp7/Osterix (1/1000; Abcam) on
308 tumour section.

309 Tibias were decalcified with 4.13% EDTA and 0.2% paraformaldehyde in PBS using
310 the KOS microwave histostation (Milestone) before embedding in paraffin. Sections
311 (Leica Microsystems) were analysed by Osterix and TRAP staining. Quantification of
312 relative OC surface (TRAP+ cells) and OB number (Osterix+ cells) was evaluated by
313 ImageJ (NIH, Bethesda, MD) software. All analyses were assessed by slide scanning
314 using nanozoomer 2.0 RS (Hamamatsu).

315 **Cellular Thermal Shift Assay (CETSA)**

316 CETSA is a target engagement assay detecting potential interaction between a
317 specific target and studied compounds and has been described previously(32).
318 Briefly, frozen tumours from the TH1579 and vehicle treated animals were grinded in
319 liquid nitrogen then resuspended in 600µl TBS-T complete protease inhibitor cocktail
320 (Roche). Aliquots were incubated for 3 min at a temperature gradient of 50 - 66° C.
321 Samples were frozen (-80° C) and thawed (25° C) three times to lyse the cells,
322 followed by centrifugation at 17000 g for 30 min. The supernatant (35µl/sample) was
323 transferred to a 96-well PCR plate (Agilent) and 12µl of 4x Laemmli sample buffer
324 were added to each sample. Proteins were denatured by heating the PCR plate at

325 95° C for 10 min. Samples were run on SDS-PAGE and transferred to nitrocellulose
326 membranes and incubated with rabbit anti MTH1 antibody (Novus Biological).
327 Membranes were developed using Super Signal West Femto Substrate (Thermo
328 Scientific), and measurements were performed using Odyssey Imaging System (LI-
329 COR) and the bands signals were quantified using Odyssey image software.

330

331 **Osteoclast differentiation assays**

332 Osteoclasts were generated from human CD14+ monocytes of different donors.
333 Purified CD14+ cells were cultured in α -MEM (Invitrogen Life Technologies) with 10%
334 FBS and 25ng/mL human macrophage colony stimulating factor (hM-CSF; R&D
335 Systems). After 4 days, 100ng/mL hRANKL +/- TH1579 were added. Multinucleated
336 cells with more than three nuclei were counted after Tartrate-Resistant Acid
337 Phosphatase (TRAP) staining (Sigma, France).

338

339 **Short term in vivo study using HOS-MNNG-GFP cells**

340 Procedures involving animal handling and care were approved by the Animal Care
341 and Ethics Committee of the Home office in UK [PPL: 70/8967, Establishment license
342 n°: 50/2509] and performed by trained, licensed personnel. To assess effects of
343 short-term treatment with TH1579 on disseminated tumour cells (DTCs) to the lung
344 and circulating tumour cells (CTCs) a paratibial osteosarcoma model was used.
345 Briefly, 2.5×10^5 HOS-MNNG-GFP cells were injected paratibially into anaesthetized
346 female Balb/c nude mice (6-7 weeks old). Five days later animals received treatment
347 of either vehicle or TH1579 at 90mg/kg b.i.d three times per week for one week by

348 oral gavage. Animals were culled 24 h (TH1579 group) or 48 h (vehicle group) after
349 the last treatment. The tumour bearing leg was measured using callipers and the
350 volume was calculated before assessing DTCs, CTCs.

351

352 **Quantification of DTCs and CTCs using Parsortix**

353 Animals were culled just before sample preparation for DTC analysis. Whole blood
354 was collected via cardiac puncture into EDTA vacutainers (BD). Blood from all
355 animals in one group was pooled. Lungs were excised, washed in PBS and minced
356 using a scalpel blade before adding tumour dissociation solution (Tumour
357 Dissociation Kit, Myltenyi) for 30 min at 37° C. The single cell suspension was then
358 filtered and centrifuged before resuspending in PBS and loading onto the Parsortix
359 system (Angle) using the PX2_S50F separation program and a 6.5 µm separation
360 cassette. Briefly, the Parsortix system is a microfluidic technology, which allows
361 enrichment, isolation and quantification of small numbers of tumour cells based on
362 cell size on deformability. The separation cassette containing the enriched sample
363 was then vizualized using a fluorescent microscope and GFP-positive tumour cells
364 trapped in the cassette were counted. As indicative experiment, the same procedure
365 was performed for CTCs in whole blood (blood in each group was pooled before
366 analysis; control N=2; TH1579, N=3) using PX2_S99F separation program.

367

368 **Statistical analysis**

369 All statistical tests were performed using the Graphpad Prism software. Statistical
370 significance was assessed by Kruskal-Wallis test or Anova. * $p < 0.05$; ** $p < 0.01$,
371 *** $p < 0.001$, **** $p < 0.0001$.

372

373 **Results**

374 **MTH1 mRNA level is overexpressed in osteosarcoma**

375 We first evaluated MTH1 mRNA expression level of in 13 human OS patient
376 samples (Fig. 1a and 1b) and 7 OS cell lines (Fig. 1c and 1d). Compared to normal
377 mesenchymal stem cells (Msc) or osteoblasts derived from hMSC (OB) from healthy
378 donors, MTH1 was overexpressed in all patient samples at mRNA level, and in all OS
379 cell lines tested at mRNA and protein levels.

380

381 **TH588 and TH1579 reduce osteosarcoma cell proliferation**

382 The effects of TH588 and TH1579 were evaluated on cell growth, in 8 OS cell
383 lines (Fig. 2a). In all tested cell lines, TH588 and TH1579 inhibited cell growth in a
384 dose dependent manner, with IC50 values ranging from 4.48 $\mu\text{mol/L}$ to 17.37 $\mu\text{mol/L}$
385 for TH588, and from 0.31 $\mu\text{mol/L}$ to 16.26 $\mu\text{mol/L}$ for TH1579 (Fig. 2b).

386 Colony formation assays was performed after 48 h of TH588 or TH1579
387 treatment and showed that both drugs significantly reduced tumour cells
388 clonogenicity in HOS-MNNG and U2OS cell lines (Fig. 2c). Indeed, number of
389 colonies was reduced by 53.4 % in HOS-MNNG cells treated with 2 $\mu\text{mol/L}$ of TH588
390 and by 91.5 % when treated with 8 $\mu\text{mol/L}$ of TH588 ($p < 0.001$). Likewise, number of
391 U2OS colonies was respectively reduced by 13 % and 91 % ($p < 0.001$) when treated
392 with 2 $\mu\text{mol/L}$ and 8 $\mu\text{mol/L}$ of TH588, respectively. For TH1579, number of colonies
393 in both cell lines was reduced by over 90 % for each concentration ($p < 0.001$).
394 Similar results were observed 143B, Cal72, G292, KHOS, MG63 and SJSA-1 cell
395 lines (Supplementary Fig. S1).

396 In order to evaluate whether these results were linked to specific MTH1 inhibition, we
397 transfected or transduced HOS-MNNG cells with a mix of two specific siRNA or two
398 different shRNA targeting MTH1. The decrease of MTH1 expression was validated by
399 qPCR (Supplementary Fig. S2) and Western blot (Fig. 2d). Specific MTH1 inhibition
400 reduced cell growth by 16% compared to control siRNA ($p < 0.01$), and by 66% ($p <$
401 0.001) and 48% ($p < 0.01$) respectively, for MTH1 shRNA-1 and 2 treatment (Fig 2d).

402

403 **TH1579 induces apoptosis by increasing DNA strand breaks**

404 Since the TH1579 compound being the most potent drug in this study, we
405 focus the rest of the study on TH1579. Using this drug, we investigated the effect of
406 TH1579 on cell cycle using propidium iodide staining after 48 h of treatment (Fig. 3a).
407 At 500nM, TH1579 induces G2/M arrest in HOS-MNNG cells as shown by 49% of
408 cells in G2/M phase compared to 11% of cells in control condition. Moreover, in both
409 HOS-MNNG and U2OS cells, TH1579 increased cells death (subG0/G1 fraction) in a
410 dose-dependent manner compared to control. For HOS-MNNG cells, 4% of cells
411 were in the subG0/G1 fraction in the control condition, while 31% and 39% of cells for
412 the 2 μ M and 8 μ M TH1579 respectively. For U2OS cell line, 8%, 69% and 67% of cells
413 were in the subG0/G1 fraction for control, 2 μ M and 8 μ M of TH1579 conditions,
414 respectively. Indeed, TH1579 induced apoptosis as shown by a significant increase
415 of caspase 3/7 activity compared to non-treated control (e.g. by 2-fold after 8 μ M
416 TH1579 treatment vs control in U2OS cells, $p < 0.01$; by 6-fold after 8 μ M TH1579
417 treatment vs control in HOS-MNNG cells, $p < 0.01$; Fig. 3b). Same results were
418 obtained for TH588 (Supplementary Fig. S3a and S3b). Induction of apoptosis was
419 also confirmed by an increase in PARP cleavage in treated conditions compared to
420 the control (Fig. 3c).

421 Since MTH1 prevents oxonucleotides integration into DNA, we measured the
422 DNA damage induced by TH1579 +/- OGG1 using comet assay (Fig. 3d). OGG1 is a
423 glycosylase that excises the 8-oxo-dG nucleotide from the double helix, inducing
424 DNA breaks and initiating the DNA repair process. In U2OS cells, combination
425 TH1579+OGG1 treatment increased the tail moment compared to controls (Fig. 3d).
426 The tail moment was increased by 3,93-fold in TH1579+OGG1 compared with
427 untreated control condition ($p < 0.001$), by 3.88-fold compared with OGG1 condition
428 ($p < 0.001$), and by 1.3-fold compared with TH1579 alone ($p < 0.001$), indicating that
429 TH1579 induces DNA strand breaks probably by enhancing 8-oxo-dG integration into
430 DNA. We confirmed the incorporation of 8-oxo-dG into DNA after treatment with
431 TH1579 compared with control in OS cells as shown by an accumulation of 8-oxo-dG
432 in the nucleus (counterstained with DAPI) observed by immunofluorescence (Fig.
433 3e).

434 Consequently, we next evaluated the DNA damage induced by TH1579. We
435 analyzed the expression of phosphorylation on serine 319 of H2AX histone (γ H2AX)
436 known to be a marker of DNA double strand breaks (33, 34). TH1579 increased
437 γ H2AX expression in U2OS cells (Fig. 3f). However, we failed to show a significant
438 impact of the treatment on γ H2AX expression, using immunofluorescence, as γ H2AX
439 expression was very heterogeneous: some cells displayed a high number of foci
440 expression while other showed a low number of foci (Supplementary Fig.S4).

441

442 **TH1579 delays tumour growth and increases DNA damages in HOS-MNNG**
443 **xenograft model**

444 Therapeutic potential of TH1579 was next evaluated *in vivo* in an OS
445 xenograft model. Mice bearing HOS-MNNG tumours, which is an OS xenograft
446 model reproducing the human pathology associated with development of pulmonary
447 metastases, were randomly separated in three groups and orally treated 3 times per
448 week with vehicle, 45 mg/kg of TH1579 or 90 mg/kg of TH1579 for 48 days. No
449 significant change of body weight was observed at these concentrations. TH1579
450 treatment significantly decreased the tumour volume in both treated groups in a dose
451 dependent-manner, compared to control group (Fig. 4a and 4b). At day 29, the mean
452 tumour volume was diminished by 40.6% in the 45mg/kg group ($398 \text{ mm}^3 \pm 57,2$
453 mm^3 , $p = 0,23$), and 73.2 % in the 90mg/kg group ($179.46 \text{ mm}^3 \pm 39,4 \text{ mm}^3$, $p <$
454 0.001), compared to control ($670.3 \text{ mm}^3 \pm 99 \text{ mm}^3$). At day 48, the mean tumour
455 volumes were reduced by 41.3 % ($1105.3 \text{ mm}^3 \pm 153,7 \text{ mm}^3$, $p = 0,22$) and 80.6 %
456 ($365.96 \text{ mm}^3 \pm 81,6 \text{ mm}^3$, $p < 0,001$) in the 45 mg/kg and the 90mg/kg groups
457 respectively, compared to control ($1883.09 \text{ mm}^3 \pm 272,3 \text{ mm}^3$; Fig. 4A). At the
458 individual level, the incidence of mice with a tumour volume of 1000 mm^3 or greater
459 was significantly reduced by day 48 in both TH1579 90mg/kg-treated mice (0/8) and
460 TH1579 45mg/kg-treated mice (4/8) compared with control mice (8/8; Fig. 4b).

461 Moreover, the target engagement evaluated by Cellular Thermal Shift Assay
462 (CETSA) is increased in a dose-dependent manner in tumour tissue following
463 TH1579 treatment (Supplementary Fig.S5).

464 Immunohistochemistry staining carried out on tumour tissues indicated that
465 TH1579 increased tumour cell death as shown by a significant increase of cleaved-
466 caspase 3 expression by 6% and 8.5% in the 45 mg/kg ($p < 0.05$) and the 90 mg/kg

467 (p < 0.001) groups respectively, compared to the control group (Fig. 4c). These data
468 indicate that TH1579 induces apoptosis *in vivo*.

469 Added to this, effects of TH1579 treatment on 8-oxo-dG integration into DNA
470 and DNA damage were evaluated in tumours by immunohistochemistry, using 8-oxo-
471 dG and γ H2AX antibodies. TH1579 significantly increased 8-oxo-dG integration and
472 DNA damage in treated groups compared to control. Indeed, in TH1579 45 mg/kg
473 group for example, 8-oxo-dG expression was increased by 2-fold (p < 0.05) and
474 γ H2AX expression was increased 1.4-fold (p < 0.01) compared to control. In TH1579
475 90 mg/kg group 8-oxo-dG and γ H2AX were respectively increased by 2.6-fold (p <
476 0.01) and 1.38-fold (p < 0.05) compared to the control group (Fig. 4c).

477

478 **TH1579 reduces pulmonary metastases by inhibiting migration and** 479 **angiogenesis**

480 Thereafter, effects of TH1579 on metastases development were evaluated in
481 HOS-MNNG xenograft model. Lung metastases were counted in each experimental
482 group after H&E coloration. The average number of metastases was reduced by 45%
483 (average of 2.06 metastases) in 45 mg/kg TH1579 group, and significantly reduced
484 by 86.7% (average of 0.5 metastasis) in 90 mg/kg group (p < 0.001), compared to
485 control (average of 3.75 metastases; Fig. 5a).

486 Moreover, TH1579 also reduced the number of Circulating Tumour Cells
487 (CTC) counted in blood and Disseminated Tumour Cells (DTC) counted in
488 dissociated lungs of mice bearing HOS-MNNG-GFP tumours after one week
489 treatment of 90 mg/kg TH1579 compared to control group, suggesting an effect of
490 TH1579 on metastatic dissemination. Indeed, while the mean tumour volume of the

491 two groups (90 mg/kg TH1579 vs vehicle) was equivalent (89.6 mm³ and 96.1 mm³
492 for the control and treated conditions respectively; Supplementary Fig. S6), the
493 number was 61 CTC/mL of blood in control group, and 13 CTC/mL in 90 mg/kg
494 TH1579 group. DTC number in lungs was also reduced by 2.68-fold after 90 mg/kg
495 TH1579 (25 GFP-positive tumour cells) treatment compared to control group (68
496 GFP-positive tumour cells; Fig. 5b). One of the mice from the control group was
497 excluded from the analysis since it did not have a visible GFP+ tumour.

498 In order to verify that TH1579 could affect processes involved in metastases,
499 migration and invasion assays were performed on HOS-MNNG cells treated with
500 TH1579. In the migration assay using transwells, number of migrating cells was
501 significantly reduced by 70% ($p < 0.01$) and 73% ($p < 0.001$) after treatment with 2 μ M
502 TH1579 (113 cells) and with 8 μ M TH1579 (102 cells) respectively, compared to
503 control (388 cells). In the invasion assay, number of cells per field was significantly
504 reduced by 32.8% ($p < 0.01$) after treatment with 2 μ M TH1579, and 69.44% ($p <$
505 0.001) after treatment with 8 μ M TH1579. TH1579 significantly reduced both
506 migration and invasion capabilities of HOS-MNNG cells (Fig.5c).

507 According to migration and invasion assays, TH1579 significantly reduced
508 angiogenesis, as shown by a reduced CD146 expression in tumours treated with 90
509 mg/kg TH1579 (1.27 times lower) compared to control group ($p < 0.01$; Fig. 5d). No
510 significant change was observed between 45mg/kg TH1579 and control groups (Fig.
511 5d).

512

513 **Effect of TH1579 on tumour-associated bone lesions**

514 In order to evaluate the effect of TH1579 on tumour associated-osteolysis,
515 cortical and trabecular bone parameters of tibia bearing tumours were examined (Fig.
516 6a and b). BV was identical between TH1579 treated (45 mg/kg and 90 mg/kg) and
517 control groups. BV/TV was slightly but not significantly increased by 17% in 90 mg/kg
518 TH1579 group compared to control group (p value = 0.0508).

519 Immunohistochemical staining of osterix showed an increase of osteoblasts
520 (OB) number over bone surface (BS) in both treated groups (2.08 and 3.95 OB/BS
521 for the 45 mg/kg and 90 mg/kg conditions respectively), compared to control group
522 (1.25 OB/BS). Likewise, TRAP staining also showed a significant increase of
523 osteoclast number over bone surface in treated groups compared to control group
524 (Fig. 6c). Ocs/BS was 2.9 times more important after treatment with 45 mg/kg
525 TH1579 and 3.9 more important after treatment with 90 mg/kg TH1579 compared to
526 control. Effects of TH1579 on bone cells were also evaluated *in vitro* on CD14+
527 osteoclast precursors as well as hMSC osteoblast precursors. TH1579 reduced
528 osteoclasts differentiation, without any significant effect on their precursor's viability
529 (Fig. 6d). However, TH1579 reduced both hMSC viability and mineralization
530 (Supplementary Fig.S7).

531

532 **Discussion**

533 Osteosarcoma is a paediatric cancer with poor prognosis and high recurrence.
534 Since 30 years, treatments are limited to chemotherapy associated with resistance
535 development. Finding new therapeutic strategies for OS is urgently needed. In this
536 context, the enzyme MTH1 is an attractive target since MTH1 is “druggable” by
537 chemical compounds (TH588, TH1579, S-Crizotinib). MTH1 is described to support
538 malignant transformation, proliferation and oncogenic ability through the degradation
539 of oxidized guanine nucleotides from oxidative stress (21, 23, 35, 36). OS exhibits a
540 high level of ROS suggesting a potential constitutive activation of MTH1 in tumour
541 cells. Indeed, we observed an overexpression of MTH1 in OS biopsies as well as in a
542 large panel of OS cell lines with various genetic alterations compared to hMSC.

543 Despite controversial opinions in literature about the dependence of MTH1 by
544 cancer cells to survive and the efficiency of therapeutic drugs under development
545 (25-28), we aimed to evaluate the therapeutic efficacy of the MTH1 inhibitors, TH588
546 and TH1579 in OS model. In the present study, we clearly observed anti-tumour
547 activity of both TH588 and TH1579 in OS cell lines with higher activity of TH1579 as
548 shown by significant decrease of cell viability associated with increase of apoptosis
549 as already described in the literature (21, 22, 37, 38). Indeed, TH1579 is more potent
550 to inhibit MTH1 activity than TH588 (22), and is currently in phase 1 clinical trial in
551 various advanced solid malignancies otherwise known as Karonudib
552 (NCT03036228). TH1579 exhibits high anti-tumour activity through a dual
553 mechanism of action, by inducing toxic 8-oxodG lesions into the DNA, that can be
554 reversed by expression of the bacterial MutT enzyme (22), and also through arrest of
555 cells in mitosis by inhibition of microtubule polymerisation. The detailed mechanism
556 of action is complex as the MTH1 protein promotes tubulin polymerisation and also

557 directly binds tubulin (39). Furthermore, TH1579, but not other potent MTH1
558 inhibitors, breaks the tubulin-MTH1 interaction and arrest cells in mitosis. To
559 complicate matters even further the TH1579 compound directly interfere with tubulin
560 (39) which altogether make up the mechanism of action of Karonudib. Here, we want
561 to evaluate the effect of TH1579 in OS and detailed understanding of the mechanism
562 of action falls outside the scope of this report.

563 However, when MTH1 was specifically knock-down using siRNA or shRNA
564 approaches, a reduction of cell viability of tumour cells was shown, but less
565 dramatically than the effects observed with TH588 and TH1579, suggesting that
566 MTH1 is probably not the only target of these drugs in OS and deserved to be deeply
567 studied. Several studies reported that specific MTH1 inhibition does not affect tumour
568 cell viability and/or apoptosis such as reported in non-small cell lung cancer (25, 26,
569 28, 40). While the cytotoxic effects of MTH1 inhibition in cancer are controversial in
570 literature, TH588 has been shown to be cytotoxic in several studies (41, 42). We also
571 found that TH588 and TH1579 induce apoptosis by increasing DNA damage as
572 shown by comet assay and induction of H2AX phosphorylation in OS cells. These
573 data are consistent with early cellular response to DNA strand breaks as already
574 reported in the literature (33, 43). As reported in two studies TH588 induces a mitotic
575 arrest with cells accumulation in G2/M phases in Hela cells or in colorectal cancer
576 (25, 41), we also observed mitotic arrest (G2/M blockage) in HOS-MNNG cell line
577 after 48h of treatment with TH1579 (500nM). But we cannot exclude that the
578 observed apoptosis associated with DNA damage could also be MTH1-dependent.

579 Our study further demonstrates that TH1579 strongly delayed tumour growth
580 *in vivo* associated with an increase of apoptosis and DNA damage as shown by
581 higher expression level of toxic 8-oxo-G and γ H2AX after TH1579 treatment. By

582 using CETSA, we could show that TH1579 binds to MTH1 inside the tumours of the
583 treated mice in a dose dependent manner. The drug seems to be well tolerated with
584 no toxicity observed. Additionally, TH1579 significantly reduced lung metastases *in*
585 *vivo* in OS xenograft model associated with reduced-CD146 expression in primary
586 tumour compared to control, suggesting less vascularization in treated-primary
587 tumours. We also have shown that TH1579 decreased the number of Circulating
588 Tumour Cells (CTC) and Disseminated Tumour Cells (DTC) in lung of treated mice
589 bearing a non-advanced OS tumour, suggesting that TH1579 might have a direct
590 effect on invasion and migration of tumour cells (44, 45). We effectively confirmed *in*
591 *vitro* that TH1579 reduced invasion and migration of OS cells in line with the literature
592 showing that MTH1 is required for invasion and migration (24, 46). Indeed,
593 Arczewska et al. demonstrated that MTH1 depletion using siRNA significantly
594 reduced invasion and migration of thyroid cancer cell lines by modulating Matrix
595 Metalloproteinases (MMPs) activity and expression (46). MTH1 inhibitor (S)-crizotinib
596 was also described to reduce migratory ability of OS cells *in vitro* (24).

597 Furthermore, associated-bone lesions characterize OS development, and no
598 significant change of bone parameters was observed after TH1579 treatment even if
599 the bone integrity of the tibia is better than in the control group. These results can be
600 explained by the smaller tumours in the treated groups compared to the control.

601 While targeting MTH1 is a contentious question in cancer research, our study
602 demonstrates an anti-tumour activity of TH1579 both *in vitro* and *in vivo* in OS model
603 associated with induction of apoptosis and DNA damage. All these data are the proof
604 of principle that TH1579 could be considered as alternative therapy in metastatic
605 osteosarcoma patient.

606

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617

618 **Declaration of interest**

619 Helleday, T: A patent has been filed with TH588 and TH1579 where T.H., is listed as
620 inventor. The Intellectual Property Right is owned by the non-profit Thomas Helleday
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622 THF. U.W.B is chairman of Oxcia AB. THF is sponsor for on-going clinical trial with
623 TH1579.

624

625 **Author's contributions**

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751

Figure Legends

Figure 1: MTH1 is overexpressed in osteosarcoma. (a) Expression of MTH1 was evaluated by RT-qPCR in 13 human osteosarcoma biopsies and compared to human mesenchymal stem cells (hMSC) or osteoblast derived from differentiation of MSCs (OB) from healthy donors. (b) Clinical features of osteosarcoma patients. (c) Expression of MTH1 in human osteosarcoma cell lines was also evaluated by RT-qPCR and compared to hMSC and OB from healthy donors. Errors bars show SD. (d) Expression of MTH1 in human osteosarcoma cell lines was also evaluated by Western blot and compared to hMSC from healthy donors and analyzed using Empiria Studio software (Licor).

Figure 2: MTH1 inhibition decreases cell proliferation in human osteosarcoma cell lines. (a) Human osteosarcoma cell lines (143B, Cal72, G292, HOS-MNNG, KHOS, MG63, SJSA-1 and U2OS) were cultured for 72 h with TH588 or TH1579 at the indicated concentrations, and cell growth was determined by a crystal violet assay. The expression of an untreated population was equaled to 100%. (b) IC₅₀ for TH588 and TH1579 in tumour cell lines was calculated. (c) HOS-MNNG and U2OS osteosarcoma cells were treated with 2 or 8 μ M of the indicated drugs for 48h then plated at clonal density for colony counts. (d) HOS-MNNG cells were transfected with siRNA (30nM) or transduced with different shRNA targeting MTH1 (MOI 20). Cell viability was compared to controls after crystal violet staining (NT: non transfected, siCt: control siRNA, shLacZ: control shRNA). Error bars show SD. Statistical significance was assessed by Kruskal-Wallis test. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

Figure 3: MTH1 inhibition disrupts cell cycle and induces apoptosis in osteosarcoma cells. (a) HOS-MNNG and U2OS cells were treated with 100nM or 500nM (low dose) and 2 or 8 μ M (high dose) TH1579 for 48 h, and the proportion of cells in subG1, G0–G1, S, G2–M was determined by propidium iodide staining and flow cytometry. (b-c) Tumour cells were treated with TH1579 at the indicated concentrations and apoptosis was evaluated by a caspase 3/7 assay and cleaved poly (ADP-ribose) polymerase (PARP) expression using western blotting. (d) U2OS cells were treated with 8 μ M TH1579 for 24 h, and modified comet assay was performed to evaluate DNA damage (+/- OGG1). The tail moment was calculated

using Fiji and the Open comet plugin. (e) U2OS cells were treated with TH1579 at indicated concentrations for 24 h and 8-oxo-dG incorporation into DNA was evaluated by fluorescence using Alexa488-conjugated Avidin (scale bar 100µm). (f) gH2AX expression was evaluated by western blot in U2OS cells, after 48 h of treatment with TH1579 at the indicated doses. Error bars show SD. Statistical significance was assessed by Kruskal-Wallis or Anova. ** $p < 0.01$, *** $p < 0.001$.

Figure 4: TH1579 significantly delays tumour growth in a HOS-MNNG xenograft model. Mice were orally treated with vehicle, 45 mg/kg or 90 mg/kg TH1579 twice a day, 3 days a week until day 48, when the tumour volume reached 100 mm³. The mean tumour volume (a) or the individual tumour volume (b) of the treated mice were compared to the control group +/- SEM (n = 8). (c) Tumours were collected after 48 days, 8-oxo-G, cleaved-caspase-3 and gH2AX were evaluated by immunohistochemical analysis (scale bar 100µm). Statistical significance was assessed by Anova (tumour growth) or Kruskal-Wallis. Error bars show SEM. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

Figure 5: TH1579 decreases lung metastases in a HOS-MNNG xenograft model. (a) Lung metastases were counted after hematoxylin-eosin coloration (HE; scale bar 1mm, except for the zoom: scale bar 100 µm as mentioned in the figure). (b) Circulating (CTC) and disseminated (DTC) tumour cells were counted in two groups of OS mice: non treated or treated with 90 mg/kg of TH1579 for one week. (c) TH1579 effect on invasion and migration was evaluated *in vitro*, on HOS-MNNG cells, by a migration assay and an invasion assay respectively. Cells were plated in non-coated boyden chambers (migration) or coated with 2 µg of matrigel (invasion) with the indicated TH1579 concentrations, for 6 h (migration) or 24 h (invasion). (d) Neo-angiogenesis was evaluated by CD146 staining, by immunohistochemical (scale bar 100µm) and quantified using ImageJ. Error bars show s.d. Statistical significance was assessed by Kruskal-Wallis or Anova. ** $p < 0.01$, *** $p < 0.001$.

Figure 6: TH1579 effect on bone lesions. (a) Representative microCt image of tibias bearing the tumour in the 3 groups. (b) Quantification of the Bone Volume (BV) and BV/TV (Total Volume) parameters. (c) Quantification of the IHC staining of TRAP and Osterix using ImageJ (scale bar 100 μ m) and represented as number of osteoblasts on bone surface (OB number/BS) and number of osteoclasts on bone surface (OCs/BS). (d) CD14+ cells viability was assessed using crystal violet after TH1579 treatment as indicated concentration. Osteoclast differentiation was assessed after treatment of CD14+ cells with 25 ng/mL human macrophage colony stimulating factor (hM-CSF) and 100 ng/mL of hRANKL +/- TH1579 at the indicated concentrations. Osteoclasts number was evaluated after TRAP staining at day 11. Negative control (CT -) represents CD14+ cells treated with 25 ng/mL hM-CSF without human RANKL. Positive control (CT+) represents cells treated with 100 ng/mL of hRANKL. DMSO represents CT+ treated with DMSO. Error bars show s.d. Statistical significance was assessed by Kruskal-Wallis. * $p < 0.05$; ** $p < 0.01$.

SUPPLEMENTARY DATA

Supplementary Figure S1: OS cell lines clonogenicity: OS cells were treated with 2 or 8 μ M of TH1579 for 48 h then plated at clonal density. After one week, cells were stained by crystal violet and colonies were counted (+/-SD).

Supplementary Figure S2: mRNA expression of MTH1 in HOS-MNNG cells after siRNA (30nM) or shRNA (MOI 20) treatment was evaluated by RT-qPCR in tumour cells (NT : non transfected, siCt : control siRNA, shLacZ : control shRNA).

Supplementary Figure S3: HOS-MNNG and U2OS cells were treated with 2 or 8 μ M TH588 for 48 h, and the proportion of cells in subG1, G0–G1, S, G2–M phases

was determined by propidium iodide staining and flow cytometry, and apoptosis was evaluated by a caspase 3/7 assay. * $p < 0.05$; ** $p < 0.01$,

Supplementary Figure S4: γ H2AX expression in U2OS cells treated with 8 μ M TH1579 for 48 h. γ H2AX assessment in U2OS cells by Immunofluorescence. Condition IR 15 Gy is used as positive control.

Supplementary Figure S5: MTH1 engagement by TH1579 *in vivo* was evaluated by cellular thermal shift assay (CETSA) at 56, 58 and 60° C, in tumours of the three groups: non treated, treated with 45 mg/kg or 90 mg/kg TH1579.

Supplementary Figure S6: Tumour volumes comparison between two groups of OS mice: non treated and treated with 90 mg/kg of TH1579, a week after HOS-MNNG cells injection.

Supplementary Figure S7: TH1579 effect on hMSC viability and mineralization. The positive control (Ct+) represents hMSC cells cultured in a medium with heparin, platelet lysate ascorbic acid, β -glycerophosphate and dexamethasone, for osteogenic differentiation. The negative control (Ct-) represents hMSC cells cultured with heparin and, platelet lysate only. The number of colonies was counted after crystal violet staining (a) and mineralization was evaluated after coloration with alizarin red (b). Error bars show SD

Supplementary Table I: MTH1 primers sequences

Supplementary Table II: Primary antibodies and dilutions

