

Supporting information

Iron Chelators in Photodynamic Therapy Revisited: Synergistic Effect by Highly Active Thiosemicarbazones

Anna Mrozek-Wilczkiewicz^{†‡}, Maciej Serda[†], Robert Musiol[†], Grzegorz Malecki[†], Agnieszka Szurko[‡], Angelika Muchowicz[§], Jakub Golab[§], Alicja Ratuszna[‡], and Jaroslaw Polanski^{*†}

[†]Institute of Chemistry, University of Silesia, Szkolna 9, PL-40-006 Katowice, (Poland),

[‡]Institute of Physics, University of Silesia, Uniwersytecka 4, PL-40-007 Katowice, (Poland),

[§]Center of Biostructure Research, Medical University of Warsaw, Banacha 1a, PL-02-097 Warsaw, (Poland)

*polanski@us.edu.pl

KEYWORDS *photodynamic therapy, thiosemicarbazones, protoporphyrin IX, triapine, reactive oxygen species*

Experimental section.....	S2
Materials and Methods.....	S2
Synthesis	S2
Figure S1. Synthesis.....	S3
Characterization data.....	S3
Figure S2. HRMS data of 1.....	S5
Figure S3. HRMS data of 2.....	S7
Figure S4. HRMS data of 3.....	S9
Crystal structures.....	S10
Table S1. Crystal data and structure refinement details of 1.....	S11
Figure S5. An ORTEP view of 1 with ellipsoids at 50% probability level.....	S12
Cell lines	S12
Measurement of cytostatic/cytotoxic effects.....	S12
Fluorescence intensity time profile of PpIX formation.....	S13
Figure S6. Fluorescence intensity time profile of PpIX formation in HCT 116+/+.....	S14
Influence of the thiosemicarbazones on PpIX formation	S14
Pharmacological synergy	S14
Table S2. Fa fractions resulted from the respective TSC and PDT doses.....	S16
Formation of ROS	S16
Figure S7. ROS formation.....	S16

Experimental section

Materials and Methods

Reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), ACROS Organics (Belgium) or Princeton Chemicals Ltd (Luton, Bedfordshire, UK). Solvents and other common consumables were obtained from POCH (www.poch.com.pl). Silica gel 60, 0.040-0.063 mm (Merck, Darmstadt, Germany) was used for column chromatography. Thin layer chromatography (TLC) experiments were performed on alumina-backed silica gel 40 F₂₅₄ plates (Merck). The plates were illuminated under UV (254 nm) and evaluated in iodine vapor.

The melting points were determined on an Optimelt MPA100 instrument (SRS, USA) and are uncorrected. High resolution-mass spectrometry (HRMS) analysis was performed for all new compounds on a Finnigan MAT95 spectrometer (Thermo Fisher Scientific, Bremen, GmbH) or on Mariner ESI-TOF spectrometer (Applied Biosystems, USA). The purity checking for all novel compounds was carried out on Gynkotec HPLC Modular System equipped with DAAD detector UVD340U. All ¹H NMR spectra were recorded on a Bruker AM-400 spectrometer (399.95 MHz for ¹H; BrukerBioSpin Corp., Germany). Chemical shifts are reported in ppm against the internal standard, Si(CH₃)₄. Easily exchangeable signals were omitted when diffuse. Syntheses were performed on a CEM-DISCOVERY microwave reactor (CEM Corporation, Matthews, NC, USA) with temperature and pressure control using closed vessel mode.

Synthesis

Thiosemicarbazones (TSC) were synthesized by reacting the respective heteroaromatic ketone or carbaldehyde and thiosemicarbazide under microwave irradiation. Equimolar quantities of the appropriate thiosemicarbazide (0.5 mmol) and carbonyl compound (0.5 mmol) were dissolved in 4 mL of EtOH with the addition of 0.1 mL of acetic acid as the catalyst. The resulting mixture was heated in a microwave reactor at 83°C/30 min (max. microwave power 50 W). After cooling, the precipitated solid was filtered and washed with ether. The final product was purified by crystallization (ethanol or methanol) or column chromatography on silica gel.

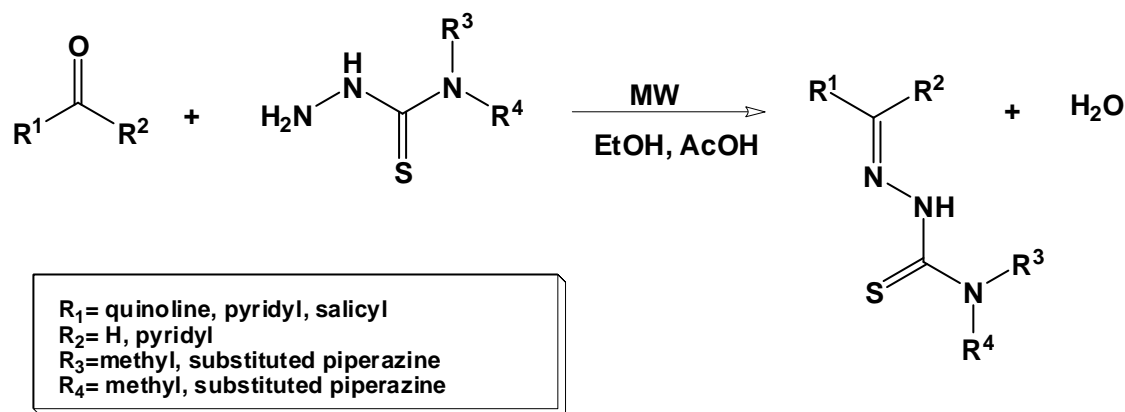


Figure S1. Synthesis.

Characterization data

The purity of all the final TSC samples was assessed using HPLC and was higher than 95%. General conditions: HPLC GynkoteK; Pump T580; Autosampler GINA50; Detector DAAD UVD340U; Column: Hilic Kinetex 100A, Phenomenex ; Flow: 0.5 mL/min (0-1 min), 0.5-1.2 mL/min (1-3 min), 1.2 mL/min (3-7 min), 1.2-0.5 mL/min (7-8) min, 90% CH₂Cl₂, 10 % CH₃OH; UV at 250 nm; Software: Chromeleon.

1 - *N'*-(di(pyridin-2-yl)methylene)-4-phenylpiperazine-1-carbothiohydrazide (Figure 1, **1**)

Yield: 78 %

¹H-NMR (*d*₆-DMSO, 400 MHz, ppm): 14.59 (bs, 1H, NH); 8.89 (d, 1H, *J* = 3.9 Hz); 8.60 (d, 1H, *J* = 4.0 Hz, pyridine); 8.01-7.91 (m, 3H); 7.60 (m, 2H); 7.49 (m, 1H); 7.25 (t, 2H, *J* = 7.7 Hz); 6.96 (d, 2H, *J* = 8.2 Hz); 6.81 (t, 1H, *J* = 7.2 Hz); 4.17 (m, 4H, CH₂, piperazine); 3.34 (m, 4H, CH₂, piperazine)

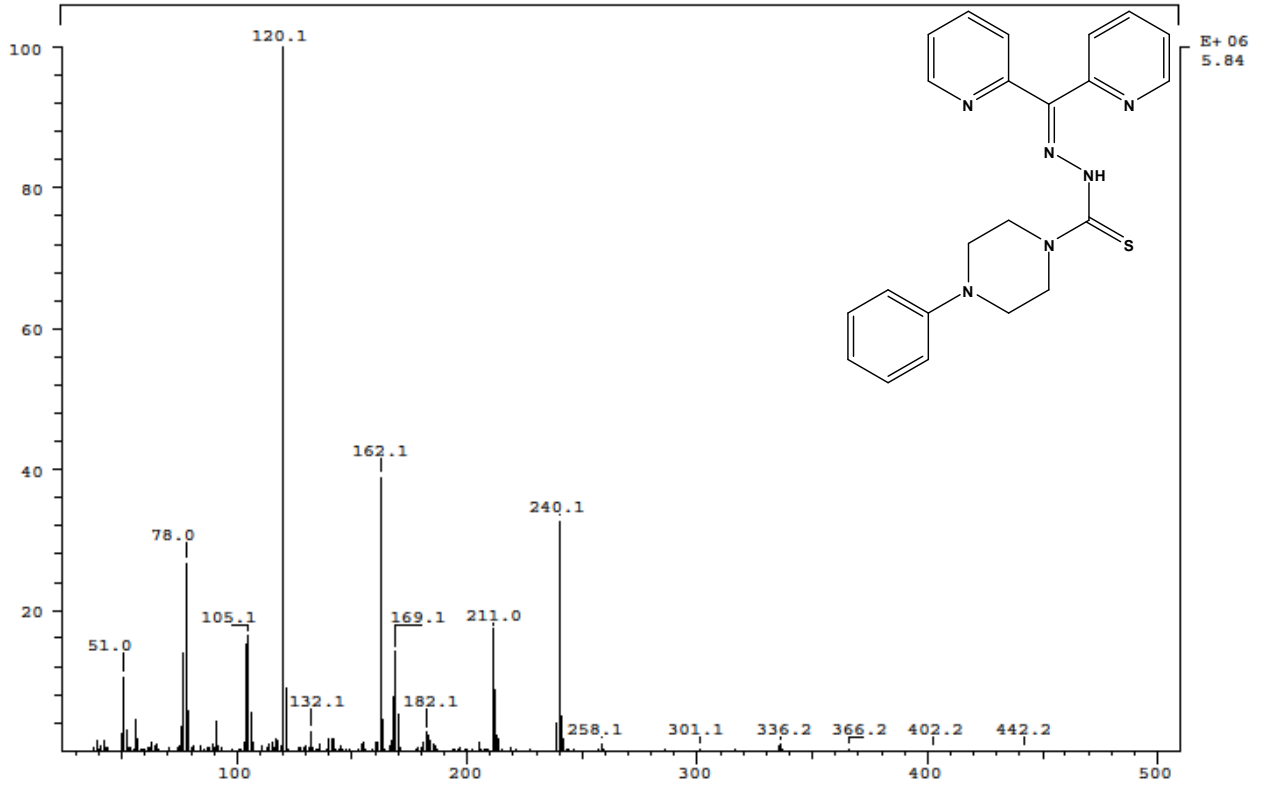
¹³C-NMR (*d*₆-DMSO, 100 MHz, ppm): 180.7; 150.8; 148.7; 148.4; 138.3; 137.7; 129.5; 127.3; 125.1; 119.4; 115.7; 49.6; 48.1.

*R*_f = 0.28 [dichloromethane: methanol 40/1 (v/v)]

HRMS-EI: 402.1627 (C₂₂H₂₂N₆S; Exact Mass: 402.1627) (Figure S2)

MP: 151-152° C

SPEC: ay132usk_a
 Samp: MS 154
 Comm: 70 eV
 Mode: EI +VE +LMR BSCAN (EXP) UP LR NRM
 Oper: ub Client: US M.Serda
 Base: 120.1 Inten: 5836319
 Norm: 120.1 RIC: 26185816
 Peak: 1000.00 mmu
 19-Nov-12 Elapse: 04:34.3 19
 Start: 09:28:51 85
 Study: MS CBM1M PAN Lodz
 Inlet: DIP
 Masses: 33 > 800
 #peaks: 396



Limit: (0) . . .
: (1417) C100.H100.S.N6
Peak: 1000.00 mmu R+D: -1.0 > 24.5

Mass of (402.16278) = 402.162780000

Abs Abun. .7359030247

Delta (ppm) R+ Formula

-28009 15.0 C₂₂H₂₂S.N₆

No. exact mass abun. %

1 402.162667 100.000000

2 403.165451 28.017525

3 404.162946 8.220301

4 405.163376 1.536025

5 406.164072 0.199690

6 407.164662 0.019966

7 408.165369 0.001586

8 409.166626 0.000101

9 410.168344 0.000005

Delta (mmu) R+ Formula

-8.6668 20.0 C₂₅H₁₈N₆

No. exact mass abun. %

1 402.159295 100.000000

2 403.162241 30.535582

3 404.165118 4.500552

4 405.167995 0.426390

5 406.170862 0.029177

6 407.173704 0.001536

7 408.176556 0.000065

8 409.179351 0.000002

Figure S2. HRMS data of 1.

2 - (E)-N'-((8-hydroxyquinolin-2-yl)methylene)morpholine-4-carbothiohydrazide (Figure 1, 2)

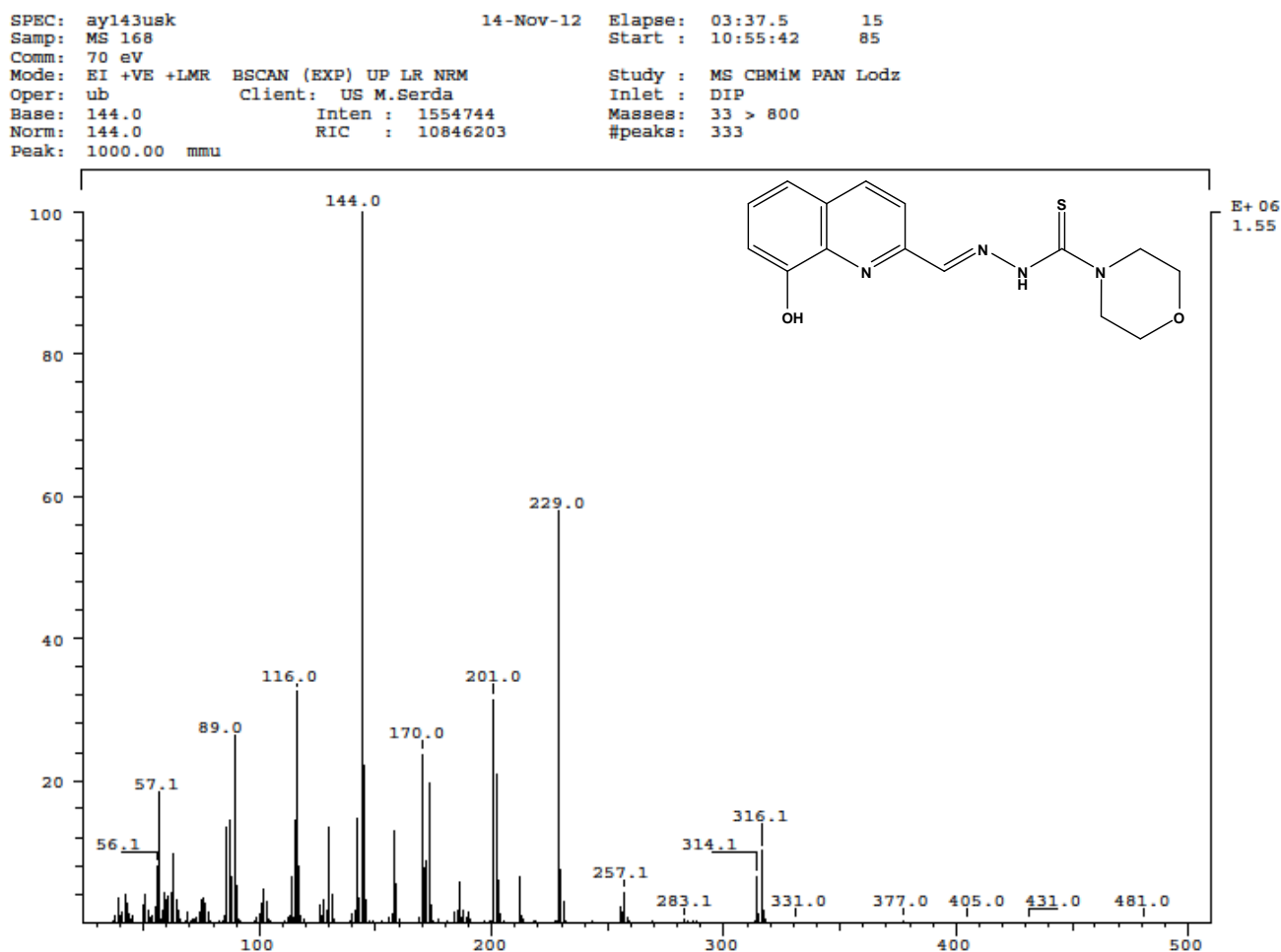
Yield: 76%

¹H-NMR (*d*₆-DMSO, 400 MHz, ppm): 11.66 (bs, 1H, NH); 9.81 (bs, 1H, OH); 8.35 (s, 1H, CH); 8.30 (d, 1H, *J* = 8.7 Hz, quinoline), 7.97 (d, 1H, *J* = 8.7 Hz, quinoline), 7.45 (t, 1H, *J* = 7.7 Hz, quinoline), 7.39 (d, 1H, *J* = 7.6 Hz, quinoline), 7.12 (d, 1H, *J* = 7.1 Hz, quinoline); 3.98 (m, 4H, piperazine); 3.72 (m, 4H, piperazine).

¹³C-NMR (*d*₆-DMSO, 100 MHz, ppm): 181.4; 153.8; 152.2; 144.3; 138.6; 137.0; 129.1; 128.6; 118.3; 117.9; 112.6; 66.5; 51.2.

HRMS-EI: 316.0994 (C₁₅H₁₆N₄O₂S; Exact Mass: 316.0994) (Figure S3)

MP: 181-182° C



Limit: (0)

:(1421) C100.H100.S.O2.N4

Peak: 1000.00 mmu R+D: -1.0 > 24.5

Mass of (316.09836) = 316.098360000

Abs Abun. .8723046184

Delta (ppm) R+ Formula

3.28499 10.0 C15.H16.S.O2.N4

No. exact mass abun. %

1	316.099398	100.000000
2	317.102163	19.411787
3	318.098306	6.625872
4	319.099694	1.007900
5	320.099833	0.123283
6	321.100554	0.011855
7	322.101608	0.000919
8	323.102906	0.000059
9	324.104424	0.000003

Delta (mmu) R+ Formula

5.34125 19.0 C23.H12.N2

No. exact mass abun. %

1	316.100048	100.000000
2	317.103236	26.731277
3	318.106439	3.426553
4	319.109551	0.280344
5	320.112773	0.016438
6	321.115989	0.000735
7	322.119132	0.000026

Delta (mmu) R+ Formula

-7.3852 15.0 C18.H12.O2.N4

Figure S3. HRMS data of 2.

3 - (*E*)-4-ethyl-*N'*-(2-hydroxybenzylidene)piperazine-1-carbothiohydrazide (Figure 1, **3**)

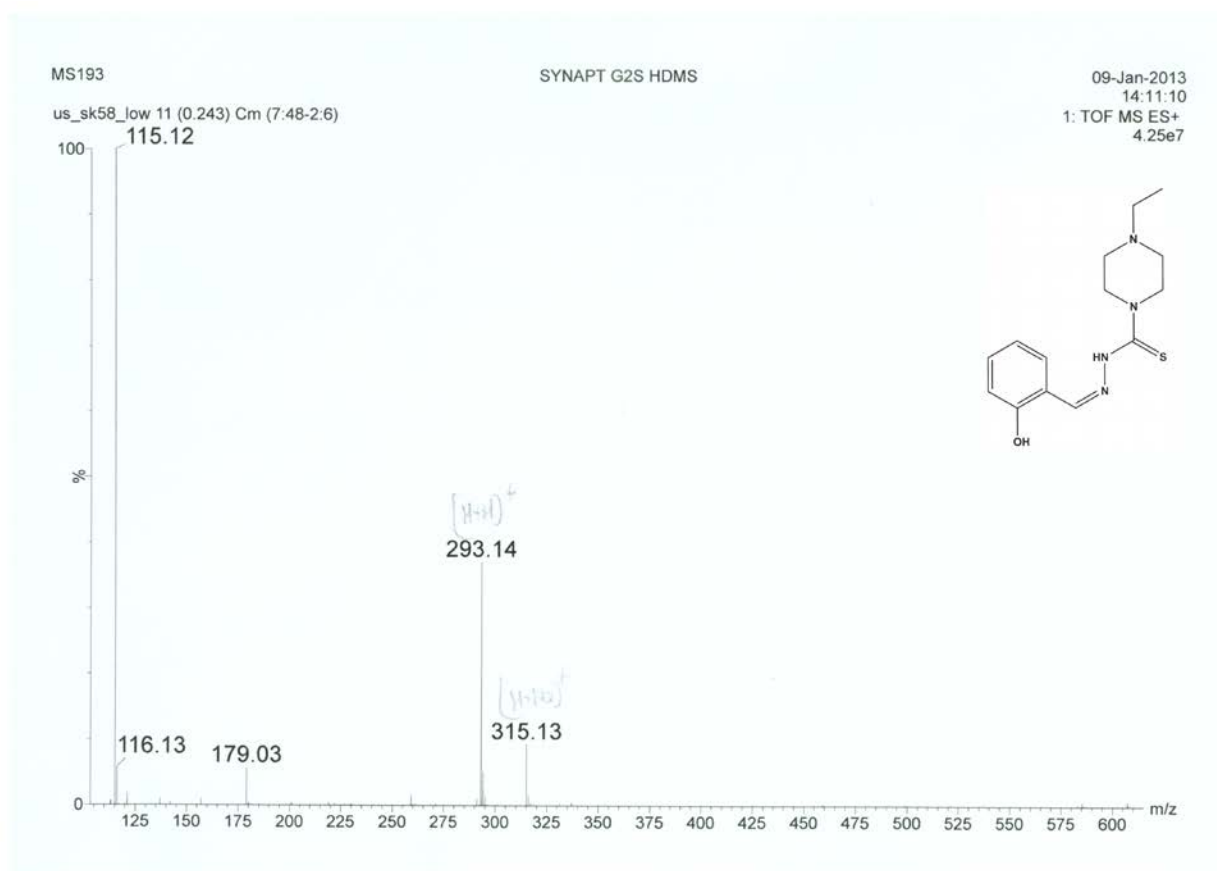
Yield: 86%

$^1\text{H-NMR}$ (d_6 -DMSO, 400 MHz, ppm): 11.60 (bs, 1H, NH), 8.46 (s, 1H, CH), 7.41 (dd, 1H $J_1 = 7.9$ Hz, $J_2 = 1.6$ Hz, phenyl), 7.27 (td, 1H, $J_1 = 7.8$ Hz, $J_2 = 7.4$ Hz, $J_3 = 1.7$ Hz, phenyl), 6.92-6.88 (m, 2H, phenyl), 3.92 (m, 4H, piperazine), 2.45 (m, 4H, piperazine), 2.35 (m, 2H, CH_2), 1.03 (t, 3H, $J = 7.2$ Hz, CH_3).

$^{13}\text{C-NMR}$ (d_6 -DMSO, 100 MHz, ppm): 183.0; 159.0; 148.0; 138.0; 128.5; 121.2; 118.6; 117.5; 57.2; 44.4; 12.5.

HRMS-ESI-TOF: 293.1434 $[\text{M} + \text{H}]^+$ ($\text{C}_{14}\text{H}_{21}\text{N}_4\text{OS}$; Exact Mass: 293.1436) (Figure S4)

MP: 160-161° C



Elemental Composition Report

Page 1

Single Mass Analysis

Tolerance = 10.0 mDa / DBE: min = -1.5, max = 100.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 4

Monoisotopic Mass, Even Electron Ions

447 formula(e) evaluated with 14 results within limits (up to 50 closest results for each mass)

Elements Used:

C: 0-200 H: 0-200 N: 0-10 O: 0-5 S: 0-1

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
293.1434	293.1436	-0.2	-0.7	6.5	2035.2	0.007	99.32	C14 H21 N4 O S
	293.1364	7.0	23.9	10.5	2041.0	5.818	0.30	C20 H21 S
	293.1396	3.8	13.0	2.5	2041.2	6.048	0.24	C9 H21 N6 O3 S
	293.1423	1.1	3.8	1.5	2041.9	6.727	0.12	C13 H25 O5 S
	293.1508	-7.4	-25.2	2.5	2043.4	8.174	0.03	C8 H21 N8 O2 S
	293.1356	7.8	26.6	-1.5	2048.0	12.822	0.00	C4 H21 N8 O5 S
	293.1468	-3.4	-11.6	-1.5	2049.5	14.302	0.00	C3 H21 N10 O4 S
	293.1389	4.5	15.4	6.5	2054.6	19.406	0.00	C16 H21 O5
	293.1501	-6.7	-22.9	6.5	2054.8	19.570	0.00	C15 H21 N2 O4
	293.1474	-4.0	-13.6	7.5	2055.0	19.845	0.00	C11 H17 N8 O2
	293.1362	7.2	24.6	7.5	2055.1	19.877	0.00	C12 H17 N6 O3
	293.1402	3.2	10.9	11.5	2055.1	19.885	0.00	C17 H17 N4 O
	293.1515	-8.1	-27.6	11.5	2055.2	19.968	0.00	C16 H17 N6
	293.1434	0.0	0.0	3.5	2055.8	20.587	0.00	C6 H17 N10 O4

Figure S4. HRMS data of **3**.

4 - *N,N*-dimethyl-2-(7-hydroxyquinolin-8-yl)-methylene)-hydrazinecarbothioamide (Figure 1, **4**)

Yield: 79%

¹H-NMR (*d*₆-DMSO, 400 MHz): 12.18 (s, 1H, NH); 11.47 (s, 1H, OH); 9.83 (s, 1H, CH); 8.85 (m, 1H, ArH); 8.31 (d, 1H, *J*= 8.0 Hz, ArH); 7.93 (d, 1H, *J*= 8.8 Hz, ArH); 7.43 (m, 1H, ArH); 7.30 (d, 1H, *J*= 8.8 Hz, ArH); 3.33(s, 6H, -CH₃).

¹³C-NMR (*d*₆-DMSO, 100 MHz): 179.4; 163.7; 150.6; 146.9; 144.8; 137.0; 131.4; 122.3; 120.5; 119.8; 111.3; 41.2

HR-MS (EI): 274.0883 (calc. for C₁₃H₁₄N₄OS: 274.0888)

MP: 172° C [lit:172-173°C]¹

Crystal structures

X-ray intensity data were collected with graphite monochromated Mo K α radiation at a temperature of 295.0(2) K, with the x scan mode using the Oxford Diffraction Gemini A Ultra diffractometer. Lorentz, polarization and empirical absorption correction using spherical harmonics implemented in the SCALE3 ABSPACK scaling algorithm [CrysAlis RED, Oxford Diffraction Ltd., Version 1.171.29.2] were applied. The structure was solved by the direct method and subsequently completed by the difference Fourier recycling. All of the non-hydrogen atoms were refined anisotropically using the full-matrix, least-squares technique. All the hydrogen atoms were found from difference Fourier synthesis after four cycles of anisotropic refinement, and refined as ‘riding’ on the adjacent carbon atom with individual isotropic temperature factor equal 1.2 times the value of equivalent temperature factor of the parent atom. The OLEX2 and SHELXS97, SHELXL97 programs were used for all the calculations. (Table S1, Figure S5). CCDC 908257; These data can be obtained free of charge via <http://www.ccdc.cam.ac.uk/conts/retrieving.html>, or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223-336-033; or e-mail:deposit@ccdc.cam.ac.uk).

Empirical formula	C ₂₂ H ₂₂ N ₆ S
Formula weight	402.52
Temperature [K]	295.0(2)
Crystal system	monoclinic
Space group	<i>P</i> 2 ₁ / <i>c</i>
Unit cell dimensions	
a [Å]	7.6772(13)
b [Å]	11.3013(9)
c [Å]	23.426(5)
β [°]	98.07(2)
Volume [Å ³]	2012.4(6)
Z	4
Calculated density [Mg/m ³]	1.329
Absorption coefficient [mm ⁻¹]	0.182
F(000)	848
Crystal dimensions [mm]	0.47 x 0.07 x 0.05
θ range for data collection [°]	3.40–25.05
Index ranges	-9 ≤ h ≤ 9, -11 ≤ k ≤ 13, -27 ≤ l ≤ 27
Reflections collected	8416
Independent reflections	3554 [R _(int) = 0.0332]
Data / restraints / parameters	3554/ 0/ 262
Goodness-of-fit on F ²	0.974
Final R indices [I > 2σ(I)]	R ₁ = 0.0426, wR ₂ = 0.0907
R indices (all data)	R ₁ = 0.0738, wR ₂ = 0.0994
Largest diff. Peak and hole	0.196/ -0.199

Table S1. Crystal data and structure refinement details of **1**.

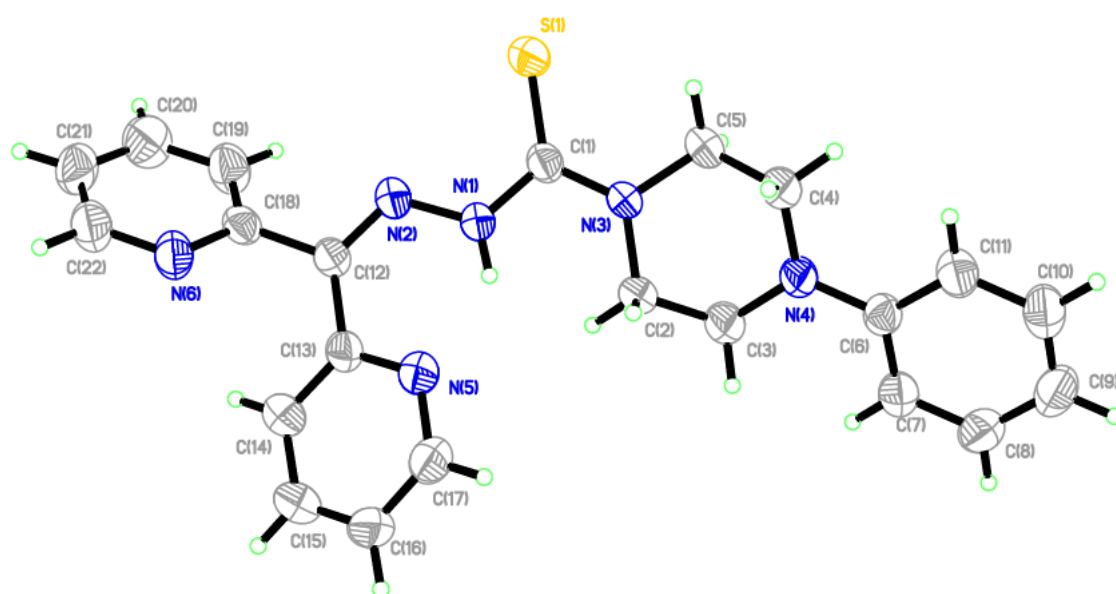


Figure S5. An ORTEP view of **1** with ellipsoids at 50% probability level.

Cell lines

The human colon cancer cell line HCT 116 with the wild type p53 (p53+/+), with a p53 gene deletion (p53-/-), normal human dermal fibroblasts (NHDF), human Burkitt's lymphoma (Raji) and human cervical carcinoma (HeLa) were obtained from ATCC. The HCT 116, HeLa and NHDF cells were grown as monolayer cultures in 75 cm² flasks (Nunc) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% (NHDF) 12% (HCT 116) or 10% (HeLa) Fetal Bovine Serum (Sigma) and standard antibiotics. Raji cells were cultured in RPMI -1640 with the addition of 10% Fetal Bovine Serum (Invitrogen, Carlsbad, CA) and antibiotic/antimycotic solution (Sigma, St. Louis, MO). For all cell types (except NHDF) we used heat inactivated Fetal Bovine Serum. Cells were cultured under standard conditions at 37°C in a humidified atmosphere at 5% CO₂ and, passaged every 3-4 days as required.

Measurement of cytostatic/cytotoxic effects

The cells were seeded in 96-well plates twenty four hours before addition of the tested compounds. The assays were performed following a 96h (HCT 116, NHDF) or 72h (Raji, HeLa) incubation with varying concentrations of the tested agents. The results were expressed as a percentage of the control and calculated as IC₅₀ values (using GraphPad Prism 5). IC₅₀ was defined as the compound concentration that was necessary to reduce the relative cell number to 50% of the untreated controls. Each individual compound was tested in triplicate in a single experiment, with each experiment being repeated three times. After incubation of

HCT 116 and NHDF cells with the tested compounds, 20 μL of the CellTiter 96® AQueous One Solution - MTS (Promega) solution was added to each well (with 100 μL of DMEM without phenol red) and incubated for 1h at 37°C. The optical densities of the samples were analyzed at 490 nm. MTT Formazan (Sigma) was used to evaluate cytostatic/cytotoxic effect in Raji cells. Following the incubation with the investigated compounds a MTT solution at a concentration of 5 mg/mL was added. After the next 4h, the plates were centrifuged, and the cells were lysed with DMSO. The absorbance was measured at 570 nm. The cytostatic/cytotoxic effect on HeLa cells was estimated using crystal violet staining. After incubation with investigated compounds cells were rinsed with PBS and stained with 0,5% crystal violet solution for 10 minutes. Finally, the plates were washed with tap water and the cells were lysed with 2% SDS. The results are presented in Table 1.

Fluorescence intensity time profile of PpIX formation

The cells (HCT 116+/+) were seeded into 96-well plates (Black Clear Bottom, Corning), ($17 \cdot 10^3$ cells/well) 48h before the experiment. The next day the medium was removed and the cells were washed with PBS (Immuniq). A solution of 1mM ALA was freshly prepared before the experiments by dissolving it into DMEM (minus phenol red and without FBS). Cells were stored in the dark at 37°C during incubation. After the appropriate incubation period, a fluorescence plate reader (Synergy4; BIO-TEK) was used to measure the fluorescence of the PpIX. A wavelength of 407 nm was used for the excitation of the PpIX and the emission spectrum had a maximum for a wavelength of 638nm. The obtained spectra were compared with the fluorescence spectrum of PpIX dissolved in DMEM (without phenol red). The comparison of spectra confirmed that PpIX was the main fluorophore that was detected. The experiments were stopped after reaching a slight decrease in the intensity of the fluorescence and achieving a maximum degree of cell confluence. Control cells without any of the test solutions were used to compare the autofluorescence of the cells with the fluorescence of the PpIX. The light level in the laboratory was reduced from about 700 to 45 lx during the experiments to avoid photobleaching of the PpIX. The experiments were repeated four times on different days. In order to expose any potential superimposition of the spectra of TSCs with that of the PpIX, the fluorescence spectra was registered at an emission $\lambda = 407$ nm. The lack of interfering signals indicates no superimposition.

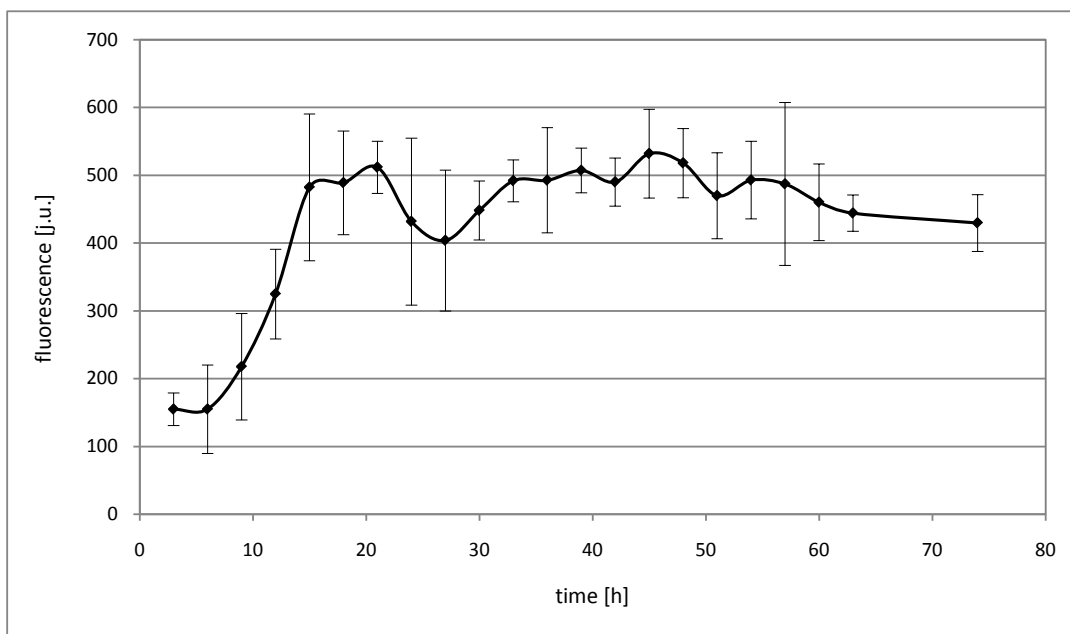


Figure S6. Fluorescence intensity time profile of PpIX formation in HCT 116+/+ cell line.

Influence of the thiosemicarbazones on PpIX formation

This experiments were performed as in former chapter (see Fluorescence intensity time profile of PpIX formation Figure S6). Solutions of 1mM ALA and the investigated compounds were freshly prepared before each experiment by dissolving them into modified DMEM (without phenol red and without FBS). After the appropriate incubation period, a fluorescence plate reader (Synergy4; BIO-TEK) was used to measure the fluorescence of PpIX. The experiments were repeated five times. Alternatively, the control experiment was performed in an iron-supplemented medium in the conditions described above. The culture medium was prepared and a 2 μ M solution of FeCl₃ was added. The experiments were carried out using the selected compounds (active and inactive). No statistically significant differences were observed in the amount of PpIX that was detected.

Pharmacological synergy

The cells (HCT 116+/+) were seeded in 3 cm Petri dishes (Nunc), ($15 \cdot 10^5$ cells/dish) 24h before the experiments. The medium was removed the next day and the cells were washed with PBS (Immuniq). Solutions of 1mM ALA and the investigated chelators were freshly prepared before each experiment by dissolving them into DMEM (minus phenol red and without FBS). Each test plate also contained cells with ALA (1mM) and cells without any of the tested compounds. The medium was replaced with PBS after 24h and irradiated with a light of 630 nm in appropriate the doses. Then buffer was replaced with a standard medium and after the next 24h, the viability of the cells was determined using the MTS test; 130 μ L of

the CellTiter 96[®] AQueous One Solution – MTS (Promega) solution was added to each dish (with 700 μ L DMEM without phenol red) and incubated for 1h at 37°C. The optical densities of the samples were analyzed at 490nm. The experiments were repeated three times. The calculation of the synergy was performed according to the Chou-Talay method (Compusyn software).^[2,3] Briefly, the concentrations scope was adjusted to cover the specified IC₅₀ value for each drug. Four concentrations were tested with four PDT doses for each drug. The doses were increased at a constant ratio according to refs^{2,3} (Table S2).

The combination index CI was calculated according to equation

$$\frac{(D)_{TSC}}{(D_x)_{TSC}} + \frac{(D)_{PDT}}{(D_x)_{PDT}} = CI$$

where D_x is the dose of PDT or TSC given alone, enough to reach effect x (x% of inhibition).

D is the portion of PDT or TSC given in combination sufficient to achieve the effect x.

Fraction affected f_a was calculated according to equation

$$f_a = \left(\frac{D}{D_m} \right)^m / f_u$$

where D is the dose of the TSC or PDT, D_m jest the dose of the TSC or PDT causes 50% effect, m describes the shape of the dose-response curve, f_u is unaffected fraction.

TSC		PDT dose (J/cm ²)				
		0	1.14	2.28	4.56	9.12
1 [nM]	0		0.18	0.25	0.22	0.49
	0.8	0.00	0.34			
	1.6	0.01		0.42		
	3.2	0.08			0.65	
	6.4	0.2				0.79
2 [nM]	0		0.1	0.13	0.28	0.38
	3.8	0.13	0.34			
	7.6	0.20		0.33		
	15.2	0.18			0.72	
	30.4	0.25				0.84
3 [μ M]	0		0.14	0.15	0.3	0.45
	5	0.24	0.49			
	10	0.42		0.57		
	20	0.48			0.7	
	40	0.49				0.7
4 [μ M]	0		0.09	0.12	0.22	0.47
	5	0.01	0.48			
	10	0.07		0.47		
	20	0.17			0.65	
	40	0.38				0.72
I [μ M]	0		0.2	0.18	0.22	0.38
	25	0.00	0.3			
	50	0.01		0.43		

	100	0.12			0.61	
	200	0.23				0.77
II [μ M]	0		0.01	0.03	0.26	0.55
	1	0.00	0.00			
	2	0.05		0.04		
	4	0.02			0.06	
	8	0.30				0.73

Table S2. Fa fractions resulted from the respective TSC and PDT doses.

Formation of ROS

24 h before experiment, the cells (HCT 116+/+) were seeded into 8-well plate (Lab-Tek), ($10 \cdot 10^3$ cells/well). The next day medium was removed and solution of tested compounds (1,6nM of **1**, 8nM of **2**, 4 μ M of **II**) was added and incubation was carried on for the next 24h. The positive control was prepared by addition of 100 μ M of H₂O₂ to the cells for 10 min. Subsequently 5 μ M of CellROX® Green Reagent (Molecular Probes®) was added. After incubation for 30 min the cells were washed with PBS and then observed with the excitation at 485nm (emission 520nm). The results of ROS formation in HCT 116+/+ cell line are presented on Figure S7 (A - negative control, B - positive control H₂O₂, C - **II**, D - **1**, E - **2**). The 50 μ m scale bar in D.

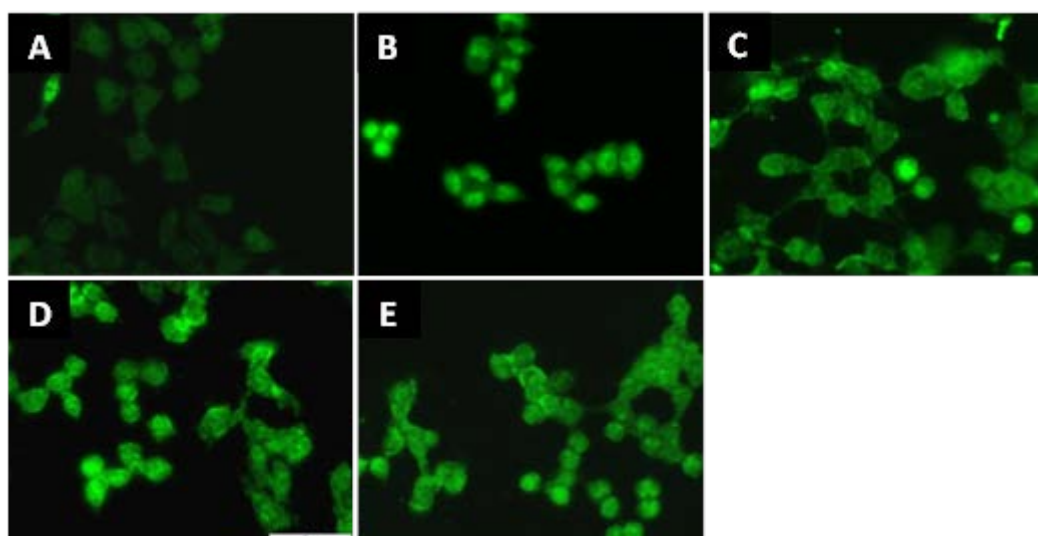


Figure S7. ROS formation in HCT 116+/+ cell line.

References

- (1) Serda, M.; Kalinowski, D. S.; Mrozek-Wilczkiewicz, A.; Musiol, R.; Szurko, A.; Ratuszna, A.; Pantarat, N.; Kovacevic, Z.; Merlot, A. M.; Richardson, D. R.; Polanski, J. Synthesis and characterization of quinoline-based thiosemicarbazones and correlation of cellular iron-binding efficacy to anti-tumor efficacy. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 5527–5531.
- (2) Chou, T.C. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol. Rev.* **2006**, *58*, 621–681.
- (3) Chou, T.C. Drug Combination Studies and Their Synergy Quantification Using the Chou-Talalay Method. *Cancer Res.* **2010**, *70*, 440–446.