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α,γ-Diketocarboxylic acids and their esters act as carbonic anhydrase IX and XII selective inhibitors

SUPPORTING INFORMATION

Content:

EXPERIMENTAL SECTION

Chemistry. Melting points were determined on a Stuart melting point apparatus SMP10. ^{1}H-NMR and ^{13}C-NMR spectra were recorded at 400 MHz and 100 MHz, respectively, by using a Bruker AC 400 spectrometer; chemical shifts are reported in δ (ppm) units relative to the internal reference tetramethylsilane (Me_4Si). Microwave-assisted reactions were performed with a Biotage Initiator (Uppsala, Sweden) high frequency microwave synthesizer working at 2.45 GHz, fitted with magnetic stirrer and sample processor; reaction vessels were Biotage microwave glass vials sealed with applicable cap; temperature was controlled through the internal IR sensor of the microwave apparatus. Mass spectra were recorded on an API-TOF Mariner by Perspective Biosystem (Stratford, Texas, USA), and samples were injected by a Harvard pump using a flow rate of 5-10 μL/min, infused in the Electrospray system. All compounds were routinely checked by TLC and ^{1}H-NMR. TLC was performed on aluminum-backed silica gel plates (Merck DC, Alufolien Kieselgel 60 F254) with spots visualized by UV light or using a KMnO4 alkaline solution. All solvents were reagent grade and, when necessary, were purified and dried by standard methods. Concentration of solutions after reactions and extractions involved the use of a rotary evaporator operating at reduced pressure of ~ 20 Torr. Organic solutions were dried over anhydrous sodium sulfate. Elemental analysis has been used to determine purity of the final compounds **1-6** that is > 95%. Analytical results are within ± 0.40% of the theoretical values. All chemicals were purchased from Sigma Aldrich srl, Milan (Italy) or from TCI Europe NV, Zwijndrecht (Belgium), and were of the highest purity. As a rule, samples prepared for physical and biological studies were dried in high vacuum over phosphorus pentoxide for 20 h at temperatures ranging from 25 to 40 °C, depending on the sample melting point.

General procedure for the synthesis of the α,γ-diketocarboxylic acids 1-3. Example: (2*Z*,5*E*)-6-(4-(2-naphthamido)phenyl)-2-hydroxy-4-oxohexa-2,5-dienoic acid (2). To a solution of (2*Z*,5*E*)-ethyl 6-(4-(2-naphthamido)phenyl)-2-hydroxy-4-oxohexa-2,5-dienoate **2** (1 equiv, 0.91

mmol, 380 mg) in a mixture of THF (4 mL) and methanol (2 mL), a 0.7 M lithium hydroxide (2.2 equiv, 2 mmol, 84.0 mg) solution (3 mL) was added while cooling at 0° C. After 6 h and 30 min at rt, the stirring was stopped, and the basic aqueous phase was acidified with 2N hydrochloric acid while cooling at 0° C. The resulting suspension was then filtered and washed over the filter with water to obtain the desired compound **2** as a pure yellow solid. (Y= 82%). Mp: 199-201 °C ^{1}H-NMR (400MHz; DMSO) δ ppm: 3.40 (bs, 2H, O*H* and COO*H*), 6.53 (s, 1H, PhCH=CHCOC*H*=COH), 7.02 (d, 1H, PhCH=C*H*COCH), 7.66-7.80 (m, 5H, PhC*H*=CHCOCH and C*H* aromatic rings), 7.94-7.96 (m, 2H, C*H* aromatic rings), 8.02-8.12 (m, 4H, C*H* aromatic rings), 8.61 (s, 1H, C*H* naphthalene ring), 10.69 (s, 1H, CON*H*). ^{13}C-NMR (100MHz; DMSO) δ ppm: 101.38, 120.69 (2C), 122.89, 124.90, 127.39, 128.16, 128.43, 128.56 (2C), 128.65, 129.46, 129.65, 129.99, 130.06, 130.17, 132.46, 132.51, 134.83, 142.17, 142.53, 163.88, 166.32. Anal. ($C_{23}H_{17}NO_5$) % Calcd: C, 71.31; H, 4.42; N, 3.62. Found (%): C, 71.40; H, 4.45; N, 3.57. MS (ESI), m/z: 386 $[M - H]^-$.

(2*Z*,5*E*)-6-(4-benzamidophenyl)-2-hydroxy-4-oxohexa-2,5-dienoic acid (1) (Y= 79 %). Mp: 218-220 °C. ^{1}H-NMR (400MHz; DMSO) δ ppm: 3.60 (bs, 2H, O*H* and COO*H*), 6.50 (s, 1H, PhCH=CHCOC*H*=COH), 7.01 (d, 1H, PhCH=C*H*COCH), 7.52-7.62 (m, 3H, PhC*H*=CHCOCH and C*H* phenyl rings), 7.69-7.76 (m, 3H, C*H* phenyl rings), 7.85-7.97 (m, 4H, C*H* phenyl rings), 10.47 (s, 1H, CON*H*). Anal. ($C_{19}H_{15}NO_5$) % Calcd: C, 67.65; H, 4.48; N, 4.15. Found (%): C, 67.74; H, 4.51; N, 4.10. MS (ESI), m/z: 336 $[M - H]^-$.

(2*Z*,5*E*)-6-(4-(1-naphthamido)phenyl)-2-hydroxy-4-oxohexa-2,5-dienoic acid (3). (Y= 76 %). Mp: 208-210 °C. ^{1}H-NMR (400MHz; DMSO) δ ppm: 3.67 (bs, 2H, O*H* and COO*H*), 6.52 (s, 1H, PhCH=CHCOC*H*=COH), 7.04 (d, 1H, PhCH=C*H*COCH), 7.60-8.29 (m, 12H, PhC*H*=CHCOCH and C*H* aromatic rings), 10.82 (s, 1H, CON*H*). Anal. ($C_{23}H_{17}NO_5$) % Calcd: C, 71.31; H, 4.42; N, 3.62. Found (%): C, 71.39; H, 4.45; N, 3.58. MS (ESI), m/z: 386 $[M - H]^-$.

General procedure for the synthesis of the α,γ-diketoesters 4-6. Example: (2*Z*,5*E*)-ethyl 6-(4-(1-naphthamido)phenyl)-2-hydroxy-4-oxohexa-2,5-dienoate (6). To a solution of sodium ethoxide

(2.2 equiv, 356 mg, 5.28 mmol) in dry THF (6 mL), diethyl oxalate (2 equiv, 4.8 mmol, 0.65 mL) and a suspension of **11** (1 equiv, 760 mg, 2.4 mmol) in dry THF (6 mL) were added dropwise, under nitrogen atmosphere while cooling at 0° C. After 3 h and 30 min at rt, the reaction was stopped, and after the evaporation of the solvent the residue was acidified with 2N hydrochloric acid while cooling at 0° C. After stirring for 30 min, the acidic suspension was filtered and the solid over filter washed with water. The obtained crude solid was finally purified by recrystallization from acetonitrile to afford compound **6** as a pure yellow solid. (Y= 62 %). Mp: 176-179 °C. ^{1}H-NMR (400MHz; DMSO) δ ppm: 1.28-1.31 (t, 3H, COOCH$_2$C*H*$_3$), 3.55-3.75 (bs, 1H, O*H*), 4.26-4.31 (q, 2H, COOC*H*$_2$CH$_3$), 6.63 (s, 1H, PhCH=CHCOC*H*=COH), 7.06 (d, 1H, PhCH=C*H*COCH), 7.59-7.64 (m, 3H, PhC*H*=CHCOCH and C*H* aromatic rings), 7.77-7.81 (m, 4H, C*H* aromatic rings), 7.90-7.93 (d, 2H, C*H* aromatic rings), 8.02-8.04 (m, 1H, C*H* naphthalene ring), 8.08-8.11 (m, 1H, C*H* naphthalene ring), 8.17-8.19 (m, 1H, C*H* naphthalene ring), 10.85 (s, 1H, CON*H*). ^{13}C-NMR (100MHz; DMSO) δ ppm: 14.36, 62.52, 101.33, 120.27 (2C), 122.51, 125.49, 126.14, 126.91, 127.60 (2C), 128.85, 130.07, 130.09, 130.15, 130.26, 130.86, 133.63, 134.82, 142.37, 143.25, 162.07, 168.01, 173.65, 185.75. Anal. ($C_{25}H_{21}NO_5$) % Calcd: C, 72.28; H, 5.10; N, 3.37. Found (%): C, 72.38; H, 5.13; N, 3.30. MS (ESI), m/z: 416 $[M + H]^+$.

(2*Z*,5*E*)-ethyl 6-(4-benzamidophenyl)-2-hydroxy-4-oxohexa-2,5-dienoate (4) (Y= 57 %). Mp: 180-182 °C. ^{1}H-NMR (400MHz; DMSO) δ ppm: 1.27-1.31 (t, 3H, COOCH$_2$C*H*$_3$), 3.52-3.70 (bs, 1H, O*H*), 4.26-4.31 (q, 2H, COOC*H*$_2$CH$_3$), 6.61 (s, 1H, PhCH=CHCOC*H*=COH), 7.04 (d, 1H, PhCH=C*H*COCH), 7.52-7.62 (m, 3H, PhC*H*=CHCOCH and C*H* phenyl rings), 7.73-7.78 (m, 3H, C*H* phenyl rings), 7.89-7.91 (d, 2H, C*H* phenyl rings), 7.95-7.97 (d, 2H, C*H* phenyl rings), 10.49 (s, 1H, CON*H*). Anal. ($C_{21}H_{19}NO_5$) % Calcd: C, 69.03; H, 5.24; N, 3.83. Found (%): C, 69.13; H, 5.27; N, 3.79. MS (ESI), m/z: 366 $[M + H]^+$.

(2*Z*,5*E*)-ethyl 6-(4-(2-naphthamido)phenyl)-2-hydroxy-4-oxohexa-2,5-dienoate (5) (Y= 59 %). Mp: 199-202 °C. ^{1}H-NMR (400MHz; DMSO) δ ppm: 1.28-1.31 (t, 3H, COOCH$_2$C*H*$_3$), 3.48-3.72

(bs, 1H, O*H*), 4.26-4.31 (q, 2H, COOC*H*$_2$CH$_3$), 6.61 (s, 1H, PhCH=CHCOC*H*=COH), 7.05 (d, 1H, PhCH=C*H*COCH), 7.64-7.67 (m, 2H, C*H* aromatic rings), 7.74-7.80 (m, 3H, PhC*H*=CHCOCH and C*H* aromatic rings), 7.94-8.10 (m, 6H, C*H* aromatic rings), 8.59 (s, 1H, C*H* naphthalene ring), 10.68 (s, 1H, CON*H*). Anal. ($C_{25}H_{21}NO_5$) % Calcd: C, 72.28; H, 5.10; N, 3.37. Found (%): C, 72.37; H, 5.14; N, 3.29. MS (ESI), m/z: 416 $[M + H]^+$.

Synthesis of (*E*)-4-(4-aminophenyl)but-3-en-2-one (8). To a solution of (*E*)-4-(4-nitrophenyl)but-3-en-2-one **7** (1 equiv, 5 g, 26 mmol) in ethanol (45 mL), a suspension of stannous chloride dihydrate (3.54 equiv, 92.6 mmol, 20.88 g) in 37% (w/w) hydrochloric acid (20 mL) was added dropwise while cooling at 0 °C. After 25 h at rt, the reaction was quenched with 2N potassium hydroxide (150 mL) and extracted with ethyl acetate (4 x 100 mL). The organic layer was then dried over sodium sulfate, filtrated and evaporated under vacuum to give a crude product that was finally purified by silica gel column chromatography eluting with a mixture ethyl acetate/chloroform 1:5, thus affording compound **8** as a pure yellow solid. (Y= 73 %). Mp: 95-98° C. ^{1}H-NMR (400MHz; $CDCl_3$) δ ppm: 2.35 (s, 3H, COC*H*$_3$), 4.02 (bs, 2H, N*H*$_2$), 6.55 (d, 1H, CHC*H*COCH$_3$), 6.65-6.68 (d, 2H, C*H* phenyl ring), 7.36-7.38 (d, 2H, C*H* phenyl ring), 7.44 (d, 1H, C*H*CHCOCH$_3$). MS (ESI), m/z: 162 $[M + H]^+$.

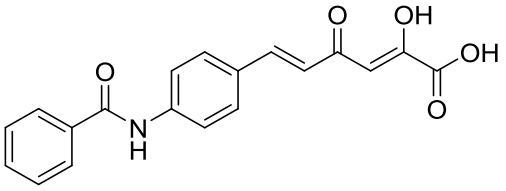
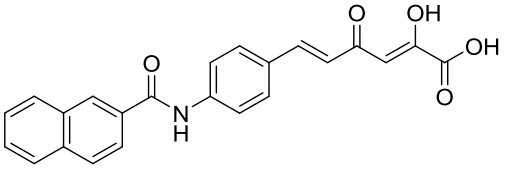
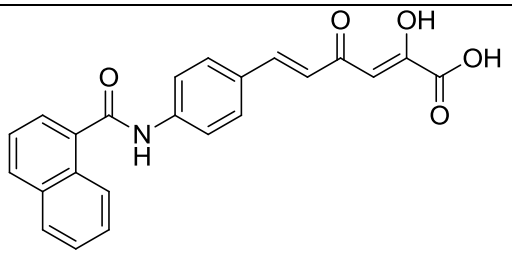
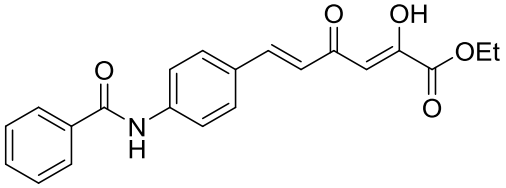
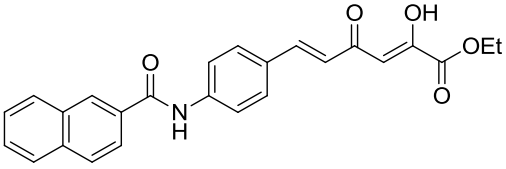
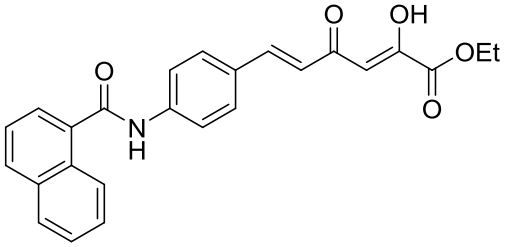
General procedure for the synthesis of the intermediate derivatives 9-11. Example: (*E*)-*N*-(4-(3-oxobut-1-en-1-yl)phenyl)benzamide (9). To a solution of (*E*)-4-(4-aminophenyl)but-3-en-2-one **7** (1 equiv, 290 mg, 1.8 mmol) in dry DCM cooled at 0° C, triethylamine (1.5 equiv, 2.7 mmol, 0.38 mL) and benzoyl chloride (1.2 eq, 2.16 mmol, 0.25 mL) were added in sequence. After stirring for 2 h at 0° C, the reaction was quenched with sodium hydrogen carbonate saturated solution (20 mL), and extracted with DCM (4 x 20 mL). The organic phase was then washed with 2N hydrochloric acid and brine, dried over sodium sulfate, filtered and concentrated under reduced pressure. The resulting crude was then triturated with DCM and filtered off to obtain compound **9** as a pure beige solid (Y= 78 %). Mp: 204-206 °C. ^{1}H-NMR (400MHz; DMSO) δ ppm: 2.31 (s, 3H, COC*H*$_3$), 6.73 (d, 1H, CHC*H*COCH$_3$), 7.51-7.61 (m, 4H, C*H*CHCOCH$_3$ and C*H* phenyl rings), 7.69-7.71 (d, 2H,

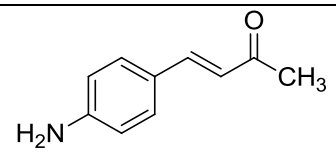
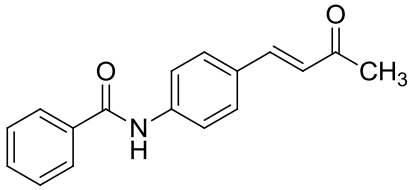
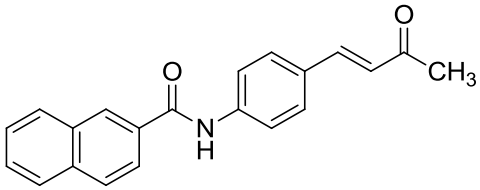
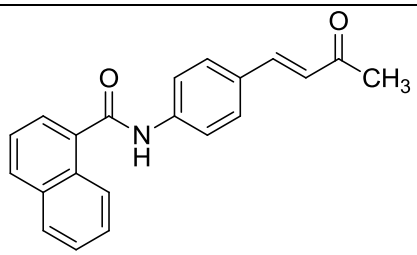
C*H* phenyl rings), 7.86-7.88 (d, 2H, C*H* phenyl rings), 7.93-7.95 (d, 2H, C*H* phenyl rings), 10.44 (s, 1H, CON*H*). MS (ESI), m/z: 266 $[M + H]^+$.

(*E*)-*N*-(4-(3-oxobut-1-en-1-yl)phenyl)-2-naphthamide (10) (Y= 75 %). Mp: 230-232 °C. ^{1}H-NMR (400MHz; DMSO) δ ppm: 2.33 (s, 3H, COCH_3), 6.76 (d, 1H, CHC*H*COCH$_3$), 7.58-7.65 (m, 3H, C*H*CHCOCH$_3$ and C*H* aromatic rings), 7.73-7.75 (d, 2H, C*H* aromatic rings), 7.92-7.94 (d, 2H, C*H* aromatic rings), 8.00-8.11 (m, 4H, C*H* aromatic rings), 8.59 (s, 1H, C*H* naphthalene ring), 10.64 (s, 1H, CON*H*). MS (ESI), m/z: 316 $[M + H]^+$.

(*E*)-*N*-(4-(3-oxobut-1-en-1-yl)phenyl)-1-naphthamide (11). (Y= 81 %). Mp: 201-203 °C. ^{1}H-NMR (400MHz; DMSO) δ ppm: 2.33 (s, 3H, COCH_3), 6.76 (d, 1H, CHC*H*COCH$_3$), 7.59-7.64 (m, 4H, C*H*CHCOCH$_3$ and C*H* aromatic rings), 7.75-7.78 (m, 3H, C*H* aromatic rings), 7.88-7.90 (d, 2H, C*H* aromatic rings), 8.01-8.04 (m, 1H, C*H* naphthalene ring), 8.08-8.10 (m, 1H, C*H* naphthalene ring), 8.16-8.19 (m, 1H, C*H* naphthalene ring), 10.80 (s, 1H, CON*H*). MS (ESI), m/z: 316 $[M + H]^+$.

Table S1. Chemical and physical data of novel intermediate and final compounds **1-6**, **8-11**.

Cpd	Structure	Mp	Yield (%)	Crystallization solvent[a]
1		218-220	79	a
2		199-201	82	a
3		208-210	76	a
4		180-182	57	c
5		199-202	59	b
6		176-179	62	c

8		110-114	73	d
9		204-206	78	e
10		230-232	75	f
11		201-203	81	e
[a]a: ethanol; b: acetonitrile/methanol; c: acetonitrile; d: cyclohexane; e: DCM; f: DCM/chloroform.				

Carbonic anhydrase inhibition

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity.[1] Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as buffer, and 20 mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10-100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5-10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng-Prusoff equation, as reported earlier,[2] and represent the mean from at least three different determinations. All CA isofoms were recombinant ones obtained in-house as reported earlier.[3]

***In silico* studies**

The crystal structure of hCA II (pdb 5LJT) and hCA IX (pdb 5FL4) were prepared using the Protein Preparation Wizard tool implemented in Maestro - Schrödinger suite, assigning bond orders, adding hydrogens, deleting water molecules, and optimizing H-bonding networks.[4] Energy minimization protocol with a root mean square deviation (RMSD) value of 0.30 was applied using an Optimized Potentials for Liquid Simulation (OPLS3) force field. 3D ligand structures were prepared by Maestro[4a] and evaluated for their ionization states at pH 7.4 ± 0.5 with Epik.[4b] OPLS3 force field in Macromodel[4e] was used for energy minimization for a maximum number of 2500 conjugate gradient iteration and setting a convergence criterion of 0.05 kcal $mol^{-1}Å^{-1}$. The docking grid was centered on the center of mass of the co-crystallized ligands and Glide used with default settings. Ligands were docked with the standard precision mode (SP) of Glide [4f] and the best 5 poses of each molecule retained as output. The best pose for each compound, evaluated in terms of coordination, hydrogen bond interactions and hydrophobic contacts, was refined with Prime[4d] with a VSGB solvatation model considering the target flexible within 3Å around the ligand.[5-7]

Cell lines and culture

Continuous cell lines of human osteosarcoma (MG-63, HOS) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). MG-63 and HOS cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Sigma, Milan, Italy), penicillin (100 U/ml), and streptomycin (100 mg/ml) (Invitrogen) at 37°C and 5% CO_2. Only cells in exponential growth phase were used.

Expression of CA IX and CA XII by quantitative real-time PCR and western blot

Total RNA was extracted from semi-confluent OS cells by using the RNeasy Mini Kit (Qiagen GmbH). Total mRNA was reverse transcribed by the Advantage RT-for-PCR Kit (Roche). The expression of CA IX and CA XII was evaluated by RealTime PCR using the Light Cycler instrumentation (Roche Diagnostics), as previously described.[8] Probes and specific primers were selected using a web-based assay design software (ProbeFinder Software, online available at the Assay Design Center: http://lifescience.roche.com). The sequences of primers selected for the analysis are listed in Table 1. Alternative splicing isoforms of CA IX and CA XII has been reported,[9] and the primers used in this study have been selected to bind the full-length isoform of CA IX and the common assay for CA XII isoforms. The protocol of amplification was: 95 °C for 10 min; 95 °C for 10s, 60 °C for 30s, and 72°C for 1s for 45 cycles; 40°C for 30s. The results were expressed as the ratio between gene of interest and the geometric average of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide (YWHAZ), used as reference genes, according to the 2-ΔΔCT method.[10] Whole-cell extracts from semi-confluent OS cells were lysated with RIPA buffer (Tris pH 7.6 50mM, NaCl 150 mM, Triton-X 100 5%, sodium deoxycholate 0.25%, EGTA pH 8 1mM, NaF 1 mM) (Sigma-Aldrich) supplemented with protease inhibitors (Roche). Equal amount of lysate were analyzed by SDS--PAGE, followed by immunoblotting with an mouse-anti-human CA IX (1:200,

clone M75, BioScience Slovakia), rabbit-anti-human-CA XII (1:200, HPA008773, Sigma) and rabbit-anti-human-TATA-box-binding protein (TBP) (1:500, sc-204, Cell Signaling) antibodies, as reference. Goat-antimouse and goat-antirabbit antibody conjugated to horseradish peroxidase was diluted (1:1000) in 5% dry milk in T-TBS and used as secondary antibody. Immunocomplexes were detected with the ECL western blot analysis system (Bio-rad).

Table S2. Real-time polymerase chain reaction primers used in the study.

Target	**Forward (5' -- 3')**	**Reverse (5' -- 3')**
CA IX (NM_001216.2)	TGCCTATGAGCAGTTGCTGT	CCAGTCCTGGGACCTGAGT
CA XII (NM_001218.3 and NM_206925)	CCCATAGACCTGCACAGTGA	AGGGCAGGTTCAGCTTCA
YWHAZ (NM_003406.3)	CCGTTACTTGGCTGAGGTTG	TGCTTGTTGTGACTGATCGAC
GAPDH (NM_002046.3)	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC

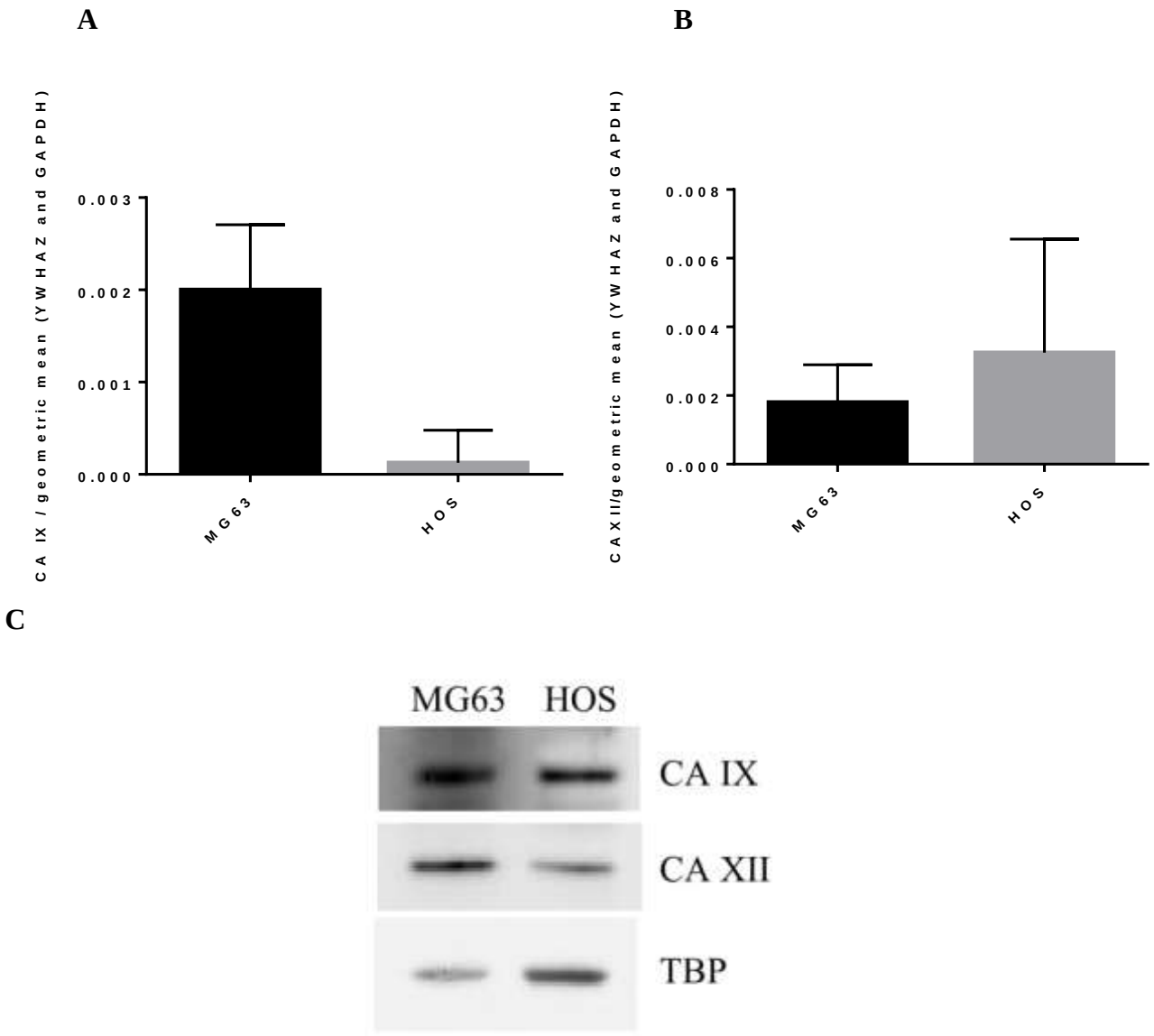


Figure S1. Real Time PCR analysis of the expression of CA IX (A) and CA XII (B) in MG63 and HOS osteosarcoma cell lines. Expression of CA IX and CA XII (C) by western blot (representative images). The expression of TATA-box-binding protein was used as reference.

Cell viability assays

The acid phosphatase assay was used to measure cell viability. Briefly, MG63 and HOS cells were seeded in triplicate in 96-well plates ($2x10^3$ cells/well). After 24 hrs for 2D cultures, cells were treated with increasing doses of **2**, **5** and **6** compounds (0-100 μM). After 72 hrs of treatment, the supernatant was discarded, cells were washed with 200 μL/well of PBS, and 100 μL/well of NaAc-buffer (Sodium Acetate, Sigma-Aldrich, Milan, Italy) containing *p*-nitrophenyl phosphate disodium hexahydrate were added. After 2 hrs of incubation at 37°C and 5% CO_2, the reaction was stopped with 10 μL/well of NaOH 1M and the absorbance at 405 nm was recorded by using a microplate-reader (Tecan Infinite F200pro, Tecan, Milan, Italy). Results were expressed as percentage of viable cells with respect to the control.

Cell proliferation assay

Cells were seeded in duplicate in 12-well plates ($2x10^4$ cells/ well) in IMDM complete medium. After 24 h, the culture medium was replaced with fresh medium containing 0-50 μM of compounds **5** and **6** dissolved in PBS. As controls, cells were incubated with medium added with the same amount of PBS. After 72 h, OS cells were harvested and the number of viable cells was evaluated by the erythrosine B dye vital staining.[11] Results were expressed as percentage of growth inhibition in respect to cells in control medium. The experiment was repeated three times, two replicates for each conditions. IC_{50} was calculated using the Graph Pad Prism software.

Apoptosis evaluation

The activity of caspase 3 and 7 was measured by a luminescence assay (Caspase-GloR 3/7 Assay, Promega) according to the manufacturer instructions. Briefly, MG63 and HOS were seeded in triplicate in 96-well plates ($2x10^3$ cells/well), in IMDM complete medium. After 24 h, the culture medium was replaced with fresh medium containing 50 μM of compound **5** or 25 μM of compound **6**. As negative control, cells were incubated with medium added with the same amount of PBS. As a

positive control of apoptosis, cells were treated with staurosporine 1 µM for 4 h. After 72 h, the Caspase-GloR 3/7 Assay was performed, and the luminescent signal, that is proportional to caspase 3/7 activities, collected using a microplate-reader (Infinite F200pro, Tecan, Milan, Italy).

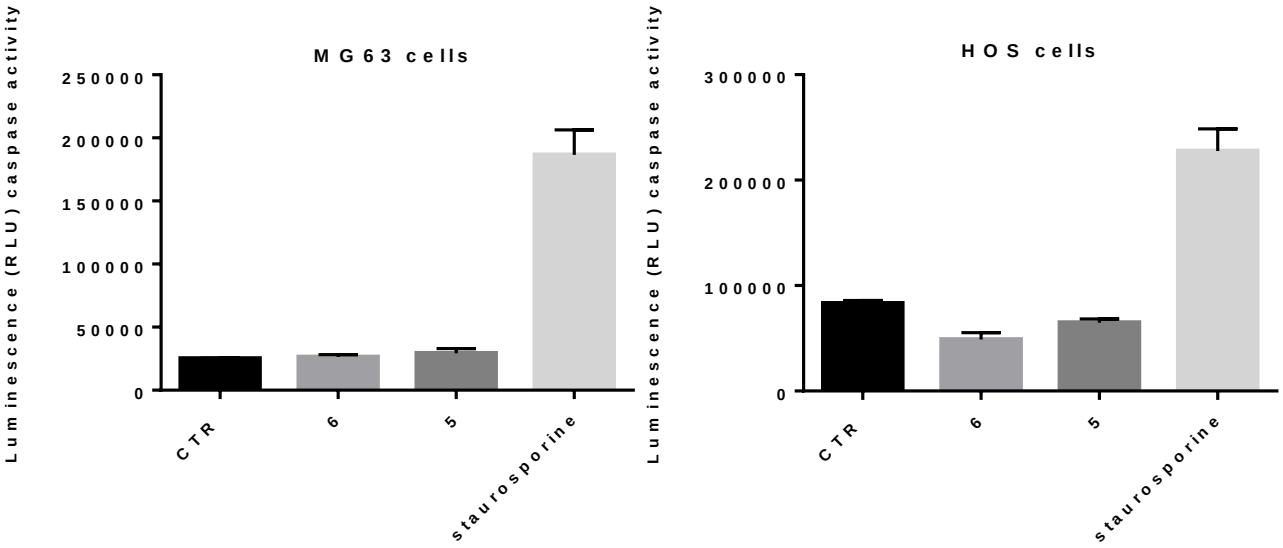


Figure S2. Apoptosis induction evaluation in MG63 and HOS OS cell lines.

Statistical analysis

Statistical analysis was performed using the StatView™ 5.0.1 software for Windows (SAS Institute, Cary, NC). Results were reported as mean ± standard deviation and the differences were analyzed using non-parametric Mann–Whitney test for the difference between groups. Only $p<0.05$ were considered significant.

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