# Supporting information for

# Discovery and synthesis of the first selective BAG domain modulator of BAG3 as an attractive candidate for the development of a new class of chemotherapeutics

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## Experimental Procedures

### Computational details

The library of compounds. The starting library of compounds was downloaded from the Otava Chemicals database (~ $3.1 \times 10^5$ compounds). The compounds were prepared using LigPrep software (Schrodinger Suite).[1] All the possible stereoisomers, tautomers, and protonation states at a pH = 7.4±1.0 were generated for each compound, and the structures were minimized using OPLS 2005 force field. Protein 3D model was prepared using the Protein Preparation Wizard workflow (Maestro, Schrödinger), using murine structure related to BAG3BD (PDB code: 1UK5).[2] All hydrogen atoms were added, and bond orders were assigned.

Virtual Screening. Molecular docking experiments were perfomed on BAG3BD. The Virtual Screening was performed using the Virtual Screening Workflow as implemented in Schrödinger Suite and using Glide software,[3-6] following the scheme (Figure 1):

- High-Throughput Virtual Screening scoring and sampling (HTVS) (input: ~ $3.1 \times 10^5$ compounds), saved first 10% of ranked compounds
- Standard Precision scoring and sampling phase (SP), saved first 10% of ranked compounds
- Extra Precision scoring and sampling phase (XP), saved first 10% of ranked compounds as final output
- finally, the obtained compounds were also re-scored using the MMGBSA method.

Due to the absence of previous information on the binding mode of a putative BAG3 modulator, the selected docking poses were those showing a favorable accommodation in the binding sites after visual inspection, establishing specific sets of interactions, such as H-bonds and/or face-to-face or edge-to-face π-π contacts protein counterpart. This workflow led to the identification of compounds **1**-**24** (Table S1).

Building of a combinatorial library and Virtual Screening. Once identified compound **2** (See Results and Discussion), we then proceeded to the building of a new "focused" virtual library of compounds

bearing the 2,4-thiazolidinedione chemical core. With this aim, *CombiGlide* software[7] was employed, allowing the decoration of the central core with a large set of substituents related to specific reagents, according to the synthetic chemical route reported in Scheme 1. In this specific case, combining commercially available synthons (aldehydes, halides, amines etc.) were accounted from Sigma-Aldrich. On the produced starting virtual library, pharmacokinetic properties were computed using QikProp software, and finally "non-drug like" compounds were discarded using Reactive filter tool, then providing a final library of $1.5 \times 10^4$ 2,3-thiazolidinedione–based derivatives.

The Virtual Screening was performed following the Virtual Screening Workflow,[3] following the scheme (Figure 1):

- High-Throughput Virtual Screening scoring and sampling (HTVS) (input: ~ $1.5 \times 10^4$ compounds), saved first 10% of ranked compounds (output: $9.0 \times 10^4$ compounds, used as input for the subsequent step)
- Standard Precision scoring and sampling phase (SP), saved first 10% of ranked compounds
- Extra Precision scoring and sampling phase (XP), saved first 10% of ranked compounds as final output
- finally, the obtained compounds were also re-scored using the MMGBSA method.

The selected compounds were ranked considering docking score (XP Glide Score) and MMGBSA score, and visually inspected as above reported, then leading to a small set of molecules as candidate items for the subsequent chemical synthesis step (Scheme 1).

**General information for compounds 1-26**

All compounds (**1-26**) have been purchased from Otava Chemicals, Otava Ltd. (http:// www.otavachemicals.com/) (Otava Codes: are reported in Table S1, while chemical structures of **1-26** are illustrated in Figure S1).

**Table S1.** Otava codes of compounds **1-26**.

| Compound | Otava Code | Compound | Otava Code |
|---|---|---|---|
| **1** | 103490002 | **14** | 1157863 |
| **2** | 5450160704 | **15** | 1101770 |
| **3** | 1665278 | **16** | 2194629 |
| **4** | 7020102417 | **17** | 1101808 |
| **5** | 701000129 | **18** | 1177455 |
| **6** | 1089672 | **19** | 7017680454 |
| **7** | 129661070 | **20** | 6138210 |
| **8** | 4355978 | **21** | 7010150281 |
| **9** | 7020721800 | **22** | 7510791436 |
| **10** | 7217221018 | **23** | 1098656 |
| **11** | 7010060072 | **24** | 6243033 |
| **12** | 7119461545 | **25** | 4352340 |
| **13** | 1110852 | **26** | 6463993 |

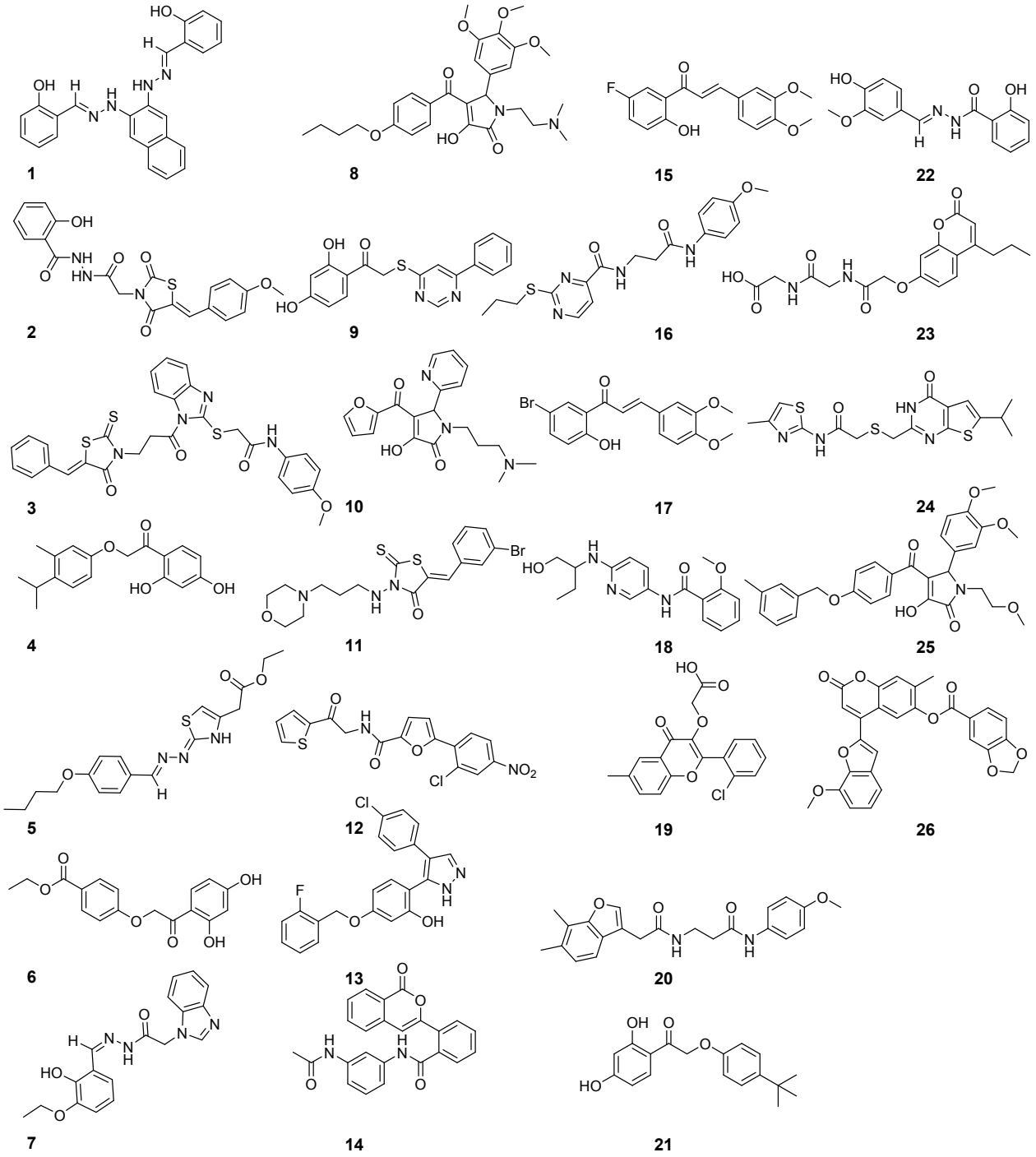



**Fig. S1.** Chemical structures of compounds **1-26**.

## Chemical synthesis and NMR data

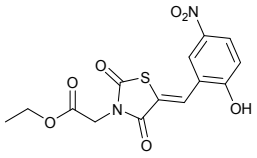
All commercially available starting materials were purchased from Sigma-Aldrich and were used without further purification. All solvents used for the synthesis were of HPLC grade (Sigma-Aldrich and VWR). NMR spectra were recorded in a Bruker Avance spectrometer at 400 MHz; and all compounds were dissolved in 0.5 mL of $CDCl_3$, DMSO-$d_6$, and $CD_3OD$ (Sigma-Aldrich, 99.8 Atom % D). The chemical shifts (δ) are expressed in parts per million (ppm) relative to the solvent peak as internal reference; coupling constants (*J*) are expressed in Hertz. $^1H$ NMR data are reported as s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Electrospray mass spectrometry (ESIMS) were obtained with a LCQ DECA TermoQuest (San Josè, California, USA) mass spectrometer. Chemical reactions were monitored on silica gel 60 F254 plates (Merck) and the spots were visualized under UV light. Analytical and semi-preparative reversed-phase HPLC was performed on Agilent Technologies 1200 Series high performance liquid chromatography using a Synergi Fusion C18 reversed-phase column (250 x 4.60mm, 4μ, 80 Å, flow rate = 1 mL/min; 250 x 10.00mm, 10μ, 80 Å, flow rate = 4 mL/min respectively, Phenomenex®). The binary solvent system (A/B) was as follows: 0.1% TFA in water (A) and 0.1% TFA in $CH_3CN$ (B). The absorbance was detected at 240-280 nm. All biologically tested compounds were determined to be >96% pure by HPLC analysis and NMR data.

### General procedure (A) for the synthesis of compounds 27-36, 44d and 44m

A mixture of thiazolidine-2,4-dione (**42**) (1.0 equiv.), aromatic aldehydes **a**-**m** (1.0 equiv.), piperidine (0.5 equiv.), and ethanol (150 mL) were placed in a 25 ml bottom flask. The reaction mixture was heated under reflux and stirred for a period of 8-9 h; then it was poured into water and acidified with acetic acid. The resulting precipitate was filtered off and recrystallized from acetic acid to give **43a**–**m** which were used without any further purification (Scheme 1A). The reaction was monitored by TLC, analytical RP-HPLC and ESI-MS.

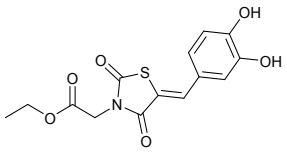
To a solution of **43a-m** (1equiv.) in dry THF (3.5 mL) sodium hydride (1.5 equiv.) was added dropwise and the mixture was stirred for 30 min/1.5h at 80 °C. Then the reaction mixture was cooled to room temperature and to the resulting suspension a solution of ethyl bromoacetate (1.1 equiv.) (for the synthesis of compounds **27**-**36**) or bromoacetic acid (1.1 equiv.) (for the synthesis of **44d** and **44m**) in dry DMF was added dropwise (Scheme 1A). The reaction was stirred at 80°C for 3 h, poured into ice-cold water and the solid product was filtered and recrystallized from EtOH/$H_2O$ to give the desired compounds **27**-**36** and **44d**, **44m**. The reaction was monitored by TLC, analytical RP-HPLC and ESI-MS. HPLC purification was performed by semi-preparative reversed-phase HPLC (Synergi Fusion C18 reversed-phase column: 250 x 10.00mm, 4μM, 80 Å, flow rate = 4 mL/min) using the gradient conditions reported below and the final products were characterized by ESI-MS and NMR spectra.

**Ethyl (*Z*)-2-(5-(5-hydroxy-2-nitrobenzylidene)-2,4-dioxothiazolidin-3-yl)acetate (27)**



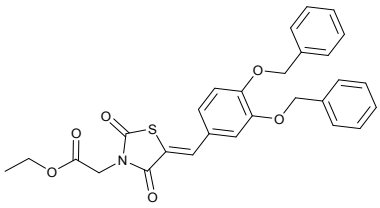

Compound **27** was obtained by following the general procedure as a pale brown powdery solid (75 mg, 44% overall isolated yield after HPLC purification); RP-HPLC $t_R$ = 32.6 min, gradient condition: from 5% B ending to 100 % B over 50 min, flow rate of 4 mL/min, λ = 240 nm. $^1$H NMR (400 MHz, $CDCl_3$): $\delta_H$ 8.24 (d, *J* = 2.6 Hz, 1H), 8.10 (dd, *J* = 9.0, 2.6 Hz, 1H), 8.21 (s, 1H), 6.96 (d, *J* = 9.0 Hz, 1H), 4.44 (s, 2H), 4.21 (q, *J* = 7.1 Hz, 2H), 1.25 (t, *J* = 7.1 Hz, 3H). $^{13}$C NMR (100 MHz, $CD_3OD$): $\delta_C$ 166.9, 166.8, 165.3, 162.5, 140.5, 127.2, 127.1, 124.5, 122.9, 120.8, 115.8, 61.8, 41.7, 12.9. ESI-MS: calcd for $C_{14}H_{12}N_2O_7S$ 352.32, found m/z = 351.2 $[M-H]^-$.

**Ethyl (*Z*)-2-(5-(3,4-dihydroxybenzylidene)-2,4-dioxothiazolidin-3-yl)acetate (28)**



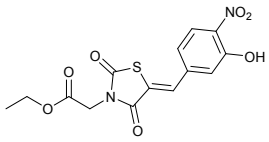
Compound **28** was obtained by following the general procedure as a bright yellow powdery solid (70 mg, 47% overall isolated yield after HPLC purification); RP-HPLC $t_R$ = 25.66 min, gradient condition: from 5% B ending to 100% B over 50 min, flow rate of 4 mL/min, λ = 240 nm.$^1$H NMR (400 MHz, DMSO-$d_6$): $\delta_H$ 7.81 (s, 1H), 7.04 (m, 2H), 6.90 (d, *J*= 8.2 Hz, 1H), 4.46 (s, 2H), 4.16 (q, *J* = 7.1 Hz, 2H), 1.21 (t, *J* = 7.1 Hz, 3H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta_C$ 167.6, 167.3, 165.6, 149.7, 146.5, 135.4, 124.9, 124.5, 117.1, 116.8, 116.1, 62.1, 42.5, 14.4. ESI-MS: calcd for $C_{14}H_{13}NO_6S$ 323.32, found m/z = 322.3 $[M-H]^-$.

**Ethyl (*Z*)-2-(5-(3,4-bis(benzyloxy)benzylidene)-2,4-dioxothiazolidin-3-yl)acetate (29)**



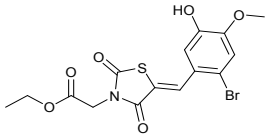
Compound **29** was obtained by following the general procedure as a grey solid (106.6 mg, 44% overall isolated yield after HPLC purification); RP-HPLC $t_R$ = 41.4 min, gradient condition: from 5% B ending to 100% B over 50 min, flow rate of 4 mL/min, λ = 240 nm. $^1$H NMR (400 MHz, $CDCl_3$): $\delta_H$ 7.72 (s, 1H), 7.40 – 7.24 (m, 10H), 7.02 (m, 2H), 6.92 (d, *J* = 8.4 Hz, 1H), 5.17 (d, *J* = 7.0 Hz, 4H), 4.38 (s, 2H), 4.17 (q, *J* = 7.1 Hz, 2H), 1.22 (t, *J* = 7.1 Hz, 3H). $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_C$ 167.5, 166.4, 165.9, 151.4, 149.9, 134.8, 128.7, 128.6, 128.5 (4C), 128.1 (6C), 127.3, 127.2, 125.4, 115.9, 114.2, 70.9, 62.2, 42.1, 29.4, 14.0. ESI-MS: calcd for $C_{28}H_{25}NO_6S$ 503.5, found m/z = 504.3 $[M + H]^+$.

**Ethyl (*Z*)-2-(5-(3-hydroxy-4-nitrobenzylidene)-2,4-dioxothiazolidin-3-yl)acetate (30)**



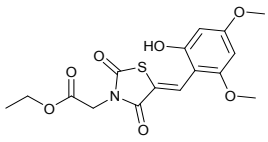
Compound **30** was obtained by following the general procedure as a pale yellow powdery solid (109.8 mg, 63.3% overall isolated yield after HPLC purification); RP-HPLC $t_R$ = 32.6 min, gradient condition: from 5% B ending to 100 % B over 50 min, flow rate of 4 mL/min, λ = 240 nm. $^1$H NMR (400 MHz, $CDCl_3$): $\delta_H$ 8.14 (d, *J* = 8.8 Hz, 1H), 7.77 (s, 1H), 7.22 (d, *J* = 1.9, 1H), 7.05 (dd, *J* = 8.8, 1.9, 1H), 4.42 (s, 2H), 4.19 (q, *J* = 7.1 Hz, 2H), 1.24 (t, *J* = 7.1 Hz, 3H). $^{13}$C NMR (100 MHz, $CD_3OD$): $\delta_C$ 166.9, 166.8, 165.3, 162.5, 140.5, 127.2, 127.1, 124.5, 122.9, 120.8, 115.8, 61.8, 41.7, 12.9. ESI-MS: calcd for $C_{14}H_{12}N_2O_7S$ 352.32, found m/z = 351.2 $[M-H]^-$.

**Ethyl-(*Z*)-2-(5-(2-bromo-5-hydroxy-4-methoxybenzylidene)-2,4-dioxothiazolidin-3-yl)acetate (31)**



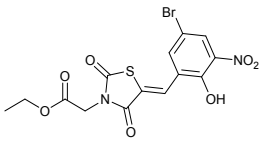
Compound **31** was obtained by following the general procedure as a pale yellow solid (124 mg, 60.5% overall isolated yield after HPLC purification); RP-HPLC $t_R$ = 31.1 min, gradient condition: from 5% B ending to 100 % B over 50 min, flow rate of 4 mL/min, λ = 240 nm. $^1$H NMR (400 MHz, $CDCl_3$): $\delta_H$ 8.17 (s, 1H), 7.27 (s, 1H), 7.14 (s, 1H), 4.50 (s, 2H), 4.27 (q, *J* = 8.0 Hz, 2H), 3.98 (s, 3H), 1.32 (t, *J* = 8.0 Hz, 3H). $^{13}$C NMR (100 MHz, $CD_3OD$): $\delta_C$ 167.1, 166.9, 165.3, 150.6, 146.5, 132.5, 124.9, 121.0, 116.3, 116.2, 114.6, 61.7, 55.5, 41.7, 12.9. ESI-MS: calcd for $C_{15}H_{14}BrNO_6S$ 416.24, found m/z = 415.2 $[M-H]^-$.

**Ethyl-(*Z*)-2-(5-(5-hydroxy-2,4-dimethoxybenzylidene)-2,4-dioxothiazolidin-3-yl)acetate (32)**



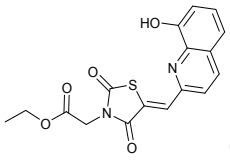
Compound **32** was obtained by following the general procedure as pale yellow solid (138.6 mg, 77% overall isolated yield after HPLC purification); RP-HPLC $t_R$ = 29.1 min, gradient condition: from 5% B ending to 100 % B over 50 min, flow rate of 4 mL/min, $\lambda$ = 240 nm. $^1$H NMR (400 MHz, $CDCl_3$): $\delta_H$ 8.24 (s, 1H), 7.24 (s, 1H), 6.09 (s, 1H), 4.48 (s, 2H), 4.26 (q, $J$ = 7.1 Hz, 2H), 3.88 (s, 3H), 3.84 (s, 3H), 1.31 (t, $J$ = 7.1 Hz, 3H). $^{13}$C NMR (100 MHz, $CD_3OD$): $\delta_C$ 171.8, 169.2, 169.1, 166.3, 161.7, 160.6, 158.6, 129.1, 105.0, 93.0, 89.8, 61.6, 54.5, 54.4, 41.3, 13.0. ESI-MS: calcd for $C_{16}H_{17}NO_7S$ 367.37, found m/z = 366.3 $[M–H]^-$.

**Ethyl-(*Z*)-2-(5-(5-bromo-2-hydroxy-3-nitrobenzylidene)-2,4-dioxothiazolidin-3-yl)acetate (33)**



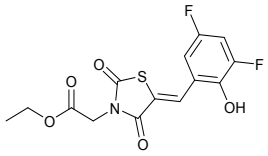
Compound **33** was obtained by following the general procedure as a pale grey solid (218.6 mg, 67% overall isolated yield after HPLC purification); RP-HPLC $t_R$ = 38.5 min, gradient condition: from 5% B ending to 100 % B over 50 min, flow rate of 4 mL/min, $\lambda$ = 240 nm. $^1$H NMR (400 MHz, $CDCl_3$): $\delta_H$ 8.35 (s, 1H), 7.99 (s, 1H), 7.85 (s, 1H), 4.52 (s, 2H), 4.28 (q, $J$ = 7.1 Hz, 2H), 1.33 (t, $J$ = 7.1 Hz, 3H). $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_C$ 165.1, 157.1, 155.4, 139.5, 138.6, 137.4, 128.8, 126.5, 125.3, 125.1, 111.8, 62.3, 42.3, 14.0. ESI-MS: calcd for $C_{14}H_{11}BrN_2O_7S$ 431.21, found m/z = 454.2 $[M + Na]^+$.

**Ethyl (*Z*)-2-(5-((8-hydroxyquinolin-2-yl)methylene)-2,4-dioxothiazolidin-3-yl)acetate (34)**



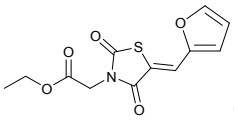
Compound **34** was obtained by following the general procedure as a light grey solid (135.2 mg, 77% overall isolated yield after HPLC purification); RP-HPLC $t_R$ = 30.5 min, gradient condition: from 5% B ending to 100 % B over 50 min, flow rate of 4 mL/min, λ = 240 nm. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta_H$ 8.42 (d, *J* = 8.4, 1H), 8.07 (s, 1H), 7.95 (d, *J* = 8.4, 1H), 7.51 (t, *J* = 7.8 Hz, 1H), 7.43 (dd, *J* = 7.8, 1.3 Hz, 1H), 7.23 (dd, *J* = 7.8, 1.3 Hz, 1H), 4.51 (s, 2H), 4.18 (q, *J* = 7.1 Hz, 2H), 1.22 (t, *J* = 7.1 Hz, 3 H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta_C$ 174.1, 167.3, 165.8, 154.3, 149.9, 138.9, 137.6, 129.8, 129.2, 129.1, 126.7, 124.9, 118.3, 113.2, 62.1, 42.0, 14.4. ESI-MS: calcd for $C_{17}H_{14}N_2O_5S$ 358.37, found m/z = 359.4 $[M + H]^+$, found m/z = 381.4 $[M + Na]^+$.

**Ethyl (*Z*)-2-(5-(3,5-difluoro-2-hydroxybenzylidene)-2,4-dioxothiazolidin-3-yl)acetate (35)**



Compound **35** was obtained by following the general procedure as a white solid (63.3 mg 37.5% overall isolated yield after HPLC purification); RP-HPLC $t_R$ = 34.75 min, gradient condition: from 5% B to ending to 100 % B over 40 min, flow rate of 4 mL/min, λ = 240 nm. $^1$H NMR (400 MHz, $CDCl_3$): $\delta_H$ 8.25 (s, 1H), 7.02 (s, 1H), 6.98 (s, 1H), 4.51 (s, 2H), 4.28 (q, *J* = 7.1 Hz, 2H), 1.33 (t, *J* = 7.1 Hz, 3H). $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_C$ 165.1, 157.1, 155.4, 139.46, 138.57, 137.35, 128.85, 126.46, 125.27, 125.03, 111.80, 62.33, 42.35, 14.5. ESI-MS: calcd for $C_{14}H_{11}F_2NO_5S$ 343.30, found m/z = 342.3 $[M - H]^-$.

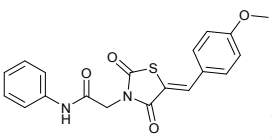
**Ethyl (*Z*)-2-(5-(furan-2-ylmethylene)-2,4-dioxothiazolidin-3-yl)acetate (36)**



Compound **36** was obtained by following the general procedure as a white solid (74 mg, 53.3% overall isolated yield after HPLC purification); RP-HPLC $t_R$ = 28.9 min, gradient condition: from 5% B ending to 100 % B over 50 min, flow rate of 4 mL/min, λ = 240 nm. $^1$H NMR (400 MHz, $CDCl_3$): $\delta_H$ 7.61 (d, *J* = 3.6 Hz, 1H), 6.74 (d, *J* = 3.6 Hz, 1H), 6.52 (m, 1H), 4.39 (s, 2H), 4.17 (q, *J* = 7.1, 2H), 1.21 (t, *J* = 7.1 Hz, 3H). $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_C$ 165.3, 149.0, 148.7, 145.6, 119.3, 117.7, 117.1, 112.2 (2C), 61.1, 41.1, 13.1. ESI-MS: calcd for $C_{12}H_{11}NO_5S$ 281.28, found m/z = 304.3 $[M + Na]^+$.

**Synthesis of (*Z*)-N-benzyl-2-(5-(3-methoxybenzylidene)-2,4-dioxothiazolidin-3-yl)acetamide (37)**

1.0 equiv. of **44m** was dissolved in DCM (4 mL), and fine disperse aniline (2.0 equiv.), hydroxybenzotriazole (1.0 equiv.), *N,N*′-diisopropylcarbodiimide (1.5 equiv.) were added (**Scheme 1B**). The mixture was stirred overnight at room temperature and the reaction was monitored by TLC, analytical RP-HPLC and ESI-MS. After completation the reaction mixture was diluted with 10 mL of water, extracted with EtOAc (3 x 20 mL) and washed with a solution of $NaHCO_3$ (1M) and brine. The combined organic layer was dried over $Na_2SO_4$ and evaporated under vacuum. A portion of the crude product was purified by semi-preparative reversed-phase HPLC (Synergi Fusion C18 reversed-phase column: 250 x 10.00mm, 4μM, 80 Å, flow rate = 4 mL/min; using the gradient conditions reported below), affording pure product **37** as a light grey solid (108.7 mg, 83.3% overall isolated yield after HPLC purification).

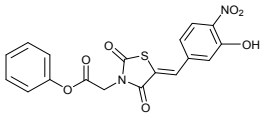


RP-HPLC $t_R$ = 42.3 min, gradient condition: from 5% B ending to 100 % B over 50 min, flow rate of 4 mL/min, λ = 240 nm. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta_H$ 7.77 (s, 1H), 7.51 (m, 2H), 7.29-7.34 (m, 5H), 7.00 (d, *J* = 8.4 Hz, 2H), 5.14 (s, 2H), 3.88 (s, 3H). $^{13}$C NMR (100 MHz,

DMSO-$d_6$): $\delta_C$ 166.3, 165.2, 160.9, 160.4, 145.6, 136.8, 136.5, 132.3, 129.8, 128.8, 128.2, 127.9, 127.2, 126.4, 124.1, 118.4, 115.3, 55.9, 45.8. ESI-MS: calcd for $C_{19}H_{16}N_2O_4S$ 368.41, found m/z = 367.4 $[M - H]^-$.

**Synthesis of Phenyl (*Z*)-2-(5-(3-hydroxy-4-nitrobenzylidene)-2,4-dioxothiazolidin-3-yl)acetate (38)**

1.0 equiv. of **44d** was dissolved in DCM (4 mL), and fine disperse 4-dimethylaminopyridine (1.0 equiv.), phenol (1.0 equiv.) and *N,N*′-diisopropylcarbodiimide (1.0 equiv.) were added (**Scheme 1B**). The mixture was stirred overnight at room temperature and the reaction was monitored by TLC, analytical RP-HPLC and ESI-MS. After completation the reaction mixture was diluted with 10 mL of water, extracted with EtOAc (3 x 20 mL) and washed with a solution of $NaHCO_3$ (1M) and brine. The combined organic layer was dried over $Na_2SO_4$ and evaporated under vacuum. A portion of the crude products was purified by semi-preparative reversed-phase HPLC (Synergi Fusion C18 reversed-phase column: 250 x 10.00mm, 4μM, 80 Å, flow rate = 4 mL/min, using the gradient conditions reported below), affording pure product **38** as a yellow solid (123.2 mg, 99.7% overall isolated yield after HPLC purification);



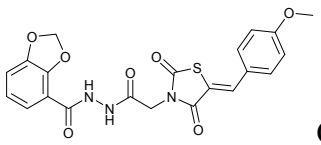

RP-HPLC $t_R$ = 34.44 min, gradient condition: from 5% B ending to 100 % B over 50 min, flow rate of 4 mL/min, λ = 240 nm. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta_H$ 8.10 (d, *J* = 8.90 Hz, 1H), 7.71 (s, 1H), 7.38 (d, *J* = 8.9 Hz, 1H), 7.34 (m, 2H), 7.11 (m, 3H), 6.93 (s, 1H), 4.33 (s, 2H). DEPT 135 NMR (100 MHz, $CD_3OD$): $\delta_C$ 165.6, 157.8, 149.1, 134.5, 130.7, 128.6 (2C), 127.7, 125.6, 123.7, 123.5, 122.4 (2C), 119.3, 114.4, 113.4, 113.3, 58.3. ESI-MS: calcd for $C_{18}H_{12}N_2O_7S$ 400.36, found m/z = 399.3 $[M-H]^-$.

**General procedure (B) for the synthesis of compounds 39-41**

1.0 equiv. of **44m** was dissolved in DCM (4 mL) and hydrazine hydrate (2.0 equiv.), hydroxybenzotriazole (1.0 equiv.) and *N,N*-diisopropylcarbodiimide (1.5 equiv.) were added. The mixture was stirred overnight at room temperature and monitored by TLC, analytical RP-HPLC and ESI-MS. Then the reaction mixture was diluted with 10 mL of water and extracted with EtOAc (3 x 20 mL) .This procedure afforded compound **45m** which was used without any further purification for the synthesis of **39-41**.

1.0 equiv. of **45m** was dissolved in DCM (4 mL) and 4-dimethylaminopyridine (1 equiv.), 1,3-benzodioxole-4-carboxylic acid (2.0 equiv.), or 2,5-dihydroxybenzoic acid (2.0 equiv.), or 3-nitrobenzoic acid (2.0 equiv.) and *N,N*- diisopropylcarbodiimide (1.0 equiv.) were added (**Scheme 1B**). The mixture was stirred overnight at room temperature and the reaction was monitored by TLC, analytical RP-HPLC and ESI-MS. Then the reaction mixture was diluted with 10 mL of water, extracted with EtOAc (3 x 20 mL) and washed with a solution of $NaHCO_3$ (1M) and brine. The combined organic layer was dried over $Na_2SO_4$ and evaporated under vacuum. A portion of the crude products was purified by semi-preparative reversed-phase HPLC (Synergi Fusion C18 reversed-phase column: 250 x 10.00mm, 4μM, 80 Å, flow rate = 4 mL/min) using the gradient conditions reported below and the final products were characterized by ESI-MS and NMR spectra. (High purity> 97% detected by HPLC analysis).

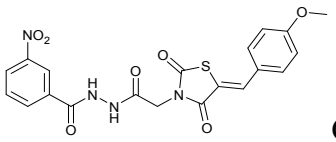
**(*Z*)-N'-(2-(5-(3-methoxybenzylidene)-2,4-dioxothiazolidin-3-yl)etyl)benzo[d][1,3]dioxole-4-carbohydrazide (39)**



Compound **39** was obtained by following the general procedure (B) as a white solid (110 mg, 74.2% overall isolated yield after HPLC purification); RP-HPLC $t_R$ = 32.1 min, gradient condition: from 5% B ending to 100 % B over 50 min, flow rate of 4 mL/min, λ = 240 nm. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta_H$ 8.00 (s, 1H), 7.97 (s, 1H), 7.72 (d, *J* = 8.1 Hz, 2H), 7.60 (s, 1H),

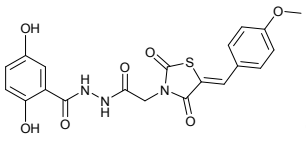
7.55 (m, 2H), 7.42 (m, 1H), 7.01 (d, $J$ = 8.1 Hz, 2H), 6.12 (s, 2H), 4.57 (s, 2H), 3.81 (s, 3H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta_C$ 167.1, 158.7, 158.4, 151.6, 147.9, 129.2, 127.8, 125.6, 125.5, 125.4, 125.2, 125.1, 119.8, 119.6, 117.7, 110.1, 109.2, 108.6, 102.4, 63.4, 41.4. ESI-MS, calcd for $C_{21}H_{17}N_3O_7S$ 455.44; found m/z = 455.3 [M].

**(*Z*)-N'-(2-(5-(3-methoxybenzylidene)-2,4-dioxothiazolidin-3-yl)acetyl)-3-nitrobenzohydrazide (40)**



Compound **40** was obtained by following the general procedure (B) as a yellow solid (69.4 mg, 46% overall isolated yield after HPLC purification); RP-HPLC $t_R$ = 29.9 min, gradient condition: from 5% B ending to 100 % B over 50 min, flow rate of 4 mL/min, λ = 240 nm. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta_H$ 7.94 (s, 1H), 7.91 (s, 1H), 7.86 (m, 2H), 7.63 (d, $J$ = 8.8 Hz, 2H), 7.46 (s, 1H), 7.44 (m, 1H), 7.13 (d, $J$ = 8.8 Hz, 2H), 4.44 (s, 2H), 3.84 (s, 3H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta_C$ 167.6, 166.9, 165.8, 164.7, 161.8, 159.2, 134.6, 134.1, 132.8 (2C), 128.9, 125.8, 119.6, 118.2, 117.7, 115.5, 115.1 (2C), 56.0, 42.5. ESI-MS: calcd for $C_{20}H_{16}N_4O_7S$ 456.43, found m/z = 474.4 $[M + H_2O]^+$.

**(*Z*)-2,5-dihydroxy-N'-(2-(5-(3-methoxybenzylidene)-2,4-dioxothiazolidin-3-yl)acetyl) benzohydrazide (41)**



Compound **41** was obtained by following the general procedure (B) as a pale grey solid (108 mg, 74.8% overall isolated yield after HPLC purification); RP-HPLC $t_R$ = 31.2 min, gradient condition: from 5% B ending to 100 % B over 50 min, flow rate of 4 mL/min, λ = 240 nm. $^1$H (400 MHz, DMSO-$d_6$): $\delta_H$ 7.94 (s, 1H), 7.92 (s, 1H), 7.72 (s, 1H), 7.46 (d, $J$ = 8.7 Hz, 2H), 7.09 (s, 1H), 7.11 (d, $J$ = 8.7 Hz, 2H), 6.89 (m, 2H), 4.45 (s, 2H), 3.98 (s, 3H). $^{13}$C NMR (100 MHz,

DMSO-$d_6$): $\delta_C$ 167.5, 158.2, 158.1, 153.7, 150.1, 142.3, 129.8, 124.8, 124.3, 123.2, 119.1, 118.9, 118.6, 116.1, 115.3, 114.6, 114.1, 113.1, 53.1, 30.0. ESI-MS: calcd for $C_{20}H_{17}N_3O_7S$ 443.43, found m/z = 442.2 $[M-H]^-$.

**Surface Plasmon Resonance Analysis**

Recombinant human r-BAG3 protein was provided by Biouniversa S.r.l. Italy, r-BAG3 domain (rBAG3BD) was purchased from ARETA International S.r.l., BAG1 and BAG4 human recombinant proteins were purchased from Abnova. Human recombinant C-terminal His-tag Heat schock protein (Hsp70) was purchased from BPS Bioscience. The polyclonal (TOS-2) antibody against human BAG3 protein was provided by Biouniversa srl, Italy. The 2-[[3-Ethyl-5-(3-methyl-2(3H)-benzothiazolylidene)-4-oxo-2-thiazolidinylidene]methyl]-1-methyl-pyridinium chloride YM-1, a modulator of Hsp70 protein, was purchased by Sigma Aldrich. SPR analyses were performed using a Biacore 3000 optical biosensor equipped with research-grade CM5 sensor chips (GE Healthcare). Using this platform, two separate recombinant rBAG proteins surfaces, a BSA surface and one unmodified reference surface were prepared for simultaneous analyses. BAG3 and BAG3 domain 100 μg $mL^{-1}$ in 10 mM $CH_3COONa$, pH 4.5, BAG1 100 μg $mL^{-1}$ in 10 mM $CH_3COONa$, pH 7.2 and BAG4 100 μg $mL^{-1}$ in 10 mM $CH_3COONa$, pH 4.5, were immobilized on individual sensor chip surfaces at a flow rate of 5 μL $min^{-1}$ using standard amine-coupling protocols to obtain densities of 8–12 kRU. Commercially available compounds **1-26**, as well as synthetic compounds **27-41**, were dissolved in 100% DMSO to obtain 4 mM solutions, and diluted 1:200 (v/v) in PBS (10 mM $NaH_2PO_4$, 150 mM NaCl, pH 7.4) to a final DMSO concentration of 0.5%. Compounds concentration series were prepared as two-fold dilutions into running buffer: for each sample, the complete binding study was performed using a six-point concentration series, typically spanning 0.025–20 μM, and triplicate aliquots of each compound concentration were dispensed into disposable vials. Binding experiments were performed at 25°C, using a flow rate of 50 μL $min^{-1}$, with 60 s monitoring of association and 300 s monitoring of dissociation. Simple interactions were suitably fitted to a single-site bimolecular interaction model (A+B = AB), yielding a single $K_D$ (Table S2 and Table 2). Sensorgram elaborations were performed using the BIA evaluation software provided by GE Healthcare.

**Table S2.** Thermodynamic constants measured by SPR for the interaction between tested compounds and immobilized rBAG proteins (BAG3, BAG4 and BAG1), rBAG3BD and rHsp70.

| Compound | BAG3 (full- length) $K_D$ (nM) ±SD | BAG4 (full- length) $K_D$ (nM) ±SD | BAG1 (full- length) $K_D$ (nM) ±SD | BAG3BD $K_D$ (nM) ±SD | Hsp70 (full- length) KD (nM) ±SD |
|---|---|---|---|---|---|
| **1** | 14.7±9.4 | No Binding | 589.0±23.2 | No Binding | No Binding |
| **2** | 5.2±0.9 | 3240±90 | - | 3.51±0.7 | No Binding |
| **3** | 6.0±0.4 | No Binding | No Binding | 287.0±13.2 | No Binding |
| **4** | No Binding | - | - | - | - |
| **5** | NoBinding | - | - | - | - |
| **6** | No Binding | - | - | - | - |
| **7** | No Binding | - | - | - | - |
| **8** | No Binding | - | - | - | - |
| **9** | No Binding | - | - | - | - |
| **10** | No Binding | - | - | - | - |
| **11** | No Binding | - | - | - | - |
| **12** | No Binding | - | - | - | - |
| **13** | No Binding | - | - | - | - |
| **14** | No Binding | - | - | - | - |
| **15** | No Binding | - | - | - | - |
| **16** | No Binding | - | - | - | - |
| **17** | No Binding | - | - | - | - |
| **18** | No Binding | - | - | - | - |
| **19** | No Binding | - | - | - | - |
| **20** | No Binding | - | - | - | - |
| **21** | No Binding | - | - | - | - |
| **22** | No Binding | - | - | - | - |
| **23** | No Binding | - | - | - | - |
| **24** | No Binding | - | - | - | - |
| **25** | 38.0 ±4.6 | No Binding | No Binding | No Binding | - |
| **26** | 19.8 ±1.49 | No Binding | No Binding | No Binding | - |
| **YM-1** | No Binding | No Binding | No Binding | No Binding | 4900±800 |
| **Anti-BAG3** | 4.8±0.5 | No Binding | No Binding | 9.8±1.5 | - |

## Limited Proteolysis

Limited proteolysis experiments[8-11] were performed on recombinant BAG3 at 37°C, PBS 0.1% DMSO, using trypsin or chymotrypsin as proteolytic agents; 30 μL of a 3 μM BAG3 solution were used for each experiment. BAG3 complexes were formed by incubating the protein with a 5:1 molar excess of ligand (2) at 37°C for 15 min. Protein and the complex were digested using a 1:100 (w/w) enzyme to substrate ratio. The extent of the reactions was monitored on a time-course basis by sampling the incubation mixture after 5, 15, and 30 min of digestion. Samples were analyzed by MALDI-TOF/MS using a MALDI micro MX (Waters). Mass data were elaborated using the Masslynx software (Waters). Preferential hydrolysis sites on BAG3 under different conditions were identified on the basis of the fragments released during enzymatic digestions.

## Biological assays

### Cell lines

A375 (human melanoma), MCF7 (human breast adenocarcinoma), PC3 (human prostate adenocarcinoma), A549 (human lung carcinoma) and Panc-1 (human pancreas adenocarcinoma) cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2mM L−glutamine, penicillin (100 U/mL) and streptomycin (100 mg/mL) purchased from Invitrogen (Carslbad, CA, USA), in 5% $CO_2$ humid atmosphere.To ensure logarithmic growth, cells were subcultured every 2 days.

As control cells, human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy donors (kindly provided by the Blood Center of the Hospital of Battipaglia, Salerno, Italy) by using standard Ficoll−Hypaque gradients.

**Cellular viability assay**

Cell growth analysis was performed with MTT conversion assay using [3-4,5-dimethyldiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich). Briefly, A375 (3000/well) cells were seeded in triplicate in 96 well/plates and incubated with increasing concentrations of compounds **1**-**3**, **25**, **26**, **28-30**, **32**, **33**, **37**, **38**, **40** and **41** (between 5 µM to 50 µM) or DMSO 0.10% (v/v) for the 72 h in DMEM medium with 10% FBS (Table S3). MCF7, PC3, Panc-1 and A549 (3000 /well) cells or PHA-stimulated PBMC were seeded in triplicate in 96 well/plates and incubated with compound **28** (concentration between 10 µM to 50 µM) or DMSO 0.10% (v/v) for the 72 h in DMEM or RPMI medium with 10% FBS.

Following the treatment, 20 µL of MTT (5mg/mL in PBS) was added and the cells were incubated for additional 3 h at 37°C. The formazan crystals thus formed were dissolved in 100 µl of buffer containing 50% (v/v) *N*,*N*-dimethylformamide, 20% SDS (pH 4.5). The absorbance was measured at 570 nm with a Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific, USA). $IC_{50}$ values were defined as the concentration resulting in 50% inhibition of cell survival compared to control cells treated with DMSO.

**Table S3.** Antiproliferative activity of the selected compounds in A375 human melanoma cancer cell line.

| Compound | $IC_{50}$ ± SD (μM) 72h |
|---|---|
| **1** | 8.3 ± 1.3 |
| **2** | 25.0 ±1.5 |
| **3** | 41.6 ±1.9 |
| **25** | NA* |
| **26** | NA* |
| **28** | 16.0 ±1.5 |
| **29** | NA* |
| **30** | 51.0 ± 1.8 |
| **32** | NA* |
| **33** | >50 |
| **37** | NA* |
| **38** | NA* |
| **40** | 48.0 ± 2.0 |
| **41** | NA* |

*: Not active.

**Co-Immunoprecipitation cell-free assay**

The A375 cells were lysed in HNTG buffer (HEPES 20 mmol/L pH 7.5, NaCl 150 mmol/L, Triton 0.1%, 10% glycerol) supplemented with a protease inhibitor cocktail (Sigma-Aldrich). Equal amount of soluble proteins (1000 μg) were subjected to immunoprecipitation with 10 μg of anti-Bag3 murine monoclonal antibody AC-2 (obtained by BIOUNIVERSA s.r.l., SA, Italy; US Patent, 537, 760) or 10 μg of mouse IgG as control with the addition of **28**, final concentration 70 μM, or equal volume of DMSO. The samples were gently rotated overnight at 4 °C, followed by a 2 hr incubation with 50 μL protein A-Sepharose Beads (Sigma Aldrich). The immunocomplexes were precipitated by centrifugation at 1000 × g, washed 3 times with PBS pH 7.4, and eluted with SDS loading dye. Obtained proteins were then separate on SDS-PAGE gels and transferred to nitrocellulose membrane. The membranes were blocked in 5% BSA in Tris buffer saline Tween-20 buffer for 1 hr and incubated with primary antibodies for anti-mouse HSC70 (Santa Cruz ) or GADPH6 (Santa Cruz sc-136467) or anti-Bag3 rabbit polyclonal (BIOUNIVERSA s.r.l., SA, Italy) overnight at 4 °C. Immunoreactivity was detected by sequential incubation with appropriate horseradish peroxidase-conjugated secondary antibody and chemiluminescence reagent Pierce ECL (Thermo Scientific, Rockford, IL, USA). Signal detection was performed using a ImageQuant LAS 4000 system (GE Healthcare Life Sciences,NY, USA). The shown blots are representative of two different experiments with similar results and the amount of co-immunoprecipitated Hsp70 was normalized to the amount of Bag3 and quantified by densitometry using ImageJ software (NIH, USA) The significance between the two groups of two different experiments was calculated by the Student's t test.

**Cell treatement with compound and Co-Immunoprecipitation assay**

The A375 cells were exposed to the compound **28** (20μM ) for 24, 48 and 72h. After the treatment the cell were collected and lysed in HNTG buffer supplemented with a protease inhibitor cocktail (Sigma-Aldrich). The co-immunoprecipitation and western blot experiments were performed as describe above. Briefly, the amount of 1000 μg of total proteins were co-immunoprecitated with 10 μg of anti-Bag3 murine monoclonal antibody AC-2 or 10 μg of mouse IgG as control and the immunocomplexes were identified by Western blot analysis.

**Cell-cycle analysis and apoptosis evaluation**

The cells were treated with DMSO or **28** 10 or 20 μM, for 72 h using DMEM medium with 10% FBS or 2% FBS. In the experiment with Staurosporine, the cells were exposed to **28** 10 or 20 μM in DMEM with 10% FBS and after 72 was added the Staurosporine 1 μM for 6h. After each treatment, the cells were harvested and incubated with a propidium iodide (PI) solution (0.1% sodium citrate, 0.1% Triton X-100 and 50 mg/ml of PI) for 30 min at 4°C. For each sample, 10 000 events were recorded using a FACS Calibur flow cytometry (Becton Dickinson, San Josè, CA) and cellular debris was excluded from analysis by raising the forward scatter threshold. The proportion of cells in each cell cycle phase was calculated using ModFit LT software (BD). Apoptosis was quantified as the proportion of cells with hypodiploid DNA (sub $G_1$ peak) using CellQuest software (BD).

## References


1 LigPrep., Schrödinger, LLC, *New York, NY*, 2017.

2 A. Arakawa, N. Handa, N. Ohsawa, M. Shida, T. Kigawa, F. Hayashi, M. Shirouzu, S. Yokoyama, *Structure*, 2010, **18***,* 309-319.

3 Glide, Schrödinger, LLC, *New York, NY*, 2017.

4 R. A. Friesner, J. L. Banks, R. B. Murphy, T. A. Halgren, J. J. Klicic, D. T. Mainz, M. P. Repasky, E. H. Knoll, M. Shelley, J. K. Perry, D. E. Shaw, P. Francis, P. S. Shenkin, *J. Med. Chem.*, 2004, **47***,* 1739-1749.

5 R. A. Friesner, R. B. Murphy, M. P. Repasky, L. L. Frye, J. R. Greenwood, T. A. Halgren, P. C. Sanschagrin, D. T. Mainz, *J. Med. Chem.*, 2006, **49***,* 6177-6196.

6 T. A. Halgren, R. B. Murphy, R. A. Friesner, H. S. Beard, L. L. Frye, W. T. Pollard, J. L. Banks, *J. Med. Chem.*, 2004, **47***,* 1750-1759.
7 CombiGlide, Schrödinger, LLC, *New York, NY*, 2017.
8 F. Dal Piaz, A. Vassallo, M. G. Chini, F. M. Cordero, F. Cardona, C. Pisano, G. Bifulco, N. De Tommasi, A. Brandi, *PloS one*, 2012, **7***,* e43316.
9 S. Orru, F. Dal Piaz, A. Casbarra, G. Biasiol, R. De Francesco, C. Steinkuhler, P. Pucci, *Protein Sci.*, 1999, **8***,* 1445-1454.
10 F. Dal Piaz, P. Ferro, A. Vassallo, M. Vasaturo, G. Forte, M. G. Chini, G. Bifulco, A. Tosco, N. De Tommasi, *Biochim. Biophys. Acta*, 2015, **1850***,* 1806-1814.
11 S. Terracciano, M. G. Chini, M. C. Vaccaro, M. Strocchia, A. Foglia, A. Vassallo, C. Saturnino, R. Riccio, G. Bifulco, I. Bruno, *Chem. Commun.*, 2016, **52***,* 13515.

**Author Contributions**

I.B. and G.B.: project administration and development of methodology
S.T. and G.L. devised the project and the main conceptual ideas
S.T. and A.R.: chemical synthesis and acquisition, analysis, and interpretation of data (Surface Plasmon Resonance)
G.L., R.R. and G.B.: in silico studies, acquisition, analysis, and interpretation of data (virtual screening)
M.C.V., M.D.M., B.R., A.R., M.C.T.: acquisition, analysis, and interpretation of data (cell experiments)
A.V.: acquisition, analysis, and interpretation of data (limited proteolysis experiments)
S.T., G.L., A.R., M.C.V., A.V., M.D.M., B.R., A.R., M.C.T., R.R., G.B., and I.B.: writing, review, and revision of the manuscript.
S.T. and G.L. contributed equally to this work.