Mechanism of Action Studies of Lomaiviticin A and the Monomeric Lomaiviticin Aglycon. Selective and Potent Activity Toward DNA Double-strand Break Repair-deficient Cell Lines.

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Supporting Information

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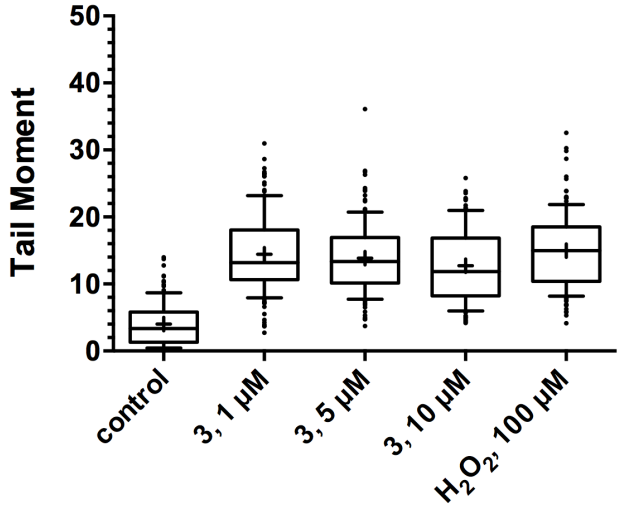


Figure S1. Tail moment in a neutral comet unwinding assay that employed (–)-MK7-206 (**3**, 1, 5, or 10 μM) and K562 cells. Drug exposure was 15 min. Cross denotes mean tail moment (117–140 cells), box denotes 2nd and 3rd quartile, lines in the middle of the box represent median. Error bars represent 10th and 90th percentile, values individually plotted are outliers. Tail moment represents the extent of DNA cleavage. Tail moment is defined as the product of the tail length and the fraction of DNA in the tail.

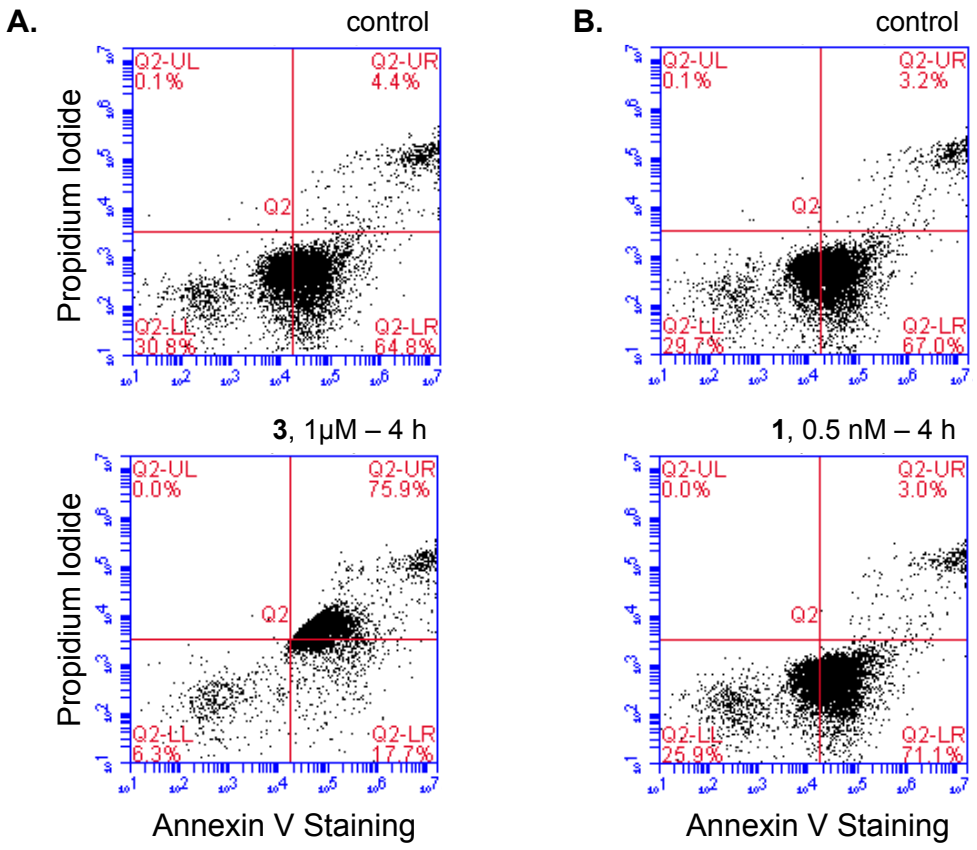
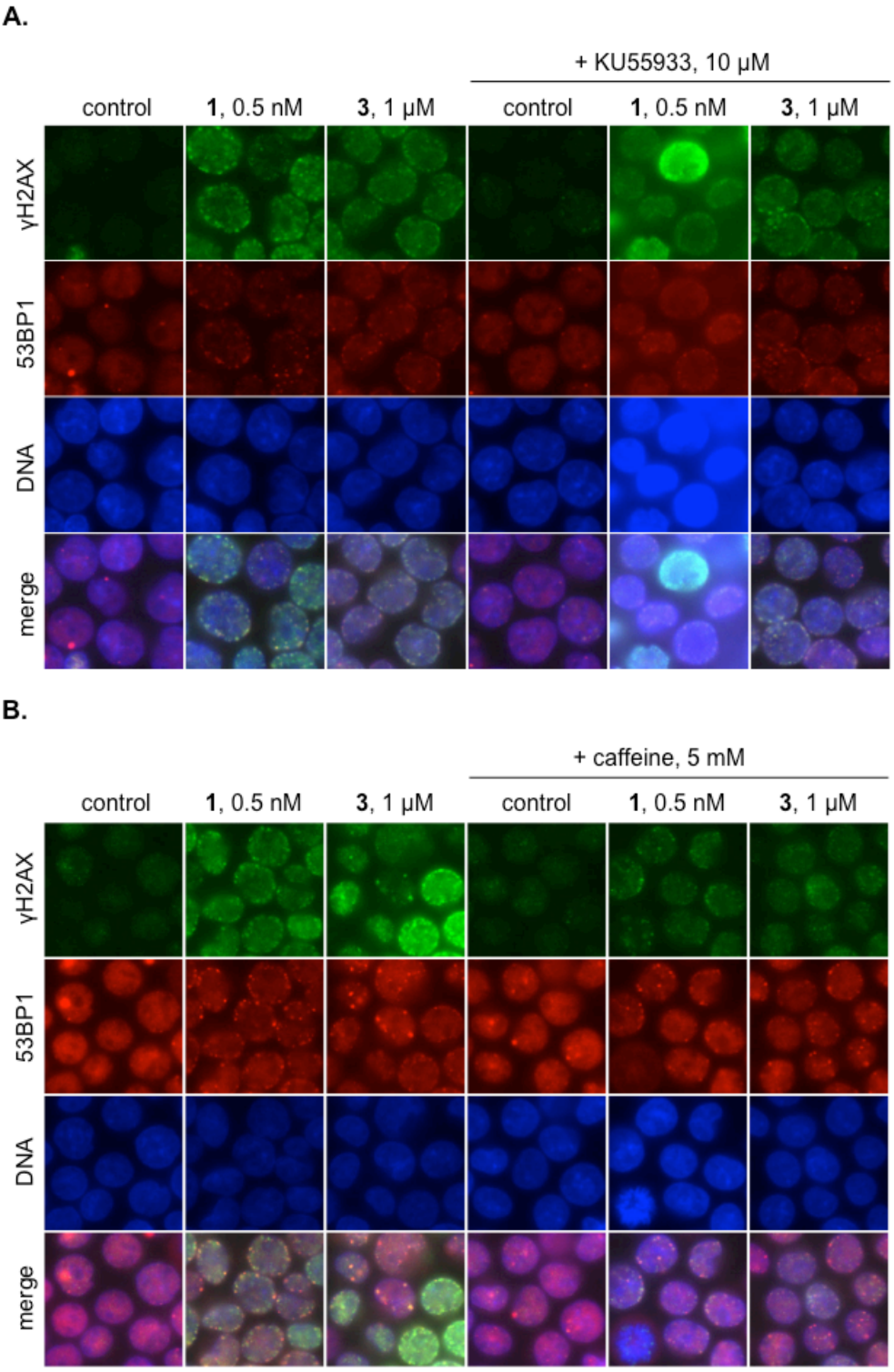


Figure S2. Annexin V/Propidium iodide staining reveals induction of apoptosis by (–)-MK7-206 (**3**) but not (–)-lomaiviticin A (**1**). K562 cells were incubated with **3** (1 μM) or **1** (0.5 nM) for 4 h. Control cells were untreated, early and late-apoptotic cells were assessed by Annexin V and Annexin V-propidium iodide staining, respectively. Dot plot on cells stained with Alexa 488-conjugated Annexin V antibody and propidium iodide. Percent early-apoptotic cells were determined by the fraction of cells bound with Alexa 488-conjugated Annexin V (LR quadrant). Percent late-apoptotic cells were determined by the fraction of cells stained with A488-conjugated Annexin V and propidium iodide (UR quadrant). The data show fast induction of late apoptosis in cells treated with **3** (A). Induction of apoptosis in cells treated with **1** was not detected (B) 4 h after treatment.


A.
+ KU55933, 10 μM
control
1, 0.5 nM
3, 1 μM
control
1, 0.5 nM
3, 1 μM
γH2AX
53BP1
DNA
merge
B.
+ caffeine, 5 mM
control
1, 0.5 nM
3, 1 μM
control
1, 0.5 nM
3, 1 μM
γH2AX
53BP1
DNA
merge

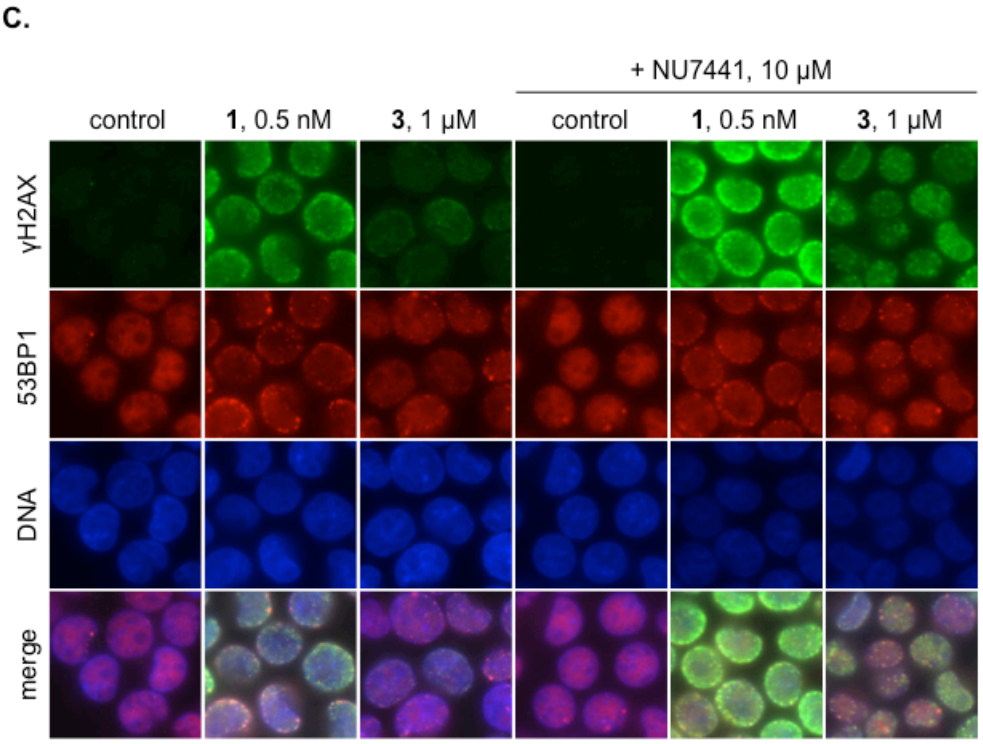


Figure S3. γH2AX foci induced by (–)-MK7-206 (**3**) and (–)-lomaiviticin A (**1**) are ATR dependent after 6 h. Immunofluorescence imaging of γH2AX and 53BP1 foci in K562 cells treated with **3** (1 μM) or **1** (0.5 nM) in the presence or absence of KU55933 (10 μM, A), caffeine (5 mM, B) or NU7441 (10 μM, C). Rows (top to bottom): γH2AX (green), 53BP1 (red), DNA (blue), and merge. Columns (left to right): control, 0.5 nM **1**, 1 μM **3**, (A) 10 μM KU55933, 0.5 nM **1** + 10 μM KU55933, 1 μM **3** + 10 μM KU55933; (B) 5 mM caffeine, 0.5 nM **1** + 5 mM caffeine, 1 μM **3** + 5 mM caffeine; (C) 10 μM NU7441, 0.5 nM **1** + 10 μM NU7441, 1 μM **3** + 10 μM NU7441. Cells were exposed to NU7441 for 1 h prior to addition of drug. Drug exposure was 1 h. K562 cells were in exponential growth phase immediately before treatment. Immunological detection was performed using a primary antibody (rabbit polyclonal anti-53BP1 antibody (Novus Biologicals) and mouse monoclonal antiphosphohistone H2AX (SER139) antibody (Upstate)) and visualized with Alexa 488 (goat-antimouse IgG) and Alexa 594 (goat-antirabbit IgG). Mounting medium contained 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclear DNA.

Table S1. Activity of (–)-lomaiviticin A (**1**) against matched cell lines deficient in DNA dsb repair factors.

	cell line	origin	deficiency	% survival at 4 pM **1** proficient	% survival at 4 pM **1** deficient	LC_{50} (pM) proficient	LC_{50} (pM) deficient	selectivity[a]
sensitive	Peo1	human ovarian adenocarcinoma	BRCA2	65	29	6 ±1	2.5 ±0.5	2.2
	DLD1	human colorectal adenocarcinoma	BRCA2	41	21	6 ±3	2 ±1	1.9
	VC8	CHO	BRCA2	58	5	3 ±2	1.5 ±0.5	11.6
	EUFA423	human fibroblasts	BRCA2	100	77	20 ±5	12 ±3	1.3
	U251	human glioblastoma	PTEN	77	0	13 ±2	2 ±0.6	n/d[b]
	XRS6	CHO	KU80	82	46	20 ±5	4.5 ±1.5	1.8
	50D	SCID mouse fibroblasts	DNA-PK	100	84	17.5 ±7.5	10 ±5	1.2
	5849	human fibroblasts	ATM	32	9	3 ±0	1 ±0.3	3.5
insensitive	HCT116	human colorectal carcinoma	MLH1	20	29	3 ±1	3 ±1	
	HCC1937	human breast ductal carcinoma	BRCA1	64	77	65 ±5	80 ±0	
	UWB1.289	human ovarian carcinoma	BRCA1	48	66	6 ±2	7 ±2	
	PD20	Human lymphoblastoid	FANCD2	59	59	9 ±2	7 ±0.5	
	XP20S	human fibroblasts	XPA	65	65	6 ±0.3	5 ±0.7	

[a]Defined as % survival (4 pM **1**) proficient cell line/% survival (4 pM **1**) deficient cell line. [b]Not defined.

Table S2. Activity of (–)-MK7-206 (**3**) against matched cell lines deficient in DNA dsb repair factors.

	cell line	origin	deficiency	% survival at 20 nM **3** proficient	% survival at 20 nM **3** deficient	LC_{50} (nM) proficient	LC_{50} (nM) deficient	selectivity[a]
sensitive	Peo1	human ovarian adenocarcinoma	BRCA2	79	22	45 ±5	11 ±5	3.6
	DLD1	human colorectal adenocarcinoma	BRCA2	89	25	63 ±19	8 ±3	3.6
	VC8	CHO	BRCA2	84	1	55 ±5	6 ±0.5	84
	EUFA423	human fibroblasts	BRCA2	83	23	40 ±0	16 ±9	3.6
	U251	human glioblastoma	PTEN	82	16	38 ±8	11 ±4	5.1
	XRS6	CHO	KU80	99	64	48 ±3	35 ±0	1.5
	5849	human fibroblasts	ATM	58	46	23 ±3	12 ±4	1.3
	UWB1.289	human ovarian carcinoma	BRCA1	38	25	15 ±0	15 ±0	1.5
	XP20S	human fibroblasts	XPA	47	31	20 ±0	15 ±0	1.5
insensitive	50D	SCID mouse fibroblasts	DNA-PK	75	82	42 ±6	63 ±7	
	HCT116	human colorectal carcinoma	MLH1	86	86	35 ±5	40 ±10	
	HCC1937	human breast ductal carcinoma	BRCA1	67	85	55 ±5	70 ±10	
	PD20	Human lymphoblastoid	FANCD2	37	52	15 ±0	20 ±0	

[a]Defined as % survival (20 nM **3**) proficient cell line/% survival (20 nM **3**) deficient cell line.

Chemical Materials.

(–)-Lomaiviticin A (**1**)[1] and (–)-MK7-206 (**3**)[2] were prepared according to the methods of Herzon and co-workers. All other chemicals were used as received.

Cell culture.

Cells were cultivated in suitable medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 ºC under an atmosphere of 5% CO_2 and constant humidity. Human chronic myelogenous leukemia K562 cells were obtained from Professor David Spiegel (Yale University) and cultivated in RPMI-1640 with L-glutamine. Peo1 (BRCA2-deficient) and Peo1-C4-2 were obtained from Dr. Toshiyasu Taniguchi (Fred Hutchinson Cancer Research Center, Seattle, WA) and were maintained in 10% FBS DMEM. DLD1 and DLD1-BRCA2-deficient were provided by Horizon Discovery (Cambridge, UK) and were maintained in 10% FBS McCoys 5A. VC8 (BRCA2-deficient) and VC8+BRCA2–complemented were provided by Dr. Graeme C.M. Smith (KuDOS Pharmaceuticals Ltd., Cambridge, United Kingdom) and were maintained in 10% FBS DMEM. EUFA423 (BRCA2-deficient) and EUFA423+BRCA2–complemented were obtained from Dr. Simon Powell (Memorial Sloan-Kettering Cancer Center, New York, NY) and were maintained in 10% FBS DMEM supplemented with 20 mmol/L HEPES, and 0.5 mg/mL G418 (complemented only). U251 (PTEN-deficient) and U251+PTEN doxycycline-inducible were provided by Dr. Amit Maity (University of Pennsylvania School of Medicine, Philadelphia, PA) and were maintained in 10% FBS DMEM and 0.4 mg/ml G418, 2 μg/ml blasticidin, 1 μg/ml doxycycline (inducible only). Xrs6 (KU80-deficent) and xrs6-hamKU80 were purchased from the European Collection of Cell Cultures and maintained in 10% FBS F12 with 0.5 mg/ml G418 (complemented only). 50D (DNA-PK –deficient) and 100E plus human DNA-PK were obtained from Dr. Cordula Kirchgessner (Stanford University, Stanford, CA) and maintained in 10% FBS DMEM. 5849 (ATM-deficient) were obtained from the Coriell Institute for Medical Research (Camden, NJ) and 5849+ATM complemented were established in the Glazer laboratory and maintained in 10% FBS DMEM with 0.8 mg/ml G418 (complemented only). HCT116 2/3 and 3/6 were obtained from Dr. Thomas Kunkel (NIEHS, Research Triangle, NC) and maintained in 10% FBS DMEM with 0.8 mg/ml G418. HCC1937 (BRCA1-deficient) and HCC1937+BRCA1-complemented were obtained from Dr. Zhong Jun (Yale University, New Haven, CT) and maintained in 15% FBS IMDM with 0.15 mg/ml G418. UWB1.289 (BRCA1-deficient) and UWB1.289+BRCA1-complemented were provided by Dr. Elizabeth Swisher (University of Washington, Seattle, WA) and maintained in 3% FBS RPMI:MEGM with 0.2 mg/ml G418. PD20 (FANCD2-deficient) and PD20+FANCD2 were obtained from the OHSU FA Cell Repository and maintained in 15% FBS DMEM. XP20S and XP20S+XPA were obtained from Dr. Kenneth Kraemer (National Cancer Institute, Bethesda, MD) and maintained in 10% FBS DMEM.

Clonogenic Survival Assays.

All cell lines, except for the HCC1937 cell lines and the DLD1 BRCA2-deficient, were seeded at 500 cells per well in 6-well dishes. The HCC1937 cells were seeded at 400 cells per well and the DLD1 BRCA2-deficient cells were seeded at 1500 cells per well. Twenty-four hours later the cells were treated with various concentrations of (–)-lomaiviticin A (**1**), 1, 2, 4, 6, 8, 10, 20, 40, 60, 80, 100 pM) or (–)-MK7-206 (**3**), 0.5, 1, 5, 10, 20, 40, 60, 80, 100, 120 nM) in triplicate for 24 hours, after which the media containing inhibitor was removed and replaced with fresh media. Colonies were fixed with 0.9% saline solution and stained with crystal violet 7–14 days later. Colonies consisting of greater than 50 cells were counted.

Comet assay.

For general procedures, see: http://www.trevigen.com/literature.php. For the neutral and alkaline comet assays, K562 cells were seeded at 5×10^5 cells per well in 6-well dishes and treated with (–)-lomaiviticin A (**1**) or (–)-MK7-206 (**3**). Concentrations of **1** and **3** and duration of exposure are specified in each figure. Incubation was conducted at 37 °C. Following treatment, the cells were immediately placed on ice and washed once with cold PBS. The cells were resuspended in cold PBS at a concentration of 3×10^5 cells/mL.

An aliquot of this cell suspension (6.0 μL) was mixed with pre-warmed (37 °C) LM Agarose (60.0 μL) and immediately pipetted onto a pre-warmed comet slide. Comet slides were allowed to gel for about 20 minutes at 4 °C. Slides were then carefully immersed in cold lysing solution and incubated for 1 h at 4 °C.

Alkaline comet assay: Following incubation in lysis solution, excess buffer was removed. The slide was immersed in freshly prepared alkaline unwinding solution (200 mM NaOH, 1 mM EDTA, pH >13) for 1 h at 4 °C. The slides were then placed in a Comet Assay ES Tank (Trevigen) containing prechilled 1X alkaline electrophoresis buffer (200 mM NaOH, 1 mM EDTA, pH >13, 850 mL). Electrophoresis was carried out for 20 min at 21V at 4 °C. Following electrophoresis, excess buffer was drained and the slides were immersed in distilled water twice for 5 minutes each. DNA was precipitated by immersing the slides in 70% ethanol for 5 minutes at room temperature.

Neutral comet assay: Following incubation in lysis solution, excess buffer was removed. The slide was then incubated in cold 1X neutral electrophoresis buffer (100 mM Tris, 300 mM sodium acetate, pH 9.0) for 30 min at 4 °C. The slides were then placed in a Comet Assay ES Tank (Trevigen) containing prechilled 1X neutral electrophoresis buffer (850 mL). Electrophoresis was carried out for 30 min at 21V at 4 °C. Following electrophoresis, excess buffer was drained from the slides, the slides were immersed in DNA precipitation solution (1 M ammonium acetate and 86% ethanol, 50 mL), and incubated for 30 min at room temperature. DNA was further precipitated by immersing in 70% ethanol (50 mL) for 30 min at room temperature.

Following DNA precipitation, the slides were dried in an incubator set at 37 °C for 30 min. DNA was stained by adding SYBR Green I solution (1X, 100 μL) in TE buffer (10 mM Tris-HCl, pH 7.5, 1mM EDTA) on each well in slide, followed by incubation for 30 min at room temperature in the dark. Excess staining solution was removed from the slides and briefly rinsed with water. Prior to scoring, slides were allowed to dry completely at 37 °C. Individual cells were scored on a two well slide to give a total of at least 50 cells per individual experiment. Comets were analyzed using a Zeiss Axiovert 200 M epifluorescence microscope at 10X magnification and the CometScore software. For the alkaline comet assay, %DNA in tail was used as a measure of DNA damage. For the neutral comet assay, tail moment, defined as the product of tail length and fraction of DNA in the tail, was used as an index of DNA damage.

Immunofluorescence.

K562 cells were seeded at a density of 5×10^4 cells per well in 12 well plates containing poly-L-lysine coated glass coverslips (BD Biosciences). At 70% confluency, cells were treated with either (–)-lomaiviticin A (**1**, 0.5 nM) or (–)-MK7-206 (**3**, 1.0 μM) at 37 °C. Duration of drug exposure is specified in each figure (5 min to 24 h). Following treatment, the cells were washed with cold PBS, fixed with 4% paraformaldehyde at room temperature (15 min), permeabilized on ice (20 min) in 0.25% Triton X-100, and blocked with 3% bovine serum albumin (BSA) in PBS, for at least 5 min at room temperature.

Coverslips were incubated with primary mouse monoclonal anti-γH2AX (SER139) (Upstate) and rabbit polyclonal anti-53BP1 (Novus Biologicals) antibodies (1:100) for 1 h at 37 °C, washed with PBS, and incubated with secondary Alexa 488 and Alexa 594 conjugated anti-mouse (1:100) and anti-rabbit (1:200) antibodies, respectively, for 1 h at 37 °C. The coverslips were washed with PBS and mounted on glass slides using Vectashield mounting medium containing DAPI (Vector Laboratories) to counterstain DNA. Foci were visualized with a Zeiss Axiovert 200 M epifluorescence microscope equipped with a 63×/1.40 Plan-Apochromat oil immersion objective. Fluorescence illumination was initiated using an EXFO X-cite Series 120 Hg arc lamp. Filters for DAPI, GFP, and Cy3 were used to acquire images. Images were captured with Zeiss Axiocam Mrm camera and AxioVision software.

For immunofluorescence assay involving DNA repair inhibitors, K562 cells on coverslips were pre-treated with KU55933 (10 μM), caffeine (5 mM), or NU7441 (10 μM) for 1 h prior to addition of either **1** (0.5 nM) or **3** (1.0 μM).

Annexin V apoptosis assay. The induction of apoptosis by (–)-lomaiviticin A (**1**) and (–)-MK7-206 (**3**) was quantified by flow cytometric analysis of Alexa Fluor 488 conjugated annexin V/dead cell apoptosis kit (Life Technologies) as per the manufacturer's instructions. K562 cells were seeded on 6 well dishes and treated with either **1** (0.5 nM) or **3** (1.0 μM) for 4 h. After incubation, the cells were collected and washed with cold PBS. Cells were resuspended in 1X annexin-binding buffer at a concentration of 1×10^6 cells/mL. AlexaFluor A488 annexin V (5.0 μL) and propidium iodide (100 μg/mL, 1.0 μL) were added to each cell suspension (100.0 μL) and incubated at room temperature for 15 min. After incubation, 1X annexin-binding buffer (400.0 μL) was added, gently mixed and immediately analyzed by flow cytometry, measuring fluorescence emission at 533/30 nm (e.g., FL1) and 585/40 nm (e.g., FL2).

Bibliography.

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2. Woo, C. M.; Gholap, S. L.; Lu, L.; Kaneko, M.; Li, Z.; Ravikumar, P. C.; Herzon, S. B. *J. Am. Chem. Soc.* **2012**, *134*, 17262.