Supplementary Information

Unraveling the mechanism of cell death induced by chemical fibrils

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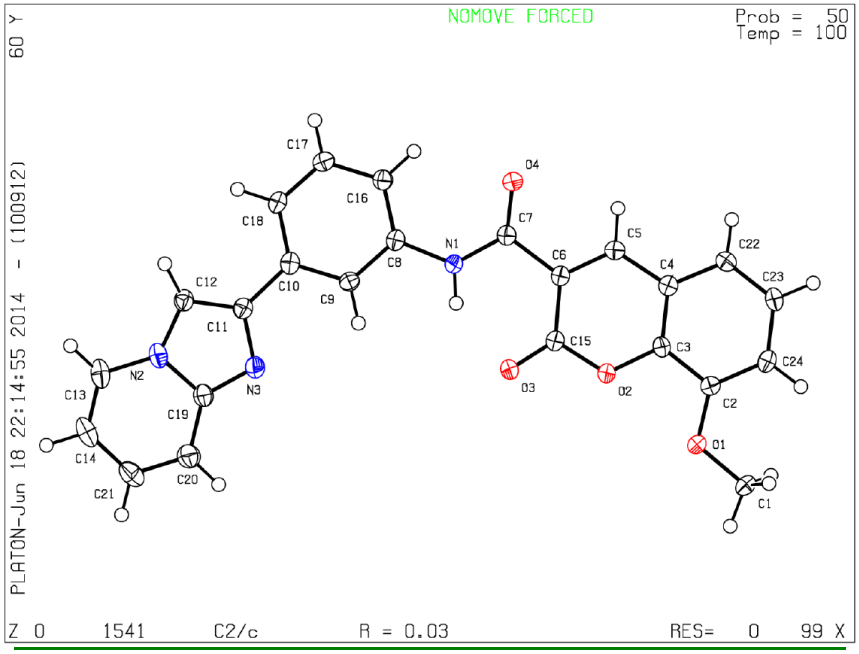
Supplementary Results



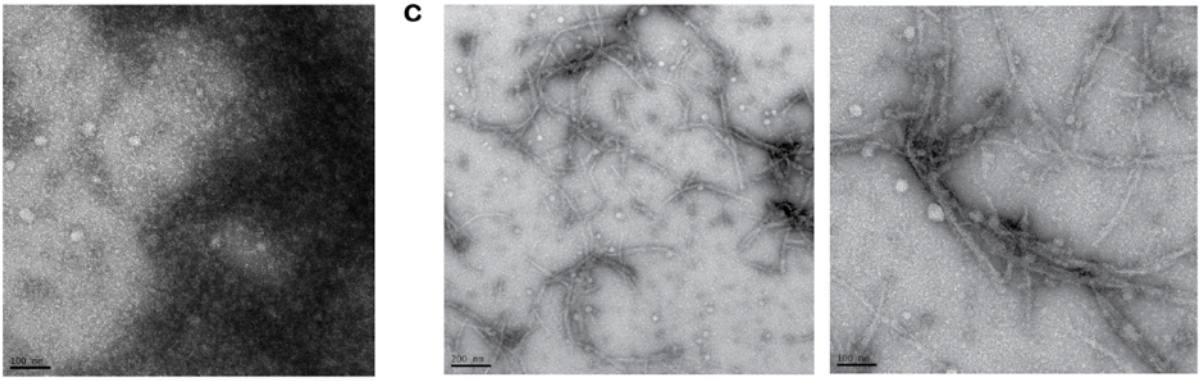
Identification code	**1541**
Empirical formula	$C_{24}\ H_{17}\ N_3\ O_4$
Formula weight	411.41
Temperature (K)	100(2)
Wavelength (Å)	1.54178
Crystal system	Monoclinic
Space group	C2/c
Cell dimensions	
a, b, c (Å)	36.299(4), 6.3737(8), 18.828(2)
α, β, γ (°)	90, 121.045(6), 90
Volume (Å^3)	3732.0(8)
Z	8
Density (calculated)	1.464 g/cm^3
Absorption coefficient (mm^{-1})	0.837
F(000)	1712
Crystal size (mm^3)	0.14 x 0.05 x 0.03
Crystal color, habit	Pale yellow needle
Theta range for data collection (°)	2.84 to 65.21
Index ranges	-42<=h<=42, -7<=k<=7, -20<=l<=21
Reflections collected	11440
Independent reflections	3076 [R(int) = 0.0222]
Completeness to theta = 62.50°	98.8 %
Absorption correction	Multi-scan
Max. and min. transmission	0.9753 and 0.8918
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	3076 / 0 / 281
Goodness-of-fit on F^2	1.020
Final R indices [I>2sigma(I)]	R1 = 0.0318, wR2 = 0.0818
R indices (all data)	R1 = 0.0393, wR2 = 0.0867
Largest diff. peak and hole	0.193 and -0.194 e Å^{-3}

Supplementary Figure 1. Crystal data and structure refinement for 1541. Crystal structure data for 1541 can be accessed from the Cambridge Crystallographic Data Centre (CCDC; www.ccdc.cam.ac.uk) and have been allocated accession number CCDC 1009102.

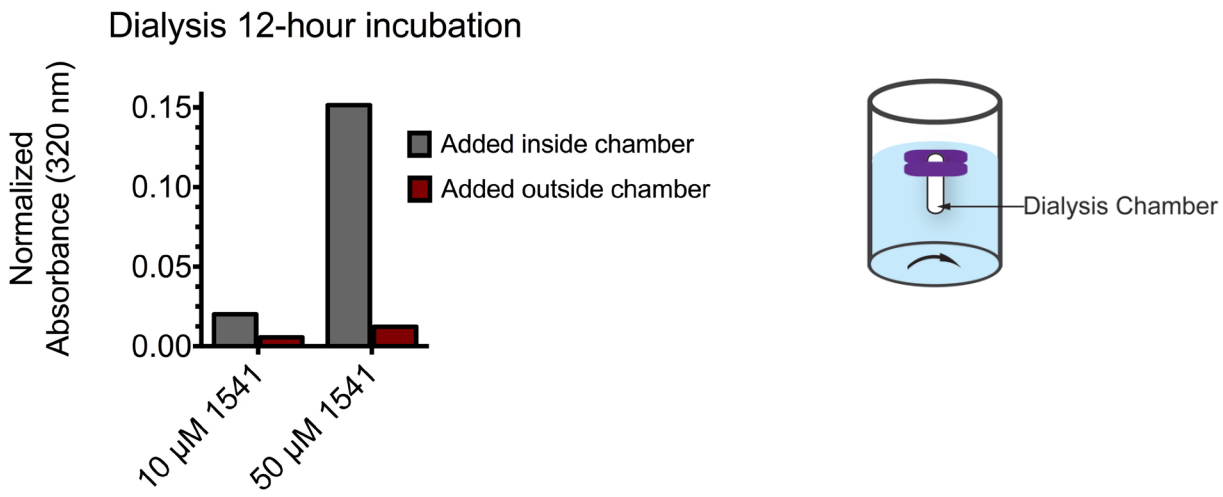
a

Compounds	BUFFER		MEDIA	
	10 uM R ± SD (nm)	50 uM R ± SD (nm)	10 uM R ± SD (nm)	50 uM R ± SD (nm)
DMSO - control	0.5 ± 0.3	0.4 ± 0.1	12.3 ± 0.7	12.3 ± 0.7
1541	1212.4 ± 299.2	1331.4 ± 683.3	18.0 ± 1.8	321.8 ± 98.3

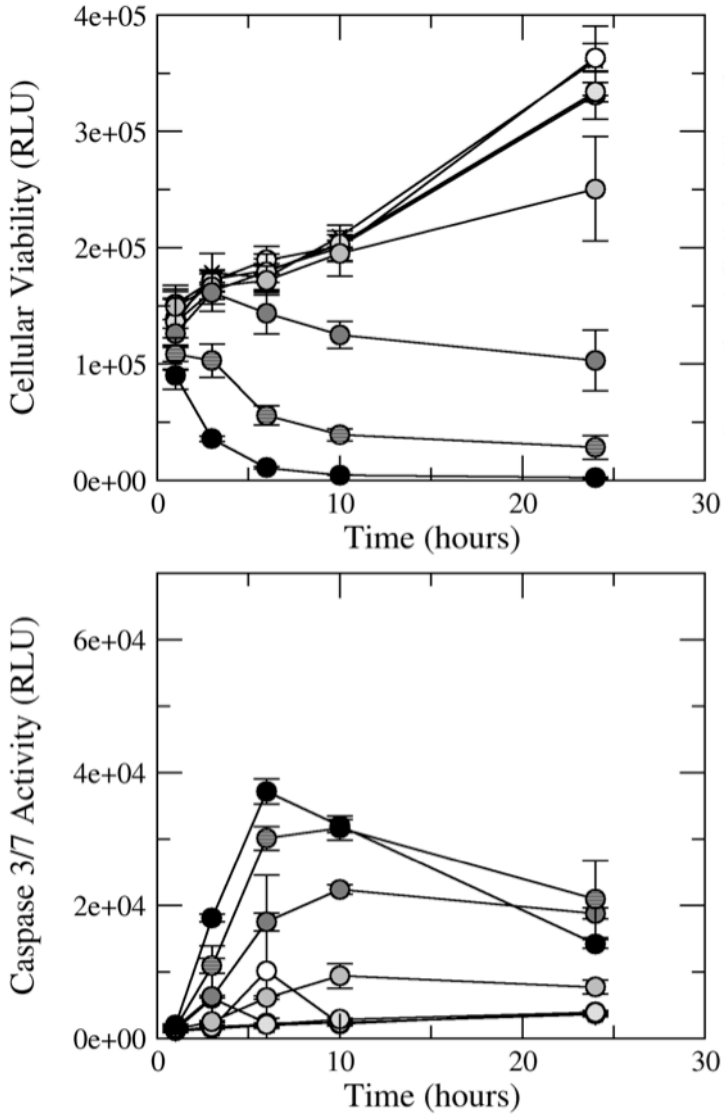
b **c**



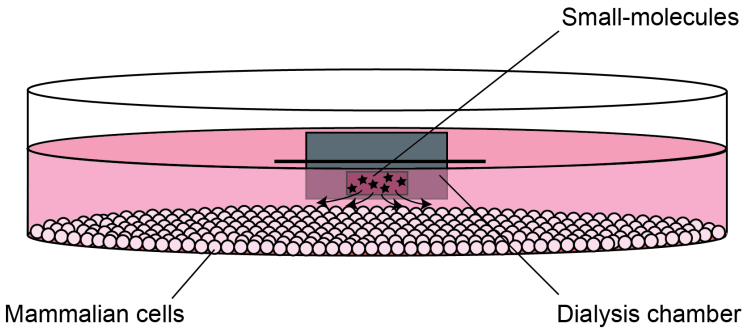
<u>Supplementary Figure 2</u>. Detection of chemi-fibrils in cell culture media. (**a**) Dynamic light scattering (DLS) of 1541 in buffer and cell culture media (RPMI containing 10% FBS, glutamine and penicillin/streptomycin) showing the formation a large particles. (**b**) TEM of cell culture media alone (**c**) TEM of 1541 fibrils in cell culture media at 21,000x (left) and 56,000x (right). The chemi-fibrils were added directly in cell culture media at 50 uM from a 5 mM DMSO stock solution. Fibrils formed at room temperature immediately without requiring an incubation time. A total of ten reads were measured per sample. Each value reported corresponds to the average of three independent experiments.



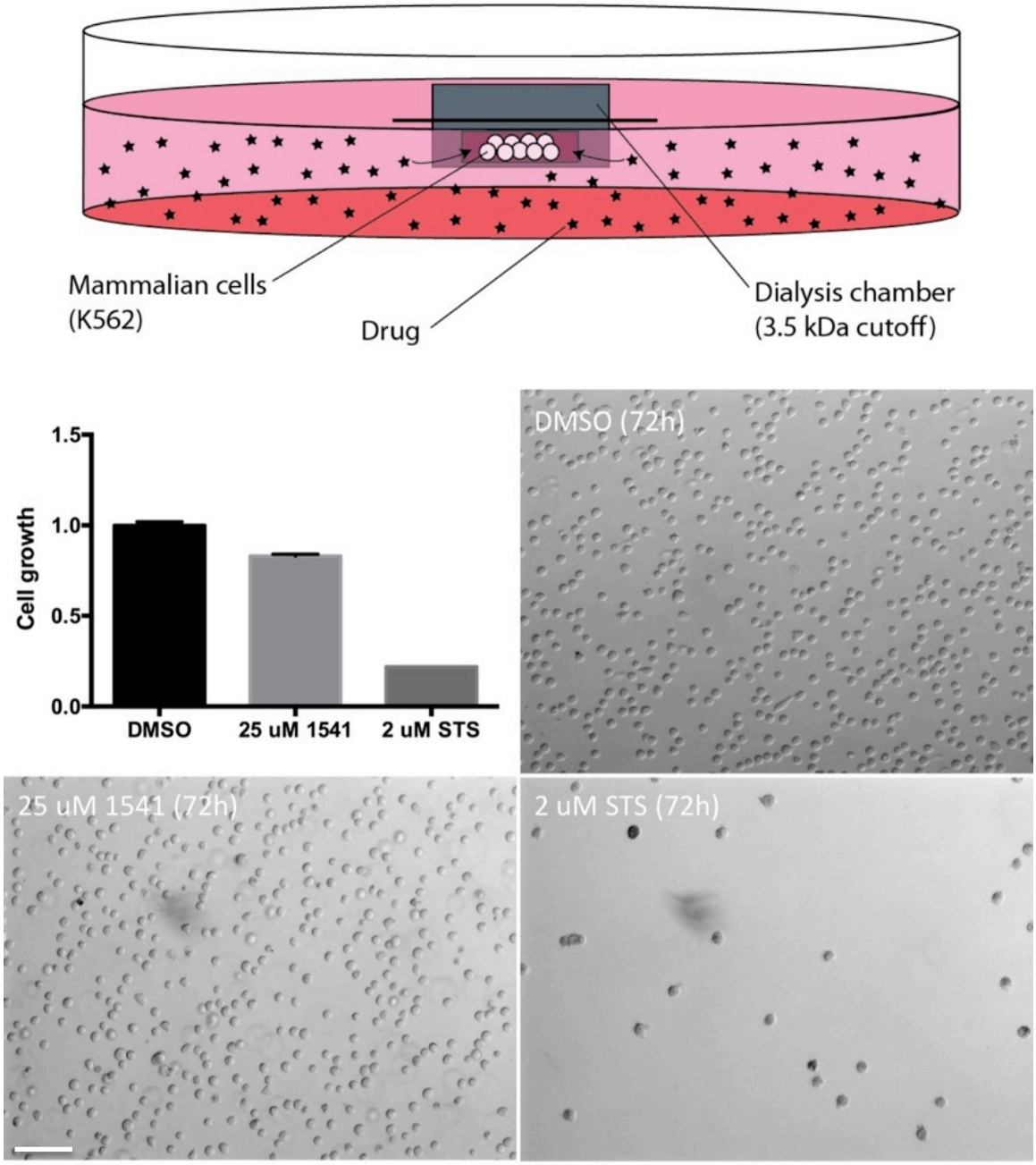
Supplementary Figure 3. The concentration of 1541 does not reach equilibrium across a dialysis membrane within 12 hours. A dialysis chamber was incubated overnight at 37°C in a 1 L solution of 1541 (10 or 50 μM). Initially, the dialysis chambers either contained buffer alone (red) or the indicated concentration of 1541 (grey). After a 12-h incubation, an absorbance measurement of the contents of the dialysis chambers was taken to estimate the concentration of compound that crossed the membrane. An estimate of the maximum absorbance is given by the sample containing 1541 both inside and outside the dialysis bag (grey). To normalize, the absorbance of buffer alone was subtracted from each sample. This experiment was performed with single absorbance measurements.



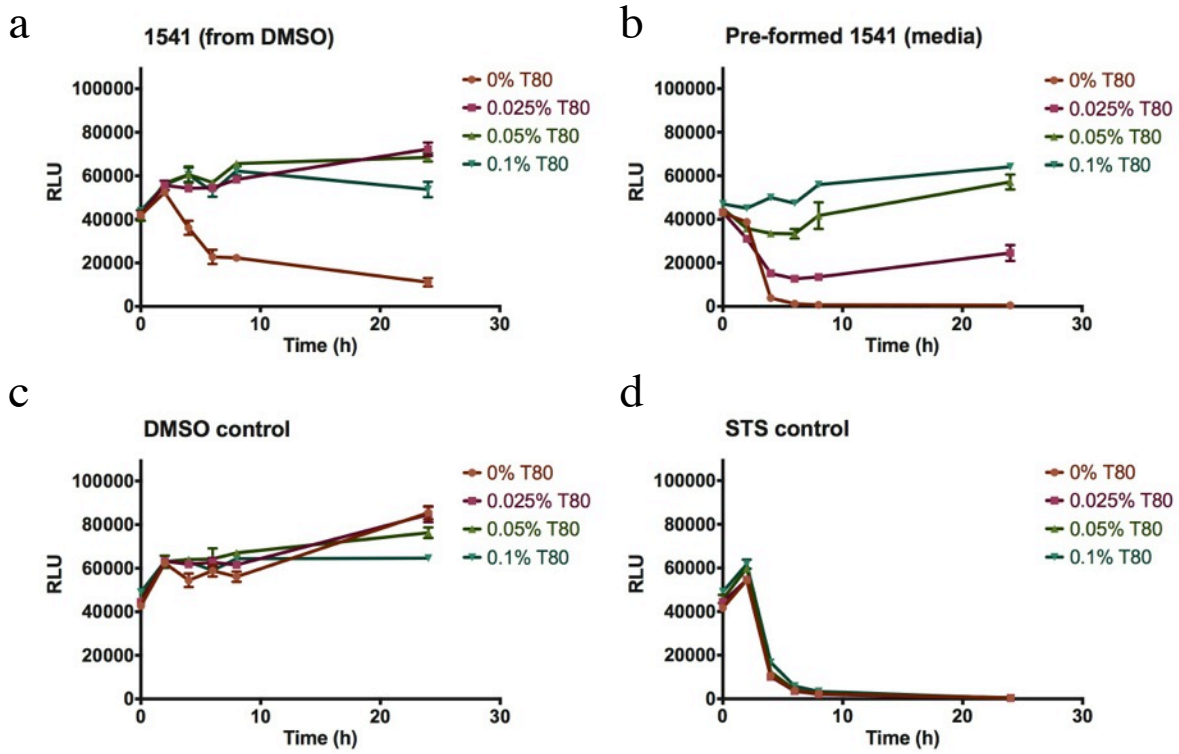
Supplementary Figure 4. Cell viability and caspase-3/7 activation in K562 cells. Cell viability and caspase-3/7 activity monitored in K562 cells at various concentrations of 1541 (from dark to white, 25, 12.5, 6.3, 3.1, 1.6, 0.8, 0.4, 0 μM), consistent with previously reported response in other cell lines by Wolan and coworkers. Cell viability and caspase activity were assessed using CellTiter-Glo and Caspase-3/7-Glo (Promega) in triplicate experiments. The mean value ± s.d. of the raw luminescence unit (RLU) is reported.



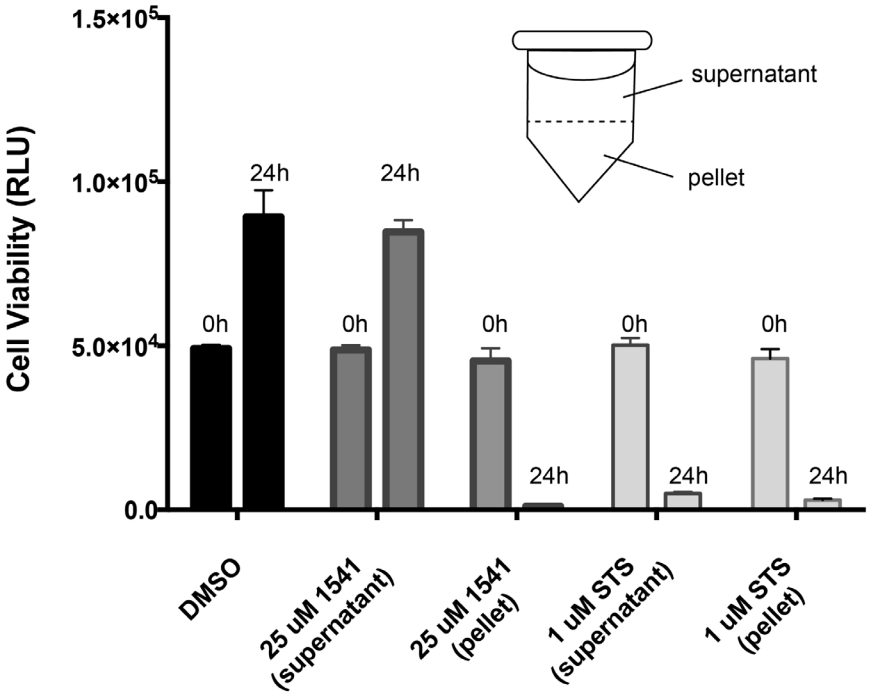
Supplementary Figure 5. Chemi-fibrils placed in a dialysis chamber are unable to induce cell death in K562 cells located on the outside. Schematic of the dialysis experiment is shown. A dialysis bag (D-tube midi, 3.5 kDa) containing 25 μM 1541, 2 μM of STS, or 0.5 % DMSO was placed in a cell culture dish filled with 15 mL of cell culture media containing K562 cells. Cell viability was assessed after 48 hours.



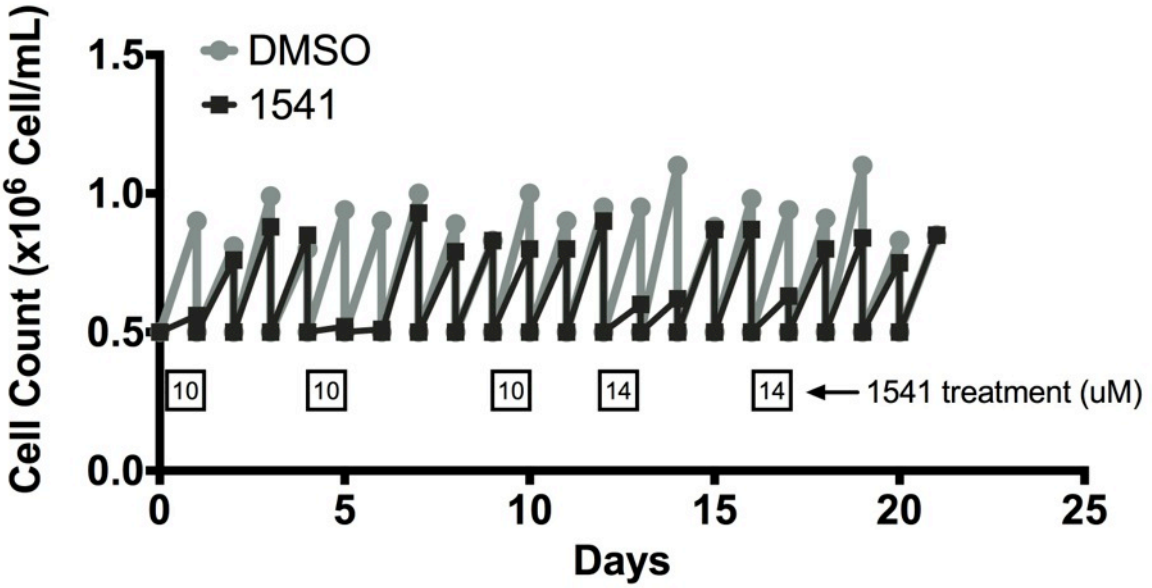
<u>Supplementary Figure 6.</u> Cells in a dialysis membrane are protected from killing from 1541 chemi-fibrils outside, but not by STS. K562 cells (750 µL at 1x10^5 cells/mL) were placed inside a dialysis bag (D-tube midi, 3.5 kDa), and the bag was placed inside a 60 mm x 15 mm cell culture dish filled with 15 mL of cell culture media containing 25 µM 1541, 2 µM of STS, or 0.5 % DMSO. Cells were taken out the bag 72 hours later and bright field images were acquired. CellTiter-Glo was then used to assess cell viability. Scale bar = 100 µm. Cell viability was assessed using CellTiter-Glo (Promega). The mean values ± s.d. of the normailized raw luminescence unit (RLU) is reported (n=3).



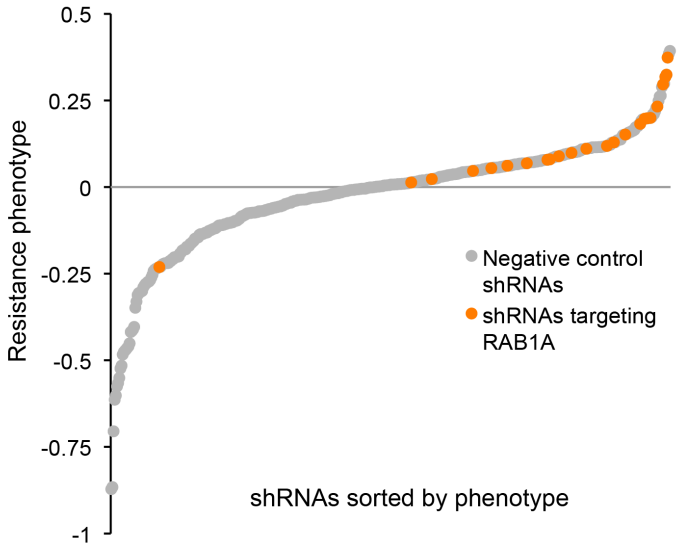
Supplementary Figure 7. Difference between pre-formed fibrils and fibril formation in the presence of detergent. Cell viability assay in HeLa cells showing the protective effect of Tween-80 (T80) detergent on 1541 chemi-fibrils. (**a**) Chemi-fibrils formation in cell culture in the presence of T80 protects cells against death (T80 added before 1541), (**b**) Concentration-dependent protection of T80 on pre-formed chemi-fibrils in cell culture media (1541 added before T80), (**c**) Detergent alone does not affect cell growth for 24h, (**d**) Detergent offers no protection against a non-fibril-forming small-molecule inducer of apoptosis (staurosporine, 2 μM). These results show that when 1541 fibrils are preformed in media, and then added to media containing T80 they are more resistant to T80 protection, than when 1541 is added directly from DMSO to media containing T80. This is consistent with the chemi-fibrils being more resistant to disruption by T80 once they are formed. Cell viability was assessed using CellTiter-Glo (Promega). The mean values ± s.d. of the raw luminescence unit (RLU) is reported (n=3).



Supplementary Figure 8. Centrifugation assay shows 1541 toxicity in the pellet, but not in the supernatant. HeLa cells were plated in 96-wells plate. Cell culture media (2 mL) containing 25 μM of 1541 or 1 μM STS was centrifuged at 15,000 rpm for 10 min. The top 1 mL of each sample was then used as “supernatant”, while the bottom 1 mL was mixed and used as “pellet”. Cell viability was assessed using CellTiter-Glo (Promega). The mean values ± s.d. of the raw luminescence unit (RLU) is reported (n=3).



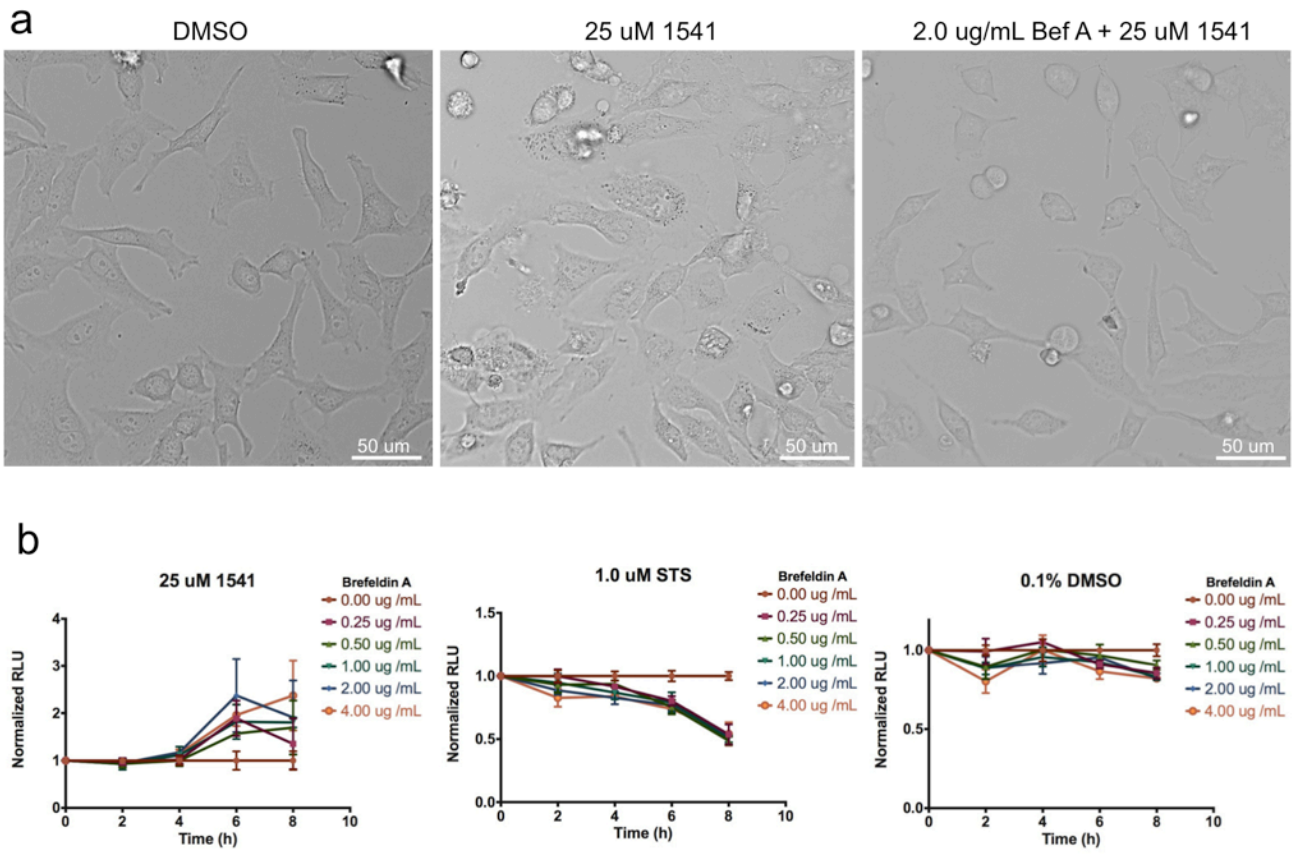
Supplementary Figure 9. Differential cell growth between treated (1541) and untreated cells (DMSO) during one replicate of the shRNA screen. Experimental cell growth of K562 for 1541 treated (black) and DMSO treated (grey) samples. The boxes below the growth chart shows the 24h treatments at the specified concentration of 1541 (μM). After each treatment, the cells were pelleted to remove the drug-containing cell culture media, and fresh media was added to the cells to let them recover several days before the next round of treatment. Every day, the cell density was measured for both samples using an automatic Scepter cell counter (Millipore) (n=1), and each sample was diluted back to $0.5x10^6$ cell/mL to monitor the growth rate differential between both samples.



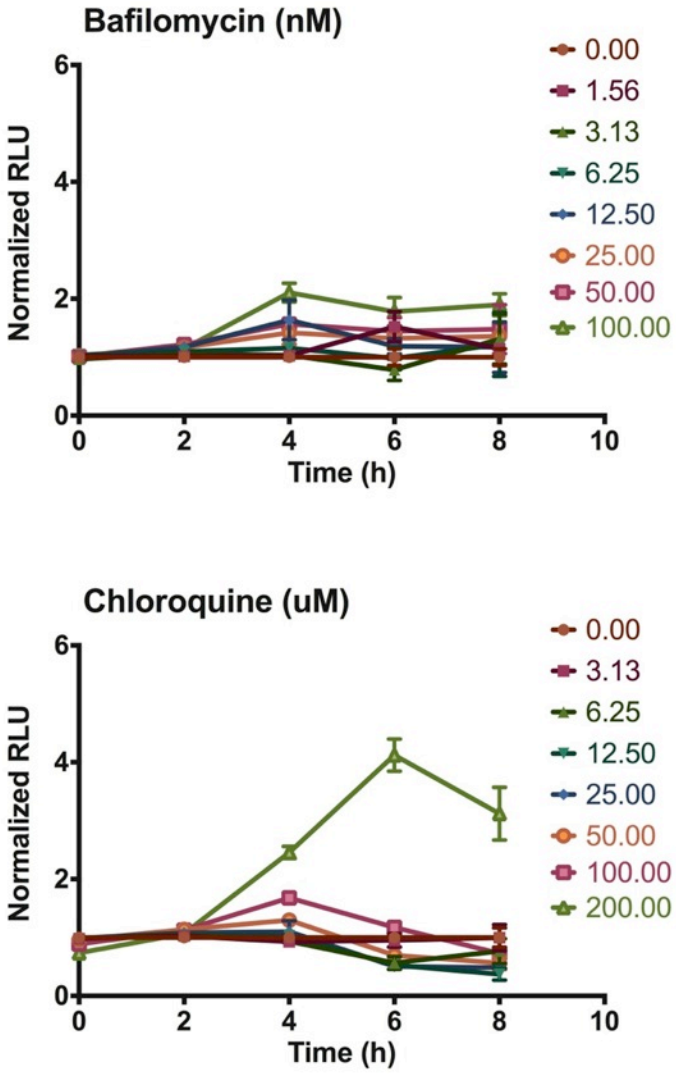
Supplementary Figure 10. Detection of statistically significant hit genes. 25 individual shRNA targeting *RAB1A* (red) are compared to the negative control shRNAs (grey).

Samples	% knockdown	Standard deviation
Rab1A_10	91%	23%
Rab1A_8	96%	16%
Rab2A_3	99%	35%
Rab2A_4	62%	20%
ATP6V0C_2	73%	12%

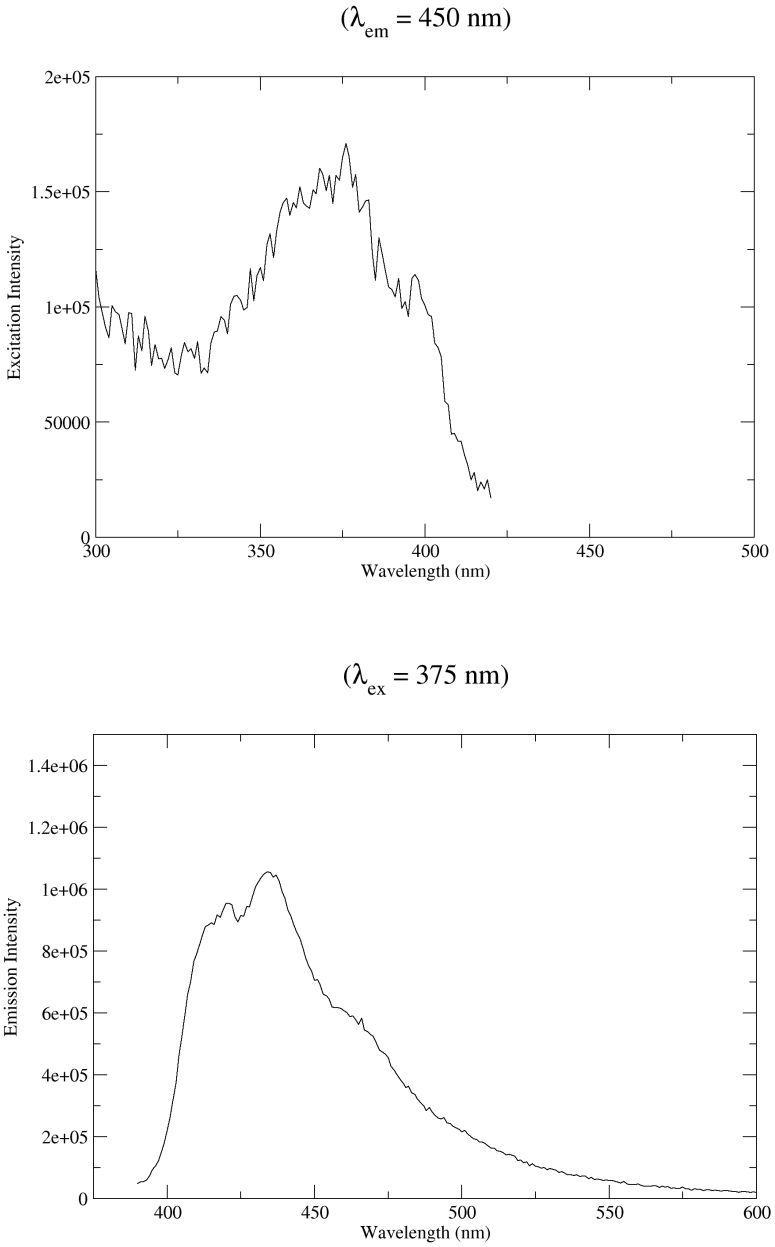
Supplementary Figure 11. qPCR of stable cell lines expressing shRNA. Knockdown of the target mRNA (mean and s.d. for 3 technical replicates).



<u>Supplementary Figure 12</u>. Partial protection of Brefeldin A treated cells against chemi-fibril induced cell death. (**a**) Bright field microscopy images of HeLa cells treated with DMSO, 25 µM of 1541, or co-treated for 4h with 25 µM of 1541 and 2.0 µg/mL of Brefeldin A (BefA). (**b**) Cell viability assay quantifying the protection effect of BefA against 1541-induced cell death compared to Staurosporine (STS) and DMSO controls. Data was normalized on 25 uM 1541 without drug. BefA delayed cell death induced by 1541, while it had a synergistic or additive effect in inducing apoptosis with STS, and had little effect on DMSO treated cells under 8h. Cell viability was assessed using CellTiter-Glo (Promega). The mean values ± s.d. are reported (n=3).

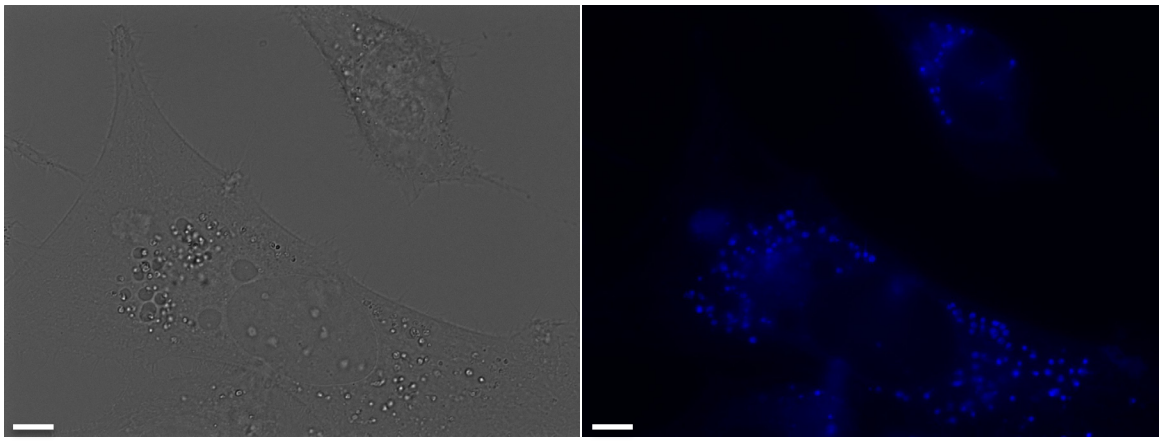


Supplementary Figure 13. Chloroquine and bafilomycin weakly protect against 1541. HeLa cells were treated with the inhibitors for 30 min before adding 20 μM of 1541. Cell growth was monitored using CellTiter-Glo, and the data was normalized on 1541 treated cells without the presence of inhibitors. N.B. At 6 hours and beyond, the majority of the cells are dead. The mean values ± s.d. are reported (n=3).

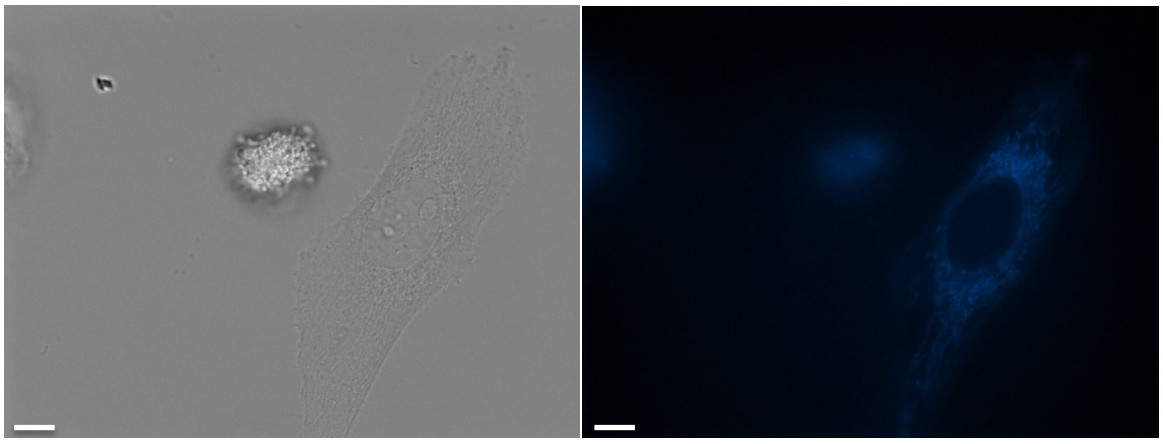


Supplementary Figure 14. Intrinsic fluorescent properties of 1541. The excitation and emission spectra of 1541 in water show that the optimal excitation and emission wavelengths are ~375 and ~440 nm, respectively.

1541

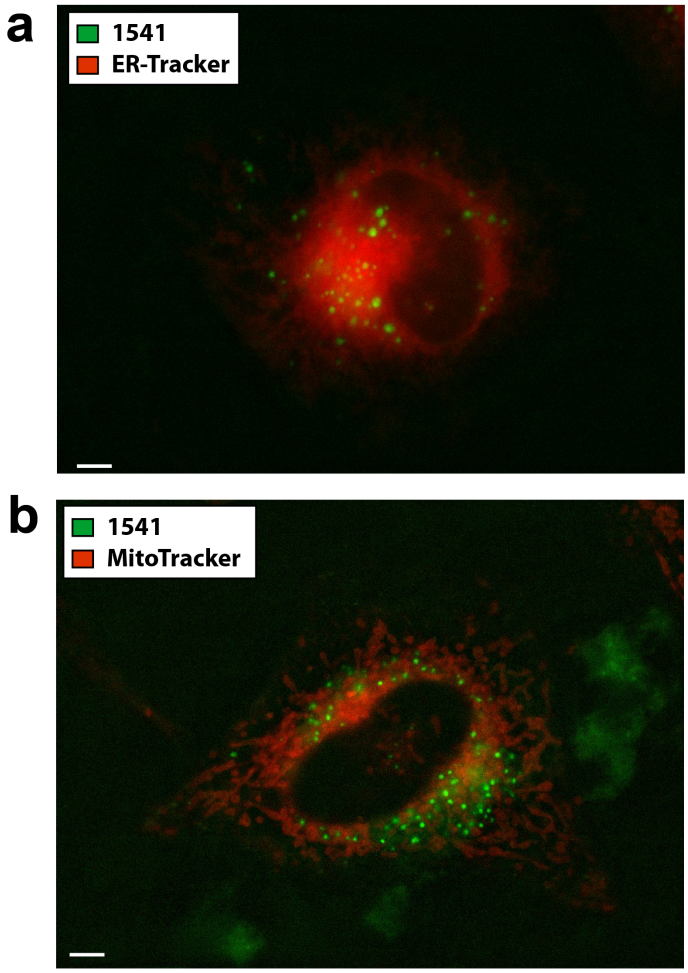


0.1 μM/mL of FasL (24h)

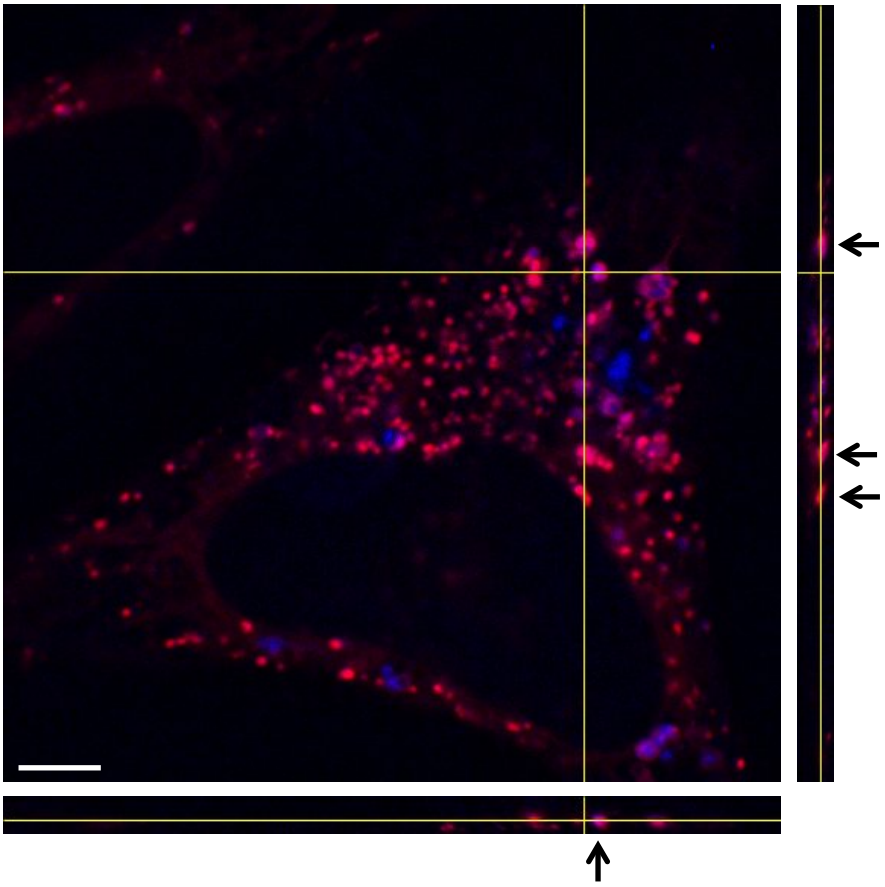


Supplementary Figure 15. Microscopy images of 1541- and FasL-induced cell death.

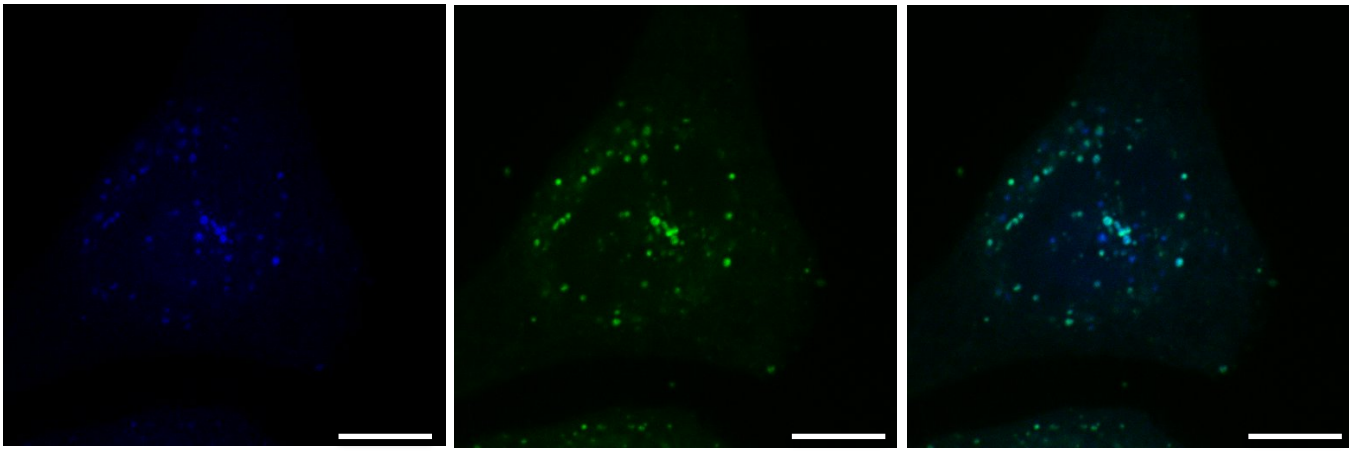
HeLa cells were treated with 20 μM 1541 (blue) or 0.1 ug/mL of FasL for 4 hours and 24 hours, respectively. The images were acquired using the same parameters on a Zeiss Observer Z1 at 63x magnification on LabTek 1.5 mm bottom glass dishes. Scale bar = 10 μm.



Supplementary Figure 16. Internalization of fluorescent aggregates in cell. (**a**) HeLa cells are treated with 1541 (green) and (**a**) ER-tracker (red) or (**b**) Mito-Tracker (red). The images show that the punctae are not co-localizing with either the ER or mitochondria. The images were acquired on live cells plated in LabTek 1.5 mm bottom glass dishes. Images were prepared using Fiji (a distribution of ImageJ, NIH). Scale bar = 5 μm.



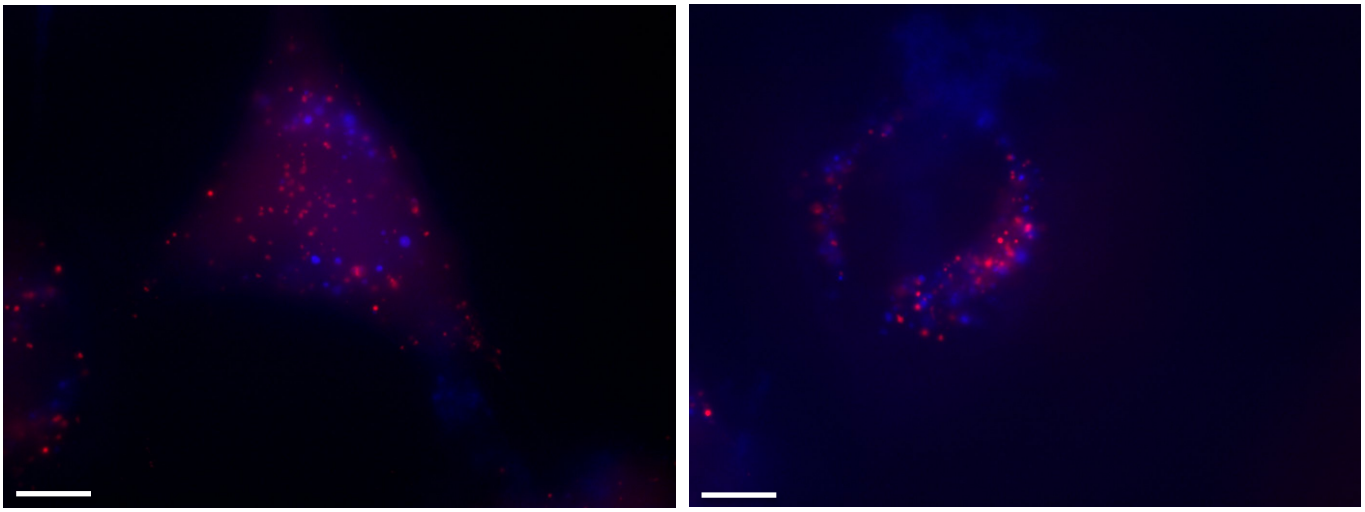
Supplementary Figure 17. Microscopy images of HeLa cells treated with 1541. Live cell imaging of HeLa cells treated with 15 μM 1541 for 2 hours. Orthogonal views of the z-stack (23 slices at 0.2 μm intervals were acquired) are shown, with the black arrows pointing to colocalized vesicles in three-dimension. The image shows that most of the 1541 punctae (blue) colocalize well with LysoTracker (red), with only a few outliers. We obtained a Manders coefficient M1 (above automatic threshold) of 0.66 between 1541 and lysotracker, suggesting that 66% of the blue signal colocalize with the red. The images were acquired on a spinning disk confocal microscope using a 100x magnification on LabTek 1.5 mm bottom glass dishes. Images were prepared using Fiji (a distribution of ImageJ, NIH). Scale bar = 5 μm.



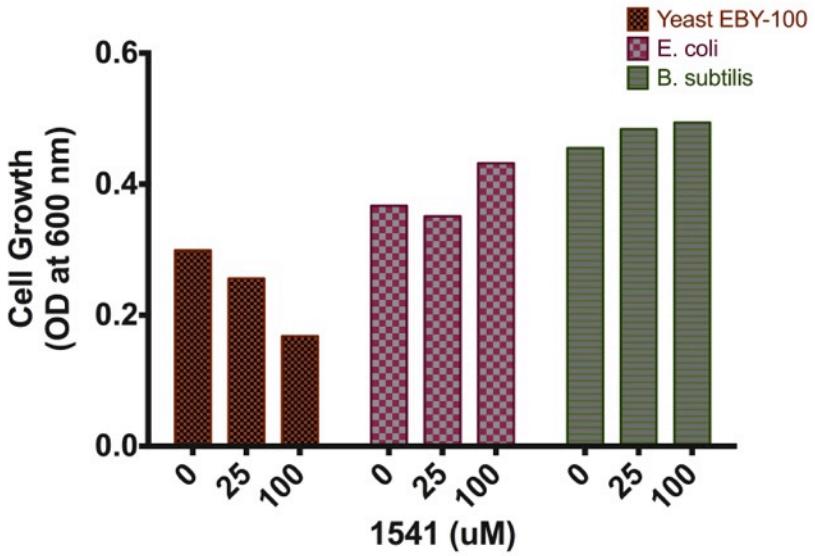
Supplementary Figure 18. Microscopy images of HeLa cells overexpressing a GFP-tagged LAMP1 and treated with 1541. HeLa cells were treated with 15 μM 1541 for 2 hours. Live cell imaging shows that most of the 1541 punctae (blue) colocalize well with LAMP1-GFP (green) in the lysosomes. The representative images were extracted from a z-stack. The images were acquired on a spinning disk confocal microscope using a 100x magnification on LabTek 1.5 mm bottom glass dishes. Images were prepared using Fiji (a distribution of ImageJ, NIH). Scale bar = 10 μm.

	R (nm)	± s.d. (nm)
DMSO	2.7	1.6
25 μM 1541	148.8	6.5
10 mM MβCD	2.5	1.4
25 μM 1541 + 10 mM MβCD	276.4	10.8
10 mM MβCD + 25 μM 1541	253.7	8.3
50 μM 1541	336.9	1.2

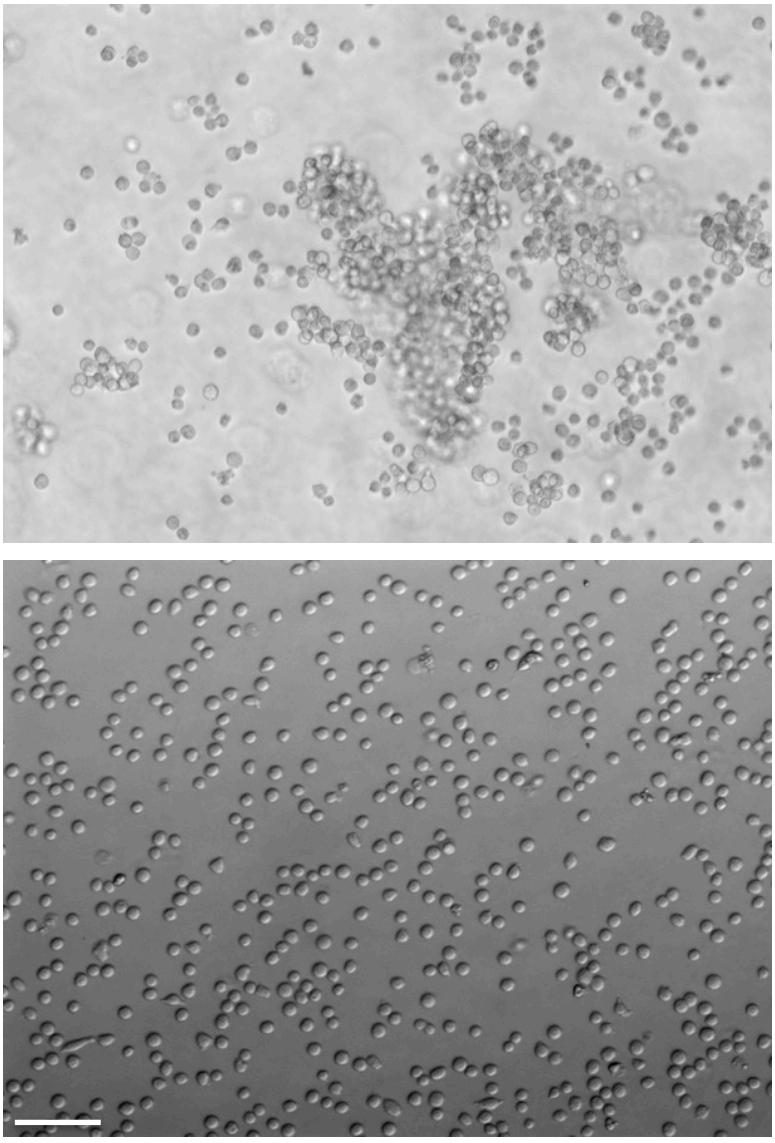
Supplementary Figure 19. Dynamic light scattering of 1541 in the presence of methyl-β-cyclo-dextrin (MβCD) shows it does not block fibril formation. The average radius of the particles in water is reported based on 10 measurements. The values correspond to the mean ± SD from three separate samples. The DMSO control (0.5%) shows the background of the filtered water, which is remains constant after the addition of 10 mM MβCD. Forming chemi-fibrils in water before the addition of MβCD (25 μM 1541 + 10 mM MβCD) resulted in comparable values to adding 1541 to a solution of 10 mM MβCD (10 mM MβCD + 25 μM 1541) suggesting MβCD neither prevent fibril formation nor dissolves them once formed.



Supplementary Figure 20. Chemi-fibrils do not colocalize with the macropinotytosis marker HIV TAT-TAMRA. In both images, HeLa cells were treated with 20 μM of 1541 (blue) and 5 μM of HIV TAT-TAMRA (red) for 4 hours. The images were acquired on a widefield Nikon Ti-E Microscope using a 100x magnification on LabTek 1.5 mm bottom glass dishes. Images were prepared using Fiji (a distribution of ImageJ, NIH). Scale bar = 10 μm.



<u>Supplementary Figure 21</u>. Effect of 1541 on yeast and gram positive and gram negative bacterial growth. Cells were treated with chemi-fibrils for 24 hours and the optical densities at 600 nm (n=1) are reported for gram-positive (*B. subtilis*), gram-negative (*E. coli*) and yeast (EBY-100). Chemi-fibrils had no effect on bacterial growth, and only a slight decrease on yeast growth at very high concentration of compound. Control experiments showed 1541 formed fibrils in both YPD and LB media, respectively, as monitored by DLS.



Supplementary Figure 22. Chemi-fibrils induces clumping of K562 cells. Cells were treated with chemi-fibrils for 4 hours with 25 μM 1541 (top), leading to the formation of large clumps of cells, compared to K562 cells treated with DMSO (bottom). Scale bar = 100 μm.

Supplementary Dataset 1. P values for all genes tested in the shRNA screen. Each row refers to a human gene; the columns list the gene ID, the official gene symbol, the official gene name, the p value for the gene calculated based on the Mann-Whitney U test, and whether knockdown gene was protective (P) or sensitizing (S).

Supplementary Dataset 2. Identification of proteolytic fragments generated during chemi-fibril induced cell death. The first tab report the recognition motifs of (a) all the substrates identified in chemi-fibril treated cells, which is subdivided into (b) caspase (P1=D) substrates and (c) non-caspase substrates (P1≠D) and compared to apoptotic and untreated datasets. The second tab contains all 2980 peptides identified in chemi-fibril treated cells, while the third tab contains the 26,044 apoptotic peptides reported in the degrabase (http://wellslab.ucsf.edu/degrabase/). Substrates common and unique to the chemi-fibrils are reported in the fourth and fifth tabs, respectively, while common substrates between chemi-fibrils and the apoptotic inducer bortezomib are reported in the sixth tab. The seventh and eight tabs contain the reference datasets for etoposide and bortezomib used in the analysis.

Supplementary Movie 1. Live cell imaging of HeLa cells treated with chemi-fibrils. HeLa cells were plated the day before in 35 mm No. 1.5 coverslip bottom dishes (MatTek Corporation). Cells were treated with 12.5 μM 1541 and pictures were taken every 3 min for 9.5 hours. Live cell imaging was performed on a Nikon Ti-E Microscope wide-field epifluorescence system equipped with a 37°C incubation chamber and CO_2 control using a 40x magnification objective.

Supplementary Movie 2. Live cell imaging of HeLa cells treated with chemi-fibrils. HeLa cells were plated the day before in 35 mm No. 1.5 coverslip bottom dishes (MatTek Corporation). Cells were treated with 25 μM 1541 for ~1h. Live cell imaging was performed on a Nikon Ti-E Microscope wide-field epifluorescence system equipped with a 37°C incubation chamber and CO_2 control using a 100x magnification objective.