ER stress and autophagy: new players in the mechanism of action and drug resistance of the cyclin-dependent kinase inhibitor flavopiridol

SUPPLEMENTAL DATA

METHODS

Confocal immunofluorescence microscopy of fixed cells: After 4 hours exposure to agents, cells were washed in phosphate-buffered saline (PBS) and made adherent on a microscope slide by centrifugation in a Cytospin3 (Shandon) centrifuge. Immediately after cytospin, cells were fixed in cold acetone. Next, the cells were incubated in blocking solution (2% bovine serum albumin in PBS) and stained for LC3 (Cell Signaling, Boston, MA) and Lamp2 (Santa Cruz Biotechnology, Santa Cruz, CA) by incubating separately with the respective primary antibodies overnight, in 4 ºC, followed by incubation with fluorescent secondary antibodies (Invitrogen, Carlsbad, CA): Alexa fluor 594 (red) anti-rabbit for LC3 and Alexa fluor 488 (green) anti-mouse for Lamp2. The LC3-II form is usually very bright due to the dotted pattern, completely overpowering the intensity of uncleaved and LC3-I forms, which are virtually comparable to the background due to the homogenous spread in the cell. Therefore, when we refer to LC3 fluorescence intensity, we are in fact presenting the LC3-II form, which localizes to the walls of the autophagosomes. Nuclei were stained blue with DAPI (Vector Laboratories, Burlingame, CA). Images were taken using a 60x objective and 4x digital zoom with Olympus Fluoview 1000 Laser Scanning Confocal microscope at the Ohio State University Campus Microscopy and Imaging Facility. ATF6 antibody was purchased from LifeSpan Biosciences (Seattle, WA) and Nrf2 was from Santa Cruz Biotechnology (Santa Cruz, CA). Nuclei were evaluated for ATF6 and Nrf2 using confocal microscopy to collect Z stacks, approximately 45 slices per cell, 0.2 µm each.

Quantification of immunofluorescence data

Integrated intensity was measured using Metamorph v.7.0. To calculate the number of "dots" (local extreme point in pixel-intensity profile at which a number of high-intensity pixels form a cluster), a Gaussian filter was first applied to reduce noise. Cells were enumerated electronically by an improved watershed-based algorithm. Quantification of co-localization was done by measuring the correlation index, which reflects the degree of overlap between red (LC3) and green (Lamp2) signals; if the index is 1, it indicates complete overlap, 0 is no overlap. Vehicle only and vehicle plus chloroquine conditions were not included in the quantification due to the fact that when autophagy is not stimulated, the signal for Lamp2 overpowers the signal for LC3 and co-localization between the two is not assessable. To quantify co-localization, two binary nuclei images - red (R) and green (G) - are obtained. The correlation value is calculated as the number of white pixels of (R&G) divided by the number of pixels of (R|G), where (R&G) is the intersection of R and G, and (R|G) is the union of R and G.

Caspase activity assays

Briefly, lysates containing approximately 3 x 10^6 cells were incubated with 50 μM LEVD-AFC (caspase 4 substrate) or LETD-AFC (caspase 8 substrate) from MP Biomedicals (Solon, OH) in 2X cytobuffer from BioVision (Exton, PA) containing 10 mM dithiothretiol (DTT). Caspase 4 activity was measured one hour after addition of substrate and caspase 8 activity was measured 30 minutes after addition of substrate. Release of free AFC was measured with a Beckman-Coulter DTX 880 multimode detector (Filters: excitation, 405/10 nm; emission, 535/25 nm). Caspase 4 inhibitor Z-YVAD-FMK was purchased from R&D Systems.

Cell viability

Percent of live cells was determined by staining with annexin V-fluorescein isothiocyanate and propidium iodide (PI). After exposure to agents, cells were washed with PBS and stained in 1X binding buffer (BD Biosciences, Franklin Lakes, NJ). Cell viability was assessed by flow cytometry using a Beckman-Coulter Cytomics FC500 cytometer. Data were analyzed with the CXP Cytometer software package (Beckman-Coulter). A total of 10,000 cells were counted for each sample. The cells treated with flavopiridol were washed after 4 hours with PBS and re-suspended in regular growth medium (RPMI 1640) supplemented with 10% human serum and antibiotics for the remainder of the incubation time. In the case of flavopiridol/chloroquine samples, chloroquine was re-added in the fresh media after flavopiridol was washed at 4 hours. For all the other conditions, cells were incubated with the respective drugs for 24 hours continuously.

Statistical analyses:

Data from experiments involving quantification of fluorescence *in vitro* data, RT-PCR, viability measured by flow cytometry and caspase assays were first log-transformed to stabilize variances. Next, linear mixed effects models were applied to the log-transformed data in order to account for the correlations among observations from the same patients. Holm's procedure was used to adjust for multiple comparisons and control overall Type I error.

For the quantification of LC3 fluorescence in serial samples (*in vivo* data), CLL patients were classified as either "no response" (n = 10) or "partial response" (n = 6). Similar to above, data from both experiments were log-transformed, and a linear mixed effects model was applied to the log-transformed data. The significance of the interaction between response category and time was tested.

SUPPLEMENTAL FIGURE LEGENDS

S FIG 1: Autophagy markers in CLL cells. Immunoblot showing autophagy regulatory protein expression in CLL cells and normal B cells; Gapdh was used as loading control.

S FIG 2: Chloroquine inhibits LC3-Lamp2 co-localization. CLL cells were incubated with 5 μM rapamycin, with or without chloroquine at several concentrations. Confocal fluorescence microscopy shows LC3 (autophagosomes) in red, Lamp2 (lysosomes) in green and DAPI (nuclei) in blue. Images were collected with 60x objective and 4x optical zoom.

S FIG 3: Expression of ATG5 and ATG7 with siRNA treatment. CLL cells were transfected with scrambled or combination of ATG5 and ATG7 siRNA. 24 hours after transfection, cells were incubated 4 hours without or with **(A)** thapsigargin (1 μM) or F-ara-A (5 μM), or **(B)** flavopiridol (2 μM). RNA was collected at the end of the treatment and analyzed by real-time RT-PCR. ATG5 and ATG7 expression was significantly reduced by siRNA ($P < 0.0001$). For (A), $n = 5$, for (B), $n = 10$. ΔCT was calculated by subtracting CT values for CD52 (our housekeeping gene) from CT values for ATG5 and ATG7. Higher values indicate lower gene expression.

S FIG 4: Effects of CDK1 and CDK5 knockdown on autophagy. (A) CLL cells ($n = 5$) were transfected with scrambled or CDK-specific siRNA, and expression level of CDK5 was assessed by real-time RT-PCR. ΔCT was calculated by subtracting CT values for CD52 (our housekeeping gene) from CT values for CDK5. Higher values indicate lower gene expression. CDK5 siRNA significantly reduced CDK5 expression ($P = 0.0003$ for CDK5 siRNA and $P = 0.0009$ for CDK1/CDK5 siRNA). **(B)**. Immunoblot for CDK1 and CDK5 in CLL cells incubated with different agents or transfected with CDK siRNA. CDK1 protein expression was undetectable in all CLL samples; lysate from Jurkat cells was used as positive control for the antibody. **(C)**. Confocal fluorescence microscopy shows increased LC3 fluorescence in the

presence of CDK5 siRNA compared to the scrambled control siRNA. Results shown are representative of 5 experiments.

S FIG 5: ER stress markers in CLL cells treated with flavopiridol. Real-time RT-PCR was used to analyze expression of **(A)** XBP1, **(B)** GRP78 and **(C)** IRE1 in CLL cells (n = 5) incubated with flavopiridol 2 μM, thapsigargin 1 μM and tunicamycin 3 μg/ml. P values were calculated for vehicle versus each reagent used and they were as follows: for IRE1: $P = 0.008$ for flavopiridol, $P = 0.005$ for thapsigargin, $P = 0.003$ for tunicamycin; for XBP1: $P = 0.053$ for flavopiridol, $P = 0.077$ for thapsigargin, $P = 0.099$ for tunicamycin; for GRP78: $P = 0.038$ for flavopiridol, $P = 0.012$ for thapsigargin, $P = 0.062$ for tunicamycin. **(D)** Real-time RT-PCR for XBP1, $P = 0.0007$, **(E)** IRE1, $P = 0.0002$ and **(F)** GRP78, $P = 0.0036$ in CLL cells (n = 5) transfected with CDK5 siRNA.

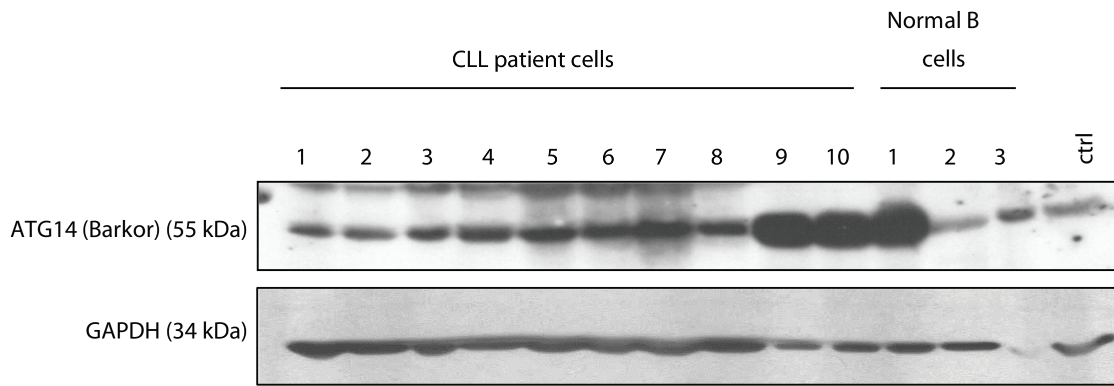
S FIG 6: PERK and IRE1 activity in CLL cells. (A). Immunoblot for phosphorylation of eIF2α in the Jurkat cell line and CLL patient cells. **(B)**. XBP1 splicing in cell lines, CLL cells and normal B cells. cDNA from various cells was subjected to XBP1-specific PCR, and products were separated on a 10% acrylamide gel. XBP1 splicing is detected with flavopiridol and thapsigargin in the cell lines 293T, RAW and 697, as well as in normal B cells, but was not detectable in CLL cells. **(C)** Immunoblot for GRP78 in CLL patient cells (representative of 3 immunoblots). Gapdh was used as loading control.

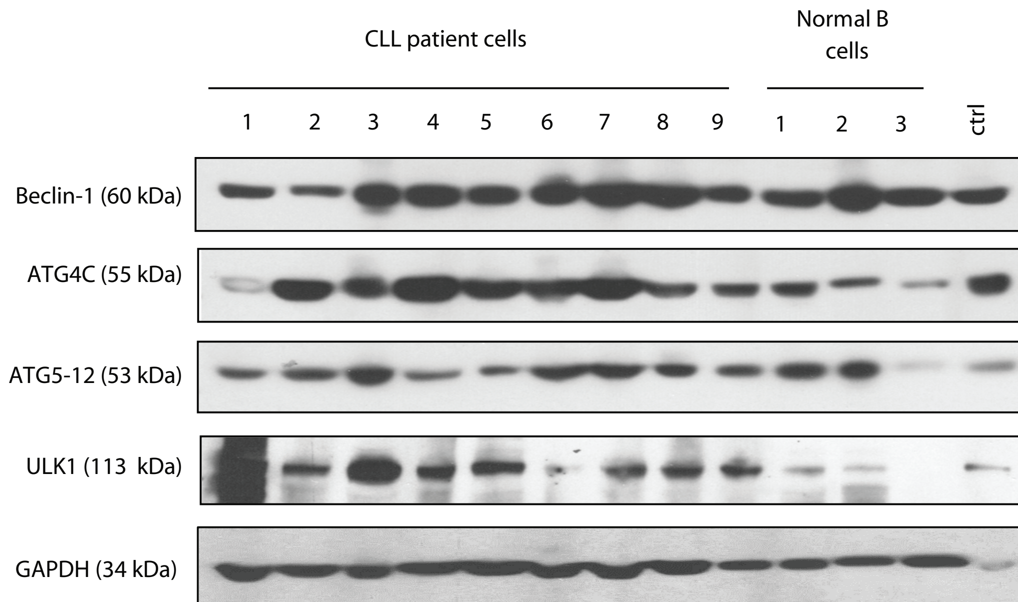
S FIG 7: Markers of cell death by ER stress. Activity of caspases 4 **(A)** and 8 **(B)** in CLL cells (n = 5) treated *in vitro* with flavopiridol (2 μM), thapsigargin (1 μM), tunicamycin (3 μg/ml) or F-ara-A (5 μM) alone or in combination with caspase 4 inhibitor Z-YVAD-FMK (20 μM). Cells were incubated with flavopiridol 4 hours, washed, and re-plated in fresh media without drug; all the other reagents were left with the cells for 24 hours. Activity is shown as free AFC/min/mg protein (fluorescence released by cleaved substrate). Caspase 4 and 8 activity was significantly

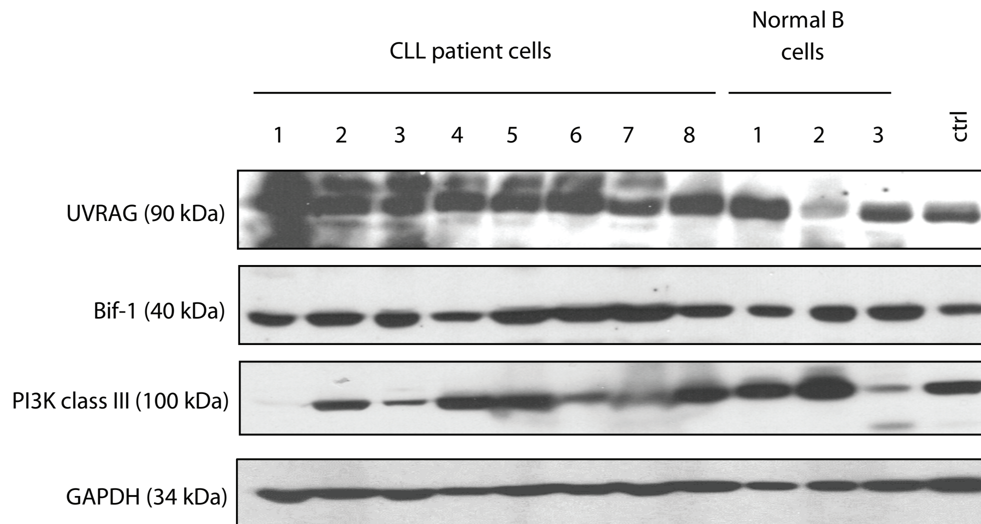
increased by each agent in the absence of Z-YVAD-FMK (*P* = 0.0001). **(C)**. Viability of CLL cells from (A) and (B), (*n* = 5). Viability is shown as percent cells negative for both annexin and PI by flow cytometry at 24 hours. Z-YVAD-FMK significantly blocked flavopiridol-mediated cell death (*P* = 0.012). **(D)** Immunoblot for ASK1 after siRNA transfection: lysates were collected after 4 hours incubation of cells with reagents described in (B). **(E)**. Activity of caspases 4 and 8 was measured in CLL cells obtained from patients before treatment, at the end of flavopiridol infusion (4.5 hours) and 24 hours after the beginning of the infusion. **(F)**. Real-time RT-PCR for ASK1 expression in CLL cells (*n* = 5) transfected with scrambled or ASK1 siRNA, then incubated 4 hours with flavopiridol 2 μM, F-ara-A 5 μM, thapsigargin 1 μM, or tunicamycin 3 μg/ml (treatments were done 48 hours post-transfection). ΔCT was calculated by subtracting CT values for CD52 (our housekeeping gene) from CT values for ASK1. Higher values indicate lower gene expression. ASK1 decreased significantly (*P* = 0.0005). **(G)** Caspase 4 assay in CLL cells (*n* = 3) untransfected or transfected with either scrambled or siRNA against ASK1.

S FIG 8: p62/SQSTM1 and LC3 gene expression does not change in the presence of autophagy inducers. Real-time RT-PCR tested p62/SQSTM **(A, B)** and LC3 **(C, D)** expression in CLL cells treated with autophagy inducers *in vitro* (*n* = 5) and *in vivo* in CLL cells harvested from 5 patients treated in clinic with flavopiridol, before infusion and at 4.5 h into the infusion.

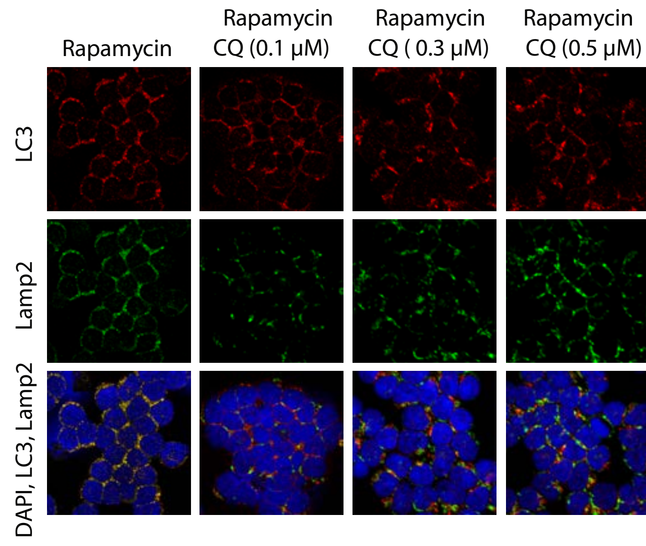
Supplemental Figure 1





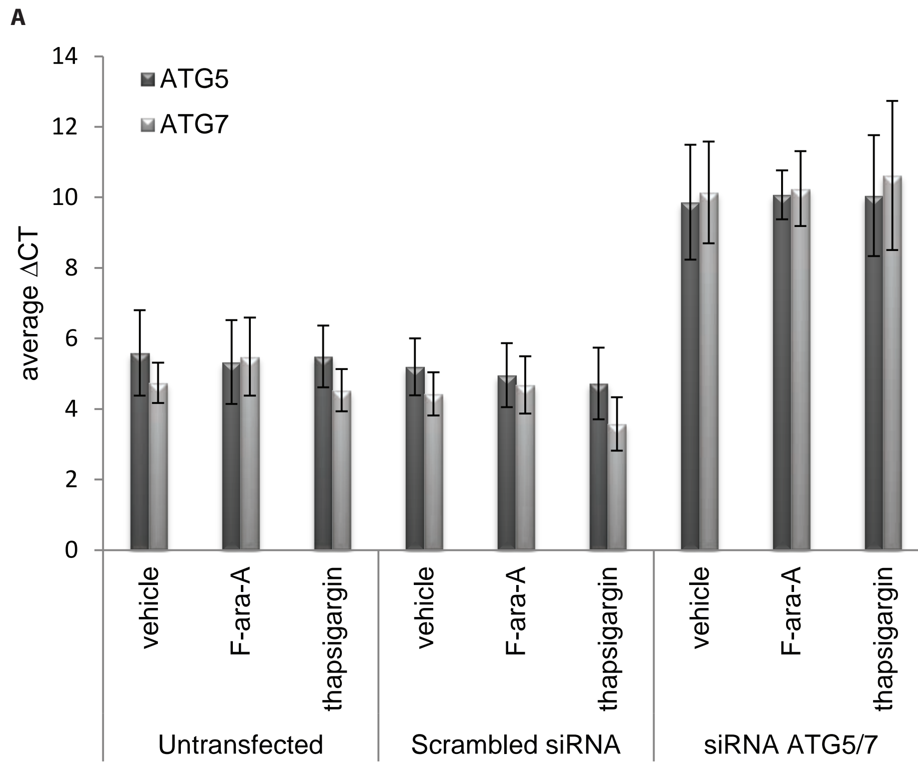


Supplemental Figure 2



Supplemental Figure 3

A



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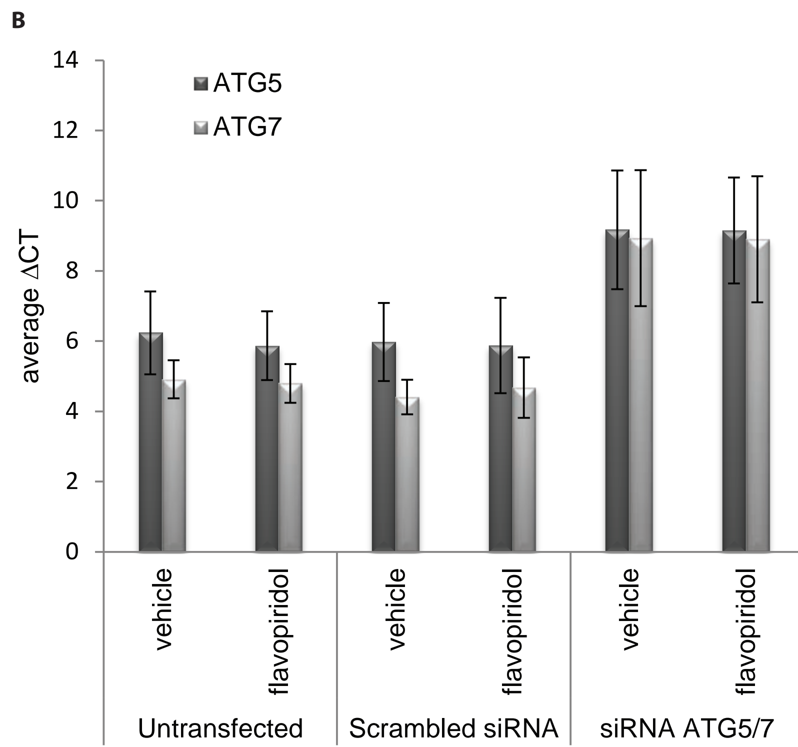

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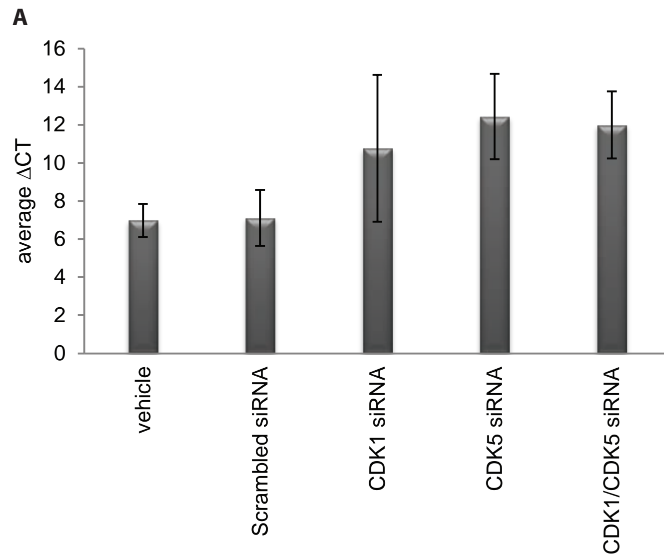
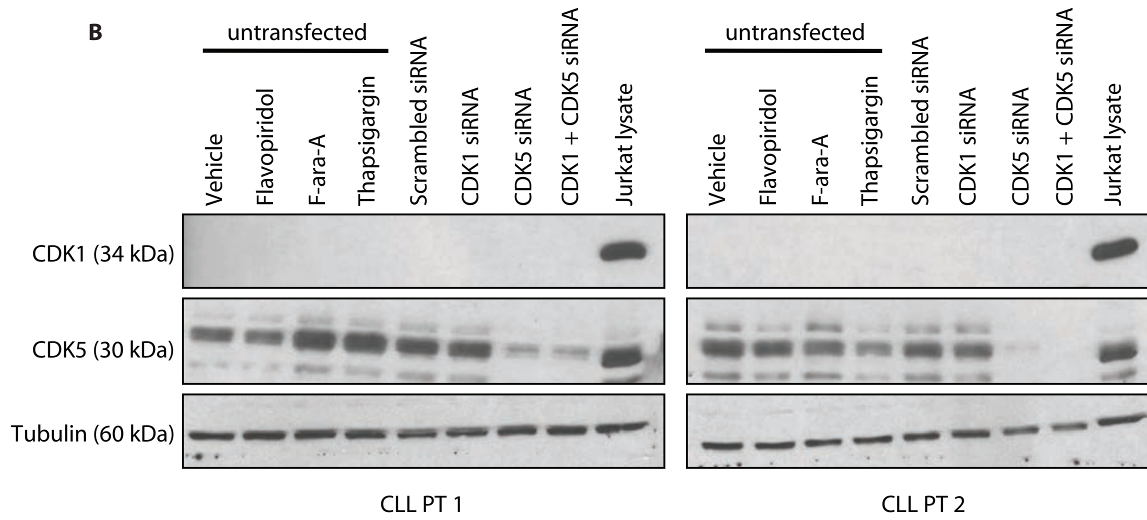
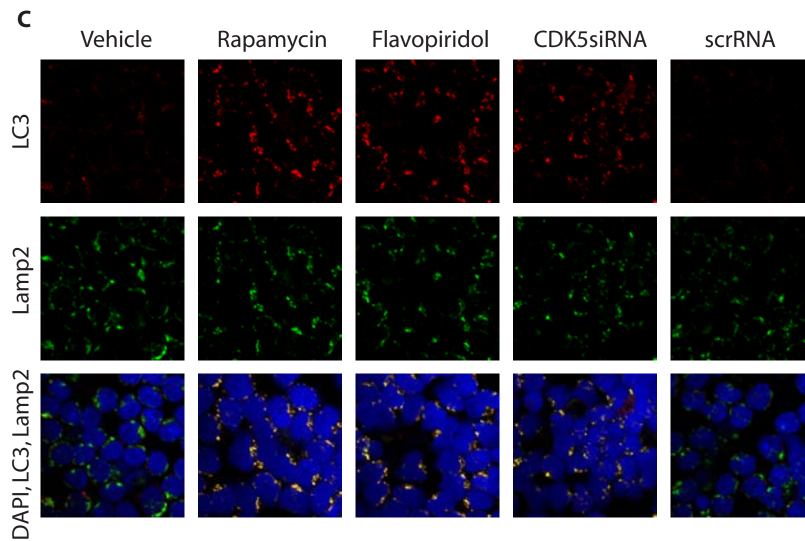
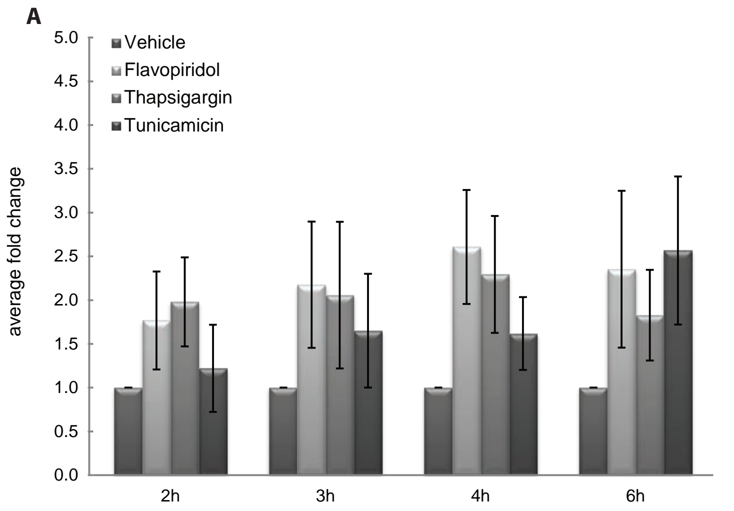






Supplemental Figure 5

A



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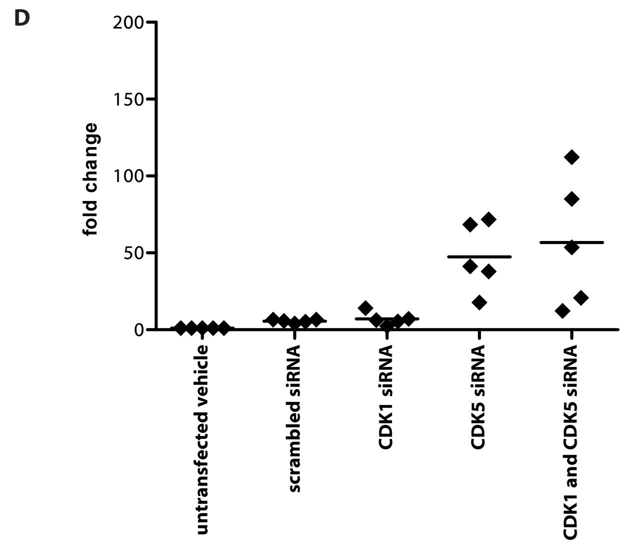

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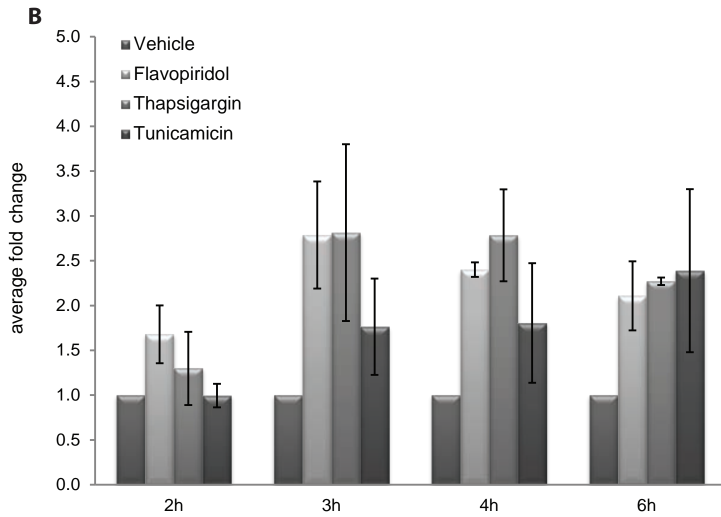

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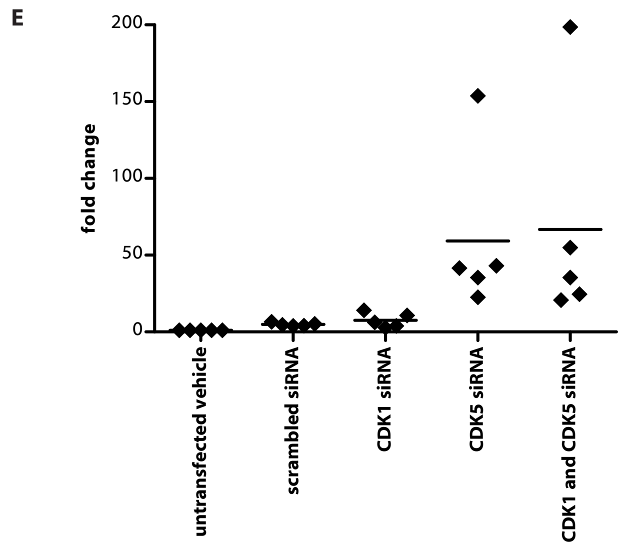

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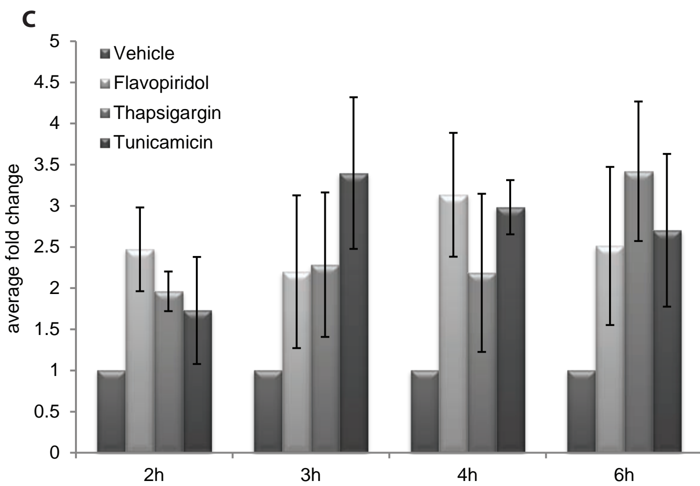

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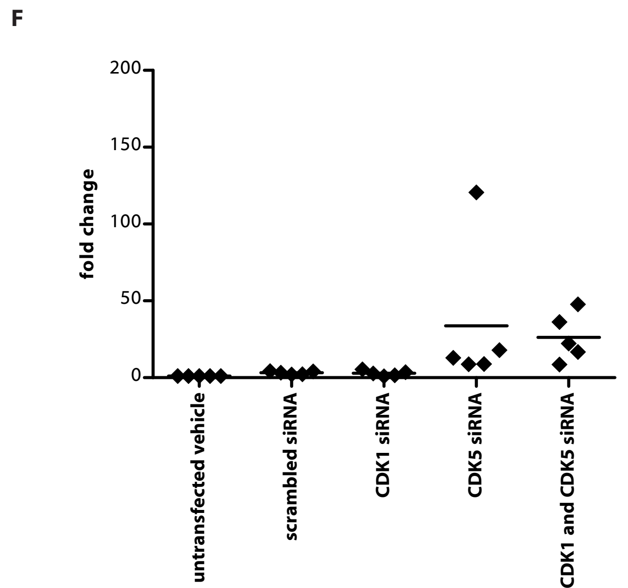


Supplemental Figure 6

A

Jurkat cell line

Vehicle 4h
Vehicle 6h
Thapsigargin 4h
Thapsigargin 6h
Tunicamycin 4h
Tunicamycin 6h

P-eIF2α (38 kDa)
Total eIF2α (38 kDa)

4h
8h

Vehicle 4h
Flavopiridol
F-ara-A
Thapsigargin
Vehicle
Flavopiridol
F-ara-A
Thapsigargin
Jurkat flavopiridol

P-eIF2 α
Total eIF2 α
PT 1

P-eIF2 α
Total eIF2 α
PT 2

P-eIF2 α
Total eIF2 α
PT 3

P-eIF2 α
Total eIF2 α
PT 4

B

293T
RAW
697

Ladder
Vehicle 4h
Flavopiridol 4h
Thapsigargin 4h
Tunicamycin 4h
Vehicle 4h
Flavopiridol 4h
F-ara-A 4h
Thapsigargini 4h
Vehicle 4h
Flavopiridol 4h
F-ara-A 4h
Thapsigargin 4h
Ladder

CLL cells

Ladder
Vehicle 4h
Control agent 4h
Flavopiridol 4h
F-ara-A 4h
Thapsigargin 4h
Tunicamycin 4h
Vehicle 8h
Control agent 8h
Flavopiridol 8h
F-ara-A 8h
Thapsigargin 8h
Tunicamycin 8h
Vehicle 8h
Thapsigargin 8h

Normal B cells
HeLa

Ladder
Vehicle 4h
Flavopiridol 4h
Thapsigargin 4h
F-ara-A 4h
Vehicle 8h
Flavopiridol 8h
Thapsigargin 8h
F-ara-A 8h
Vehicle 4h
Thapsigargin 4h

C

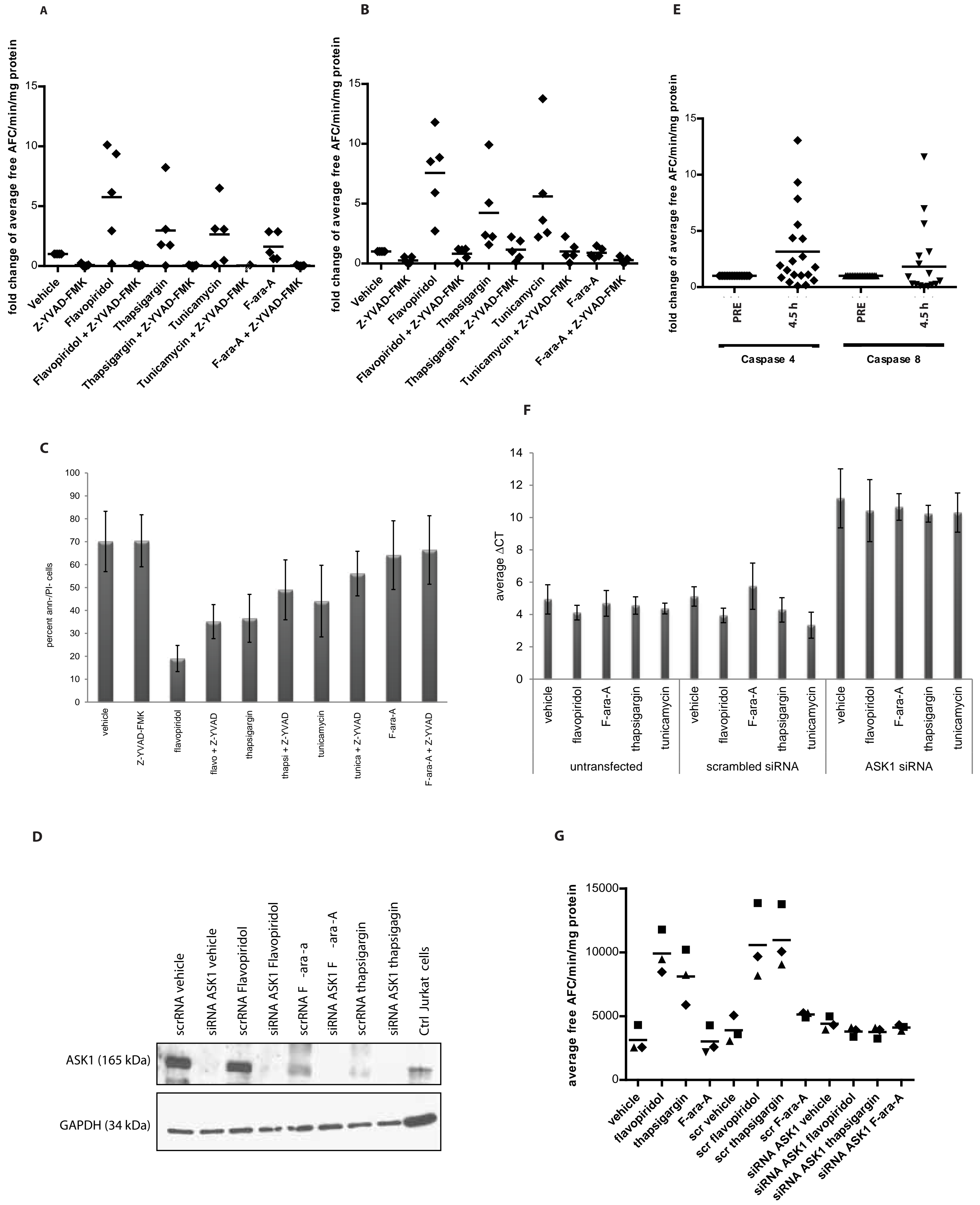
PT 1
PT 2

Ctrl
Vehicle
F-ara-A
Flavopiridol
Thapsigargin
Vehicle
F-ara-A
Flavopiridol
Thapsigargin

GRP78 (78 kDa)
GAPDH (34 kDa)

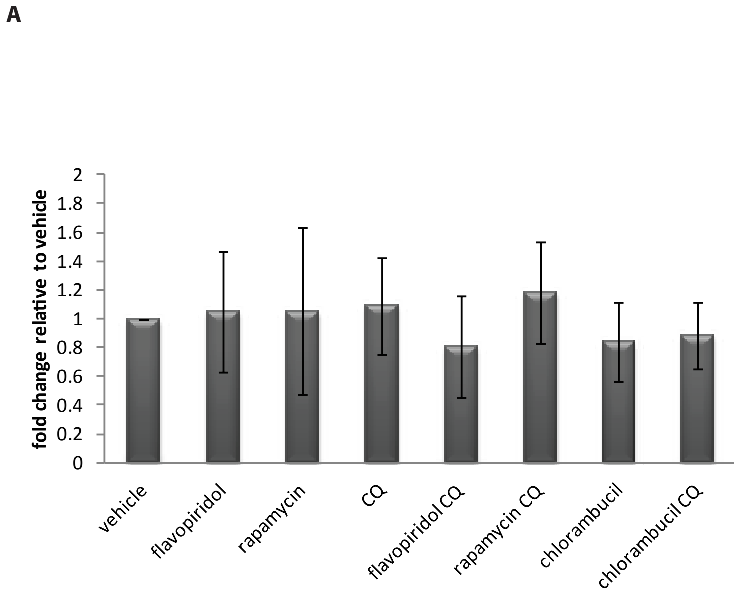
	Ctrl	Vehicle	F-ara-A	Flavopiridol	Thapsigargin	Vehicle	F-ara-A	Flavopiridol	Thapsigargin
GRP78/GAPDH	0.4	0.2	0.2	0.6	0.7	0.2	0.2	0.7	0.9

Supplemental figure 7



Supplemental Figure 8

A



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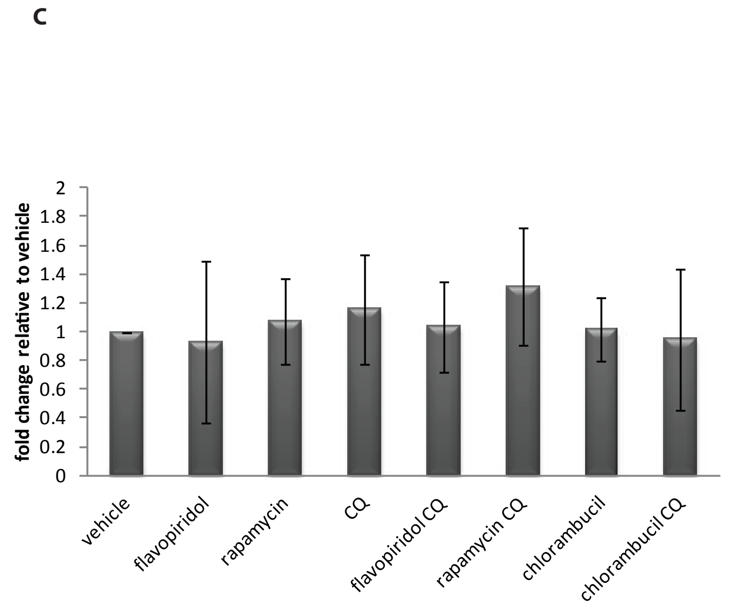

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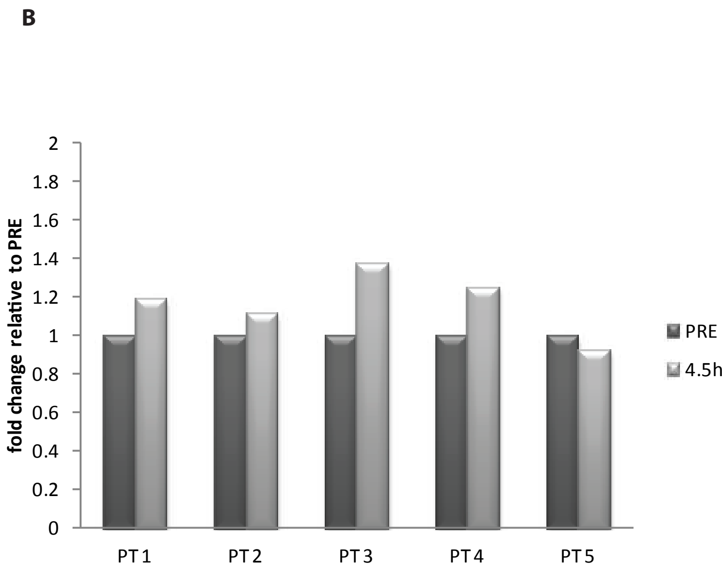

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