Supplementary Materials and Methods

menB knockout in *E. faecalis*

All chemicals were purchased from Sigma unless otherwise stated. Restriction enzymes *SalI*, *Pst*I, *Xba*I, *Xma*I, and T4 DNA ligase were purchased from New England Biolabs and Takara ExTaq DNA polymerase from Millipore. *Escherichia coli* TG1 electrocompetent and Sure2 super competent cells were purchased from Stratagene. The TA Cloning® Kit with pCR II vector was purchased from Invitrogen and DNA purification kits from Zymo Research.

DNA sequences for the *menB* gene encoding 1,4-dihydroxy-2-naphthoic acid synthase and flanking regions were obtained from the *Enterococcus faecalis* v583 genome (AE016830). Primers (menB1 and menB2) were designed for a 500 bp upstream segment (menBf1) that covered the start codon of *menB* with a *Sal*I site on the 5' end of menB1 and an *Xba*I site on the 5' end of menB2. Additional primers (menB3 and menB4) were designed for a downstream segment of 519 bp (menBf2) that also covered the stop codon of *menB* with a *Pst*I site on the upstream end of menB3 and an *Xma*I site on the downstream end of menB4. Gene fragments were designed to sandwich the chloramphenicol gene amplified using primers cat1 and cat2 with an *Xba*I restriction site on the upstream end and a *Pst*I restriction site on the downstream end.

Each segment was PCR amplified, cloned into the TA cloning vector, and transformed into TOP10F' competent cells (Invitrogen). Blue/white screening was used to pick clones containing the segments of interest. Clones containing the segments were harvested by Zyppy™ Plasmid Miniprep Kit (Zymo Research). Target segments were then digested from plasmids, gel purified, and ligated to each other to construct a double crossover cassette. The entire knockout cassette was amplified from the ligation mixture using the outermost flanking primers (menB1 and menB4) to produce a 1.75 kb PCR product. This product was then ligated into the TA

cloning vector, transformed into Sure-2 competent cells, and clones selected. Plasmids containing the knockout cassette were harvested using the Plasmid Miniprep Kit (Zymo Research), digested with *Sal*I and *Xma*I, and gel purified. The purified knockout cassette was ligated into the transposon vector pG+host10 (a derivative of pG+host9) that had been previously digested with *Sal*I and *Xma*I.[1]

The pG+host10::menBf1-cat-menbf2 knockout plasmid was electroporated into *E. coli* TG1, selected on LB with 400μg/ml erythromycin, and verified by PCR. Plasmid was purified by Plasmid Miniprep Kit and electroporated into *E. faecalis* OG1RF as described by Shepard and Gilmore.[2] Clones were selected at 28°C on brain-heart infusion (BHI) agar containing 500 μg/ml erythromycin. Clones were grown in BHI without antibiotics at 28°C for 5 hrs and then temperature shifted to 42°C overnight. Bacteria were subsequently plated on BHI agar containing 25 μg/ml chloramphenicol and grown at 37°C overnight. Chloramphenicol resistant colonies were replica-plated at 37°C on BHI-erythromycin and BHI-chloramphenicol agar. Colonies resistant to chloramphenicol and sensitive to erythromycin were chosen as *menB* knockouts and verified by PCR and DNA sequencing. One confirmed knockout strain with *menB* disrupted by insertion of *cat* was designated WY84. Deficiency in extracellular superoxide production by WY84 was confirmed using the cytochrome *c* reduction assay as previously described.[3]

Nuclear magnetic resonance

One-dimensional ^{1}H chemical shifts were collected using a Varian VNMRS-400 MHz spectrometer with 16 transients and 1 sec recycle delay. ^{13}C shifts were obtained by indirect methods. Assignments were made based on gCOSY, HSQC, and gHMBC experiments.

Concentration of 4-HNE was determined by quantitative ^{1}H NMR using 0.8 μM benzene as an internal reference for integration ratios.[4] The spectrum was collected with 32 scans, 90 degree pulse width, and recycle delay of 300 seconds to ensure total relaxation between scans. The experiment was repeated 5 times. Each spectrum was carefully phased and a baseline correction employed prior to integration to improve accuracy. Spectra were collected at 21ºC and all pulse sequences were performed using Varian VNMRJ2.2c software.

Fluorescence-activated cell sorting and immunofluorescence

Fluorescence-activated cell sorting (FACS) was used to determine ploidy, γH2AX staining, and cell cycle arrest as previously described.[5] For microtubule staining, cells were stained for tubulin using 1:100 diluted α/β tubulin antibody (Cell Signaling Technology) and Fluor® 647 goat anti-rabbit IgG (1:1,000 dilution, Invitrogen) as a secondary antibody. Images were collected using laser scanning confocal microscopy (Leica Microsystems) and analyzed using Volocity software (Improvision).

Western blotting

Protein extraction and Western blotting were performed as described.[5] Anti-phospho-stathmin (Ser^{16}) rabbit monoclonal and anti-α/β-tubulin rabbit polyclonal antibodies were purchased from Cell Signaling Technology. Anti-Gsta4 purified MaxPab mouse polyclonal antibody was purchased from Abnova and anti-4-HNE mouse monoclonal antibody from R&D Systems.

Cytotoxicity assay

4-HNE cytotoxicity was determined using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) viability assay (Sigma) as previously described.[6]

Immunohistochemistry

Immunohistochemical staining was performed as previously described.[5] For 4-HNE staining, slides were blocked with 5% normal goat serum and incubated with rabbit anti-4-HNE antiserum (Alpha Diagnostic) and HRP-goat anti-rabbit IgG conjugate (Zymed). For immunocytochemical staining, RAW264.7 cells were seeded onto chamber slides (BD Biosciences) and incubated at 37 ºC overnight. Cells were treated with *E. faecalis* OG1RF, *E. faecalis* WY84, *E. coli* DH5α, or sham at 37ºC for 2 hrs. Following fixation with 4% formalin and acetone, slides were processed and stained as previously described.[5] Slides were developed with DAB Enhanced liquid substrate and counterstained with Mayer's hematoxylin solution (Sigma).

References

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2. Shepard BD, Gilmore MS. Electroporation and efficient transformation of *Enterococcus faecalis* grown in high concentrations of glycine. In: Nickoloff JA, ed. Methods in Molecular Biology: Electroporation and Electroprofusion of Microorganisms Protocols. Volume 47. Totowa, NJ: Humana Press, Inc., 1995:217-226.
3. Huycke MM, Joyce W, Wack MF. Augmented production of extracellular superoxide production by blood isolates of *Enterococcus faecalis*. J Infect Dis 1996;173:743-746.

4. Pauli GF, Jaki BU, Lankin DC. Quantitative ^{1}H NMR: development and potential of a method for natural products analysis. J Nat Prod 2005;68:133-49.

5. Wang X, Allen TD, May RJ, Lightfoot S, Houchen CW, Huycke MM. Enterococcus faecalis induces aneuploidy and tetraploidy in colonic epithelial cells through a bystander effect. Cancer Res 2008;68:9909-17.

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Supplementary Figure legends

Figure S1. HPLC and NMR assays for 4-HNE. *A*, Chromatogram of HPLC showing 4-HNE peak at 30 min. *B*, Concentration of pure 4-HNE from *E. faecalis*-infected macrophages is determined by NMR using benzene as an internal control.

Figure S2. Survival curve for 4-HNE. Cells were exposed to serially diluted 4-HNE for 1 hr and incubated in fresh medium for 18 hrs at 37°C (HCT116) or 33°C (YAMC). Cell viability was determined by the MTT assay and survival rate calculated by untreated control as 100% survival. A, HCT116 cells; B, YAMC cells.

Figure S3. Survival curve for *trans*-2-nonenal. HCT116 (*circle*) and YAMC (*square*) cells were exposed to serially diluted *trans*-2-nonenal for 1 hr and incubated in fresh medium for 18 hrs at 37°C (HCT116) or 33°C (YAMC). Cell viability was determined by the MTT assay.

Figure S4. 4-HNE does not generate detectable 4-HNE-tubulin adducts in HCT116 and YAMC cells. *A*, western blot showing global modification of proteins in HCT116 cells treated with 4-HNE at 2 to 5 μM. *B* and *C*, immunoprecipitation of 4-HNE modified tubulins using α/β-tubulin antibody. Western blot shows positive staining for tubulin monomers in all samples (*A*), but negative staining for 4-HNE antibody (*B*).

Figure S5. 4-HNE activates stathmin in YAMC cells. *A*, Western blots show 19 and 20 kDa isoforms of p-stathmin (Ser^{16}) in untreated YAMC cells, YAMC cells treated with 4-HNE and nocodazole. *B*, After normalizing to β-actin, the 19 kDa Ser^{16}-phosphorylated isoform of p-stathmin (Ser^{16}) clearly decreased 0.5–6 hrs following 4-HNE treatment. Nocodazole, a spindle poison, produces a similar effect. *C*, Reductions in the 20 kDa isoform of stathmin were also evident in YAMC cells at early and late time points following exposure to nocodazole and 4-HNE.

Figure S6. Inactivation of *Gsta4* attenuates 4-HNE decay. 4-HNE at 1 μM was added to medium for Gsta4-silenced YAMC (*triangle*), wild-type YAMC (*open circle*), and YAMC transfected with a negative control plasmid (*closed square*) cells. 4-HNE was extracted from supernatants and cells over 60 min and measured by electrochemical detection.

Figure S7. 4-HNE does not generate tubulin dimers in HCT116 cells. Purified bovine brain tubulin (Cytoskeleton Inc.) was treated with 4-HNE for 30 min at 37°C. Western blotting using α/β-tubulin antibody shows tubulin dimers generated by 4-HNE (110 kDa) in a dose dependent

manner for pure tubulin (*left lanes*). Marked dimers are not noted in cell lysates from 4-HNE-treated HCT116 cells (*right lanes*).

Figure S1

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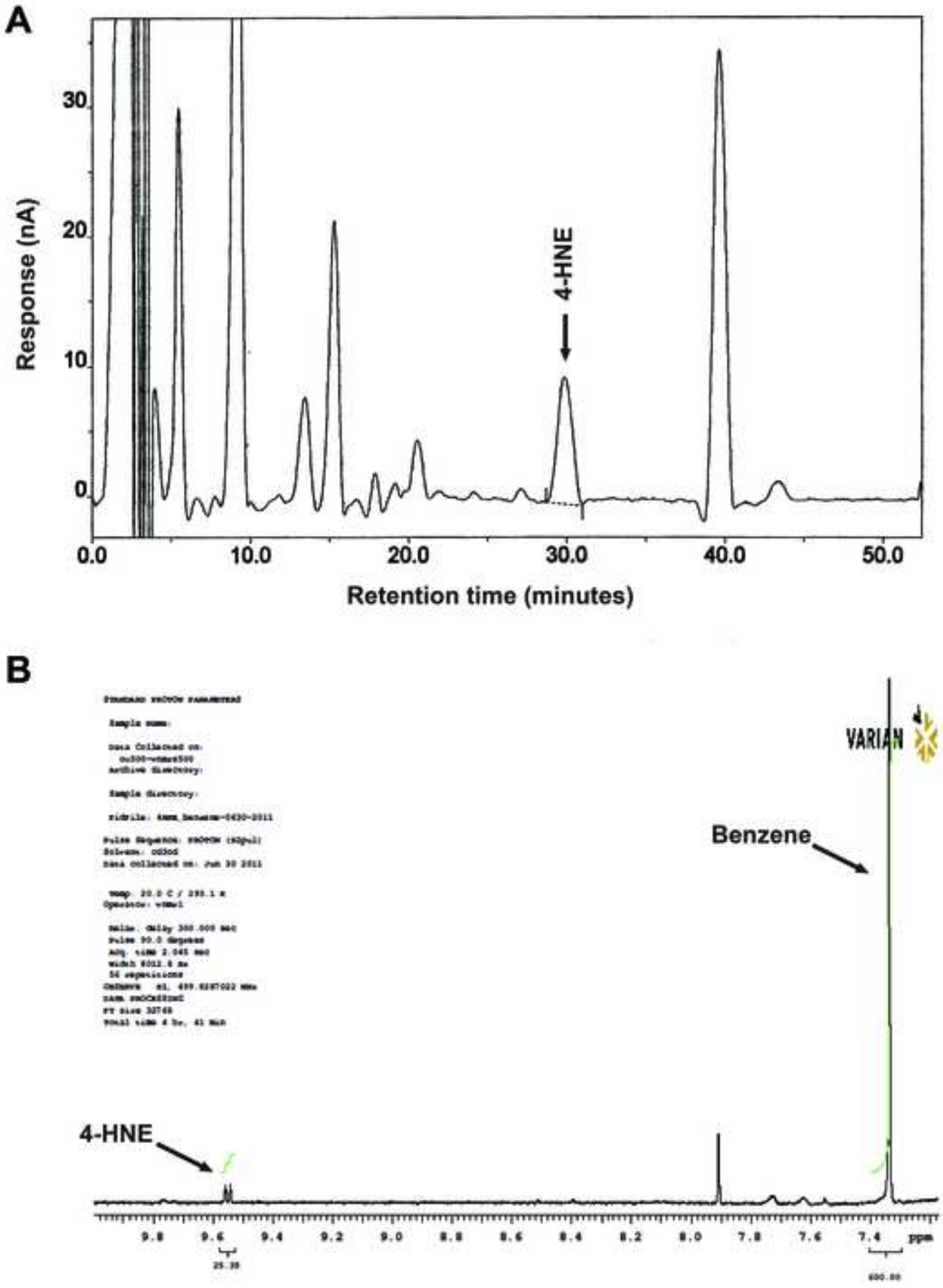

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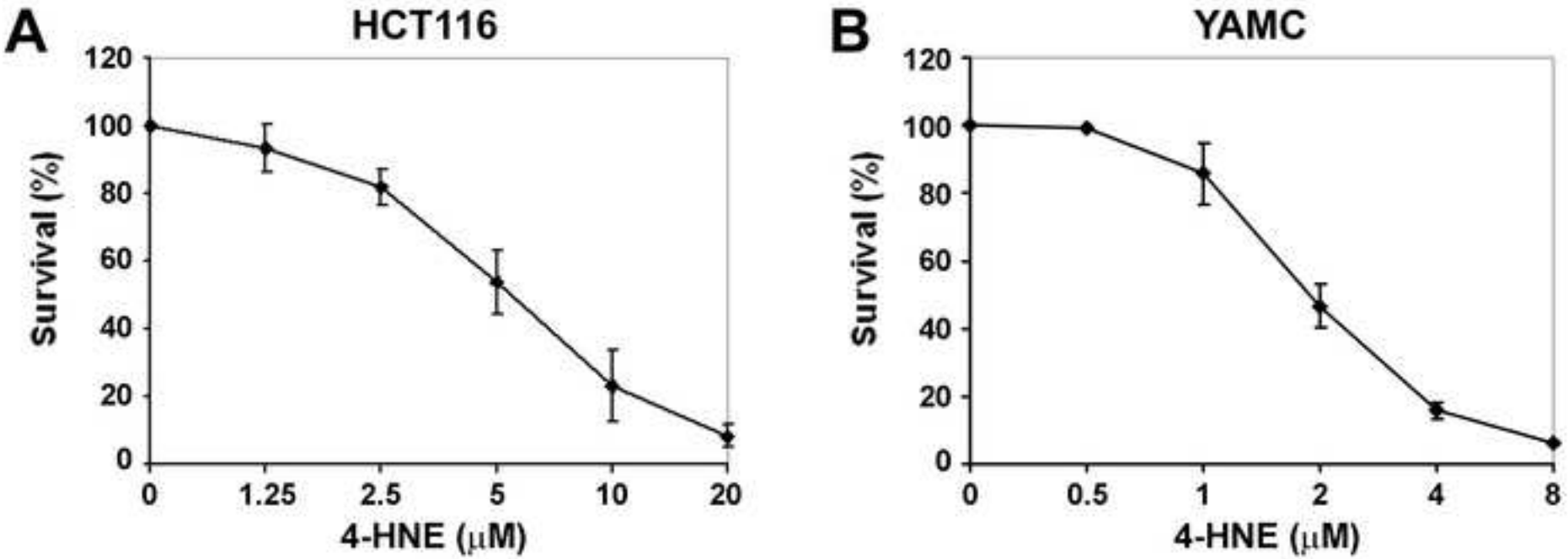

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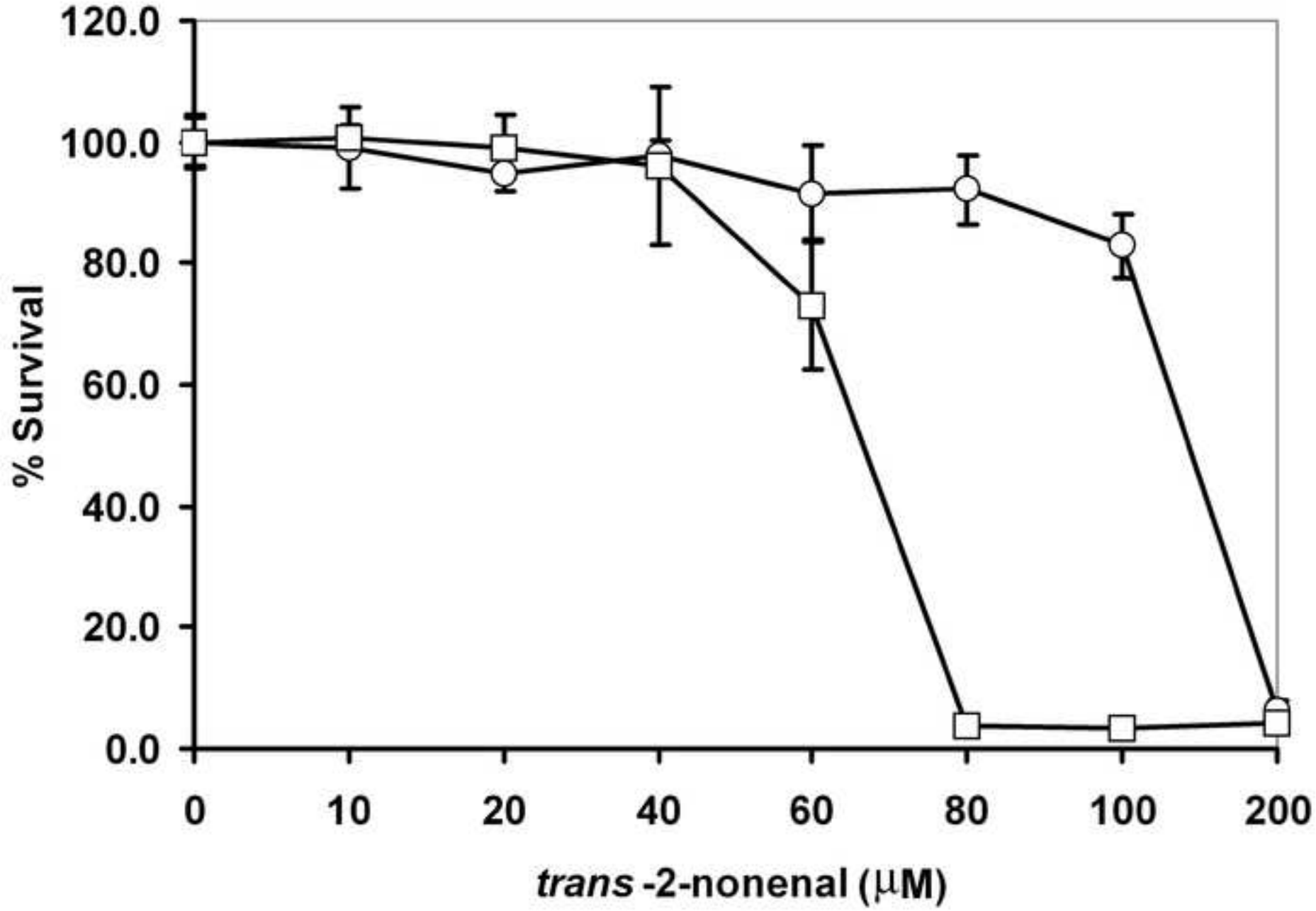

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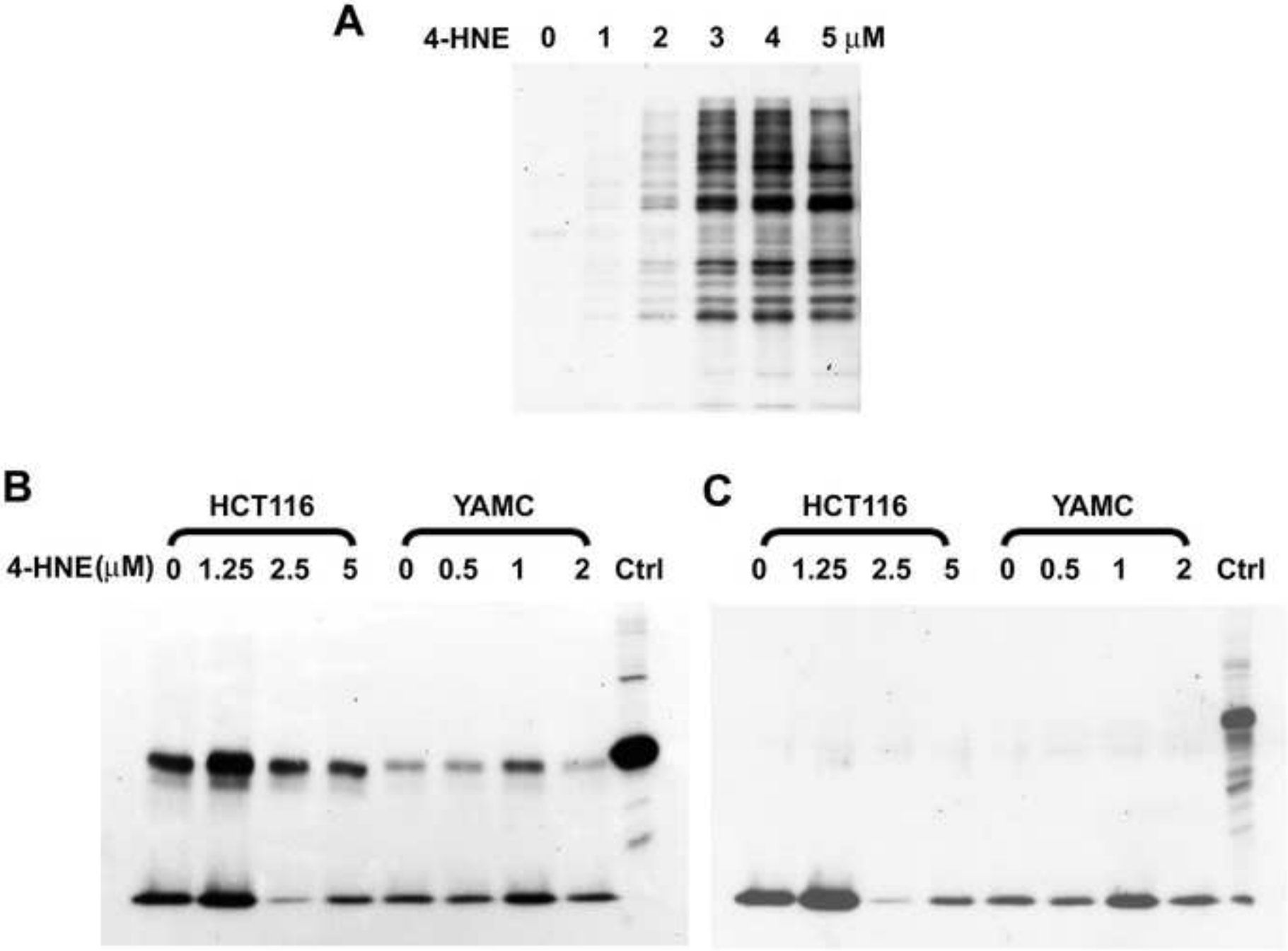

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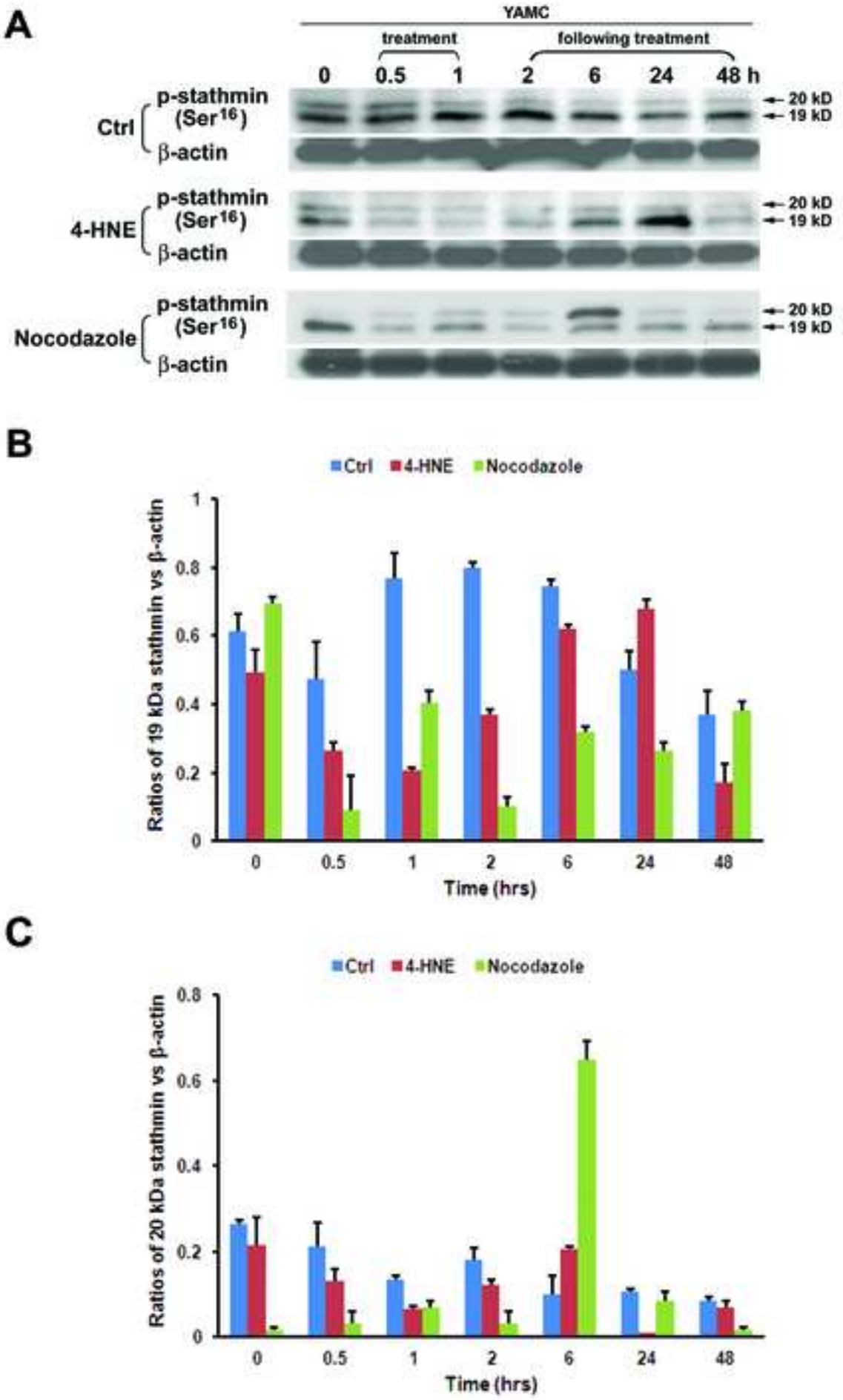

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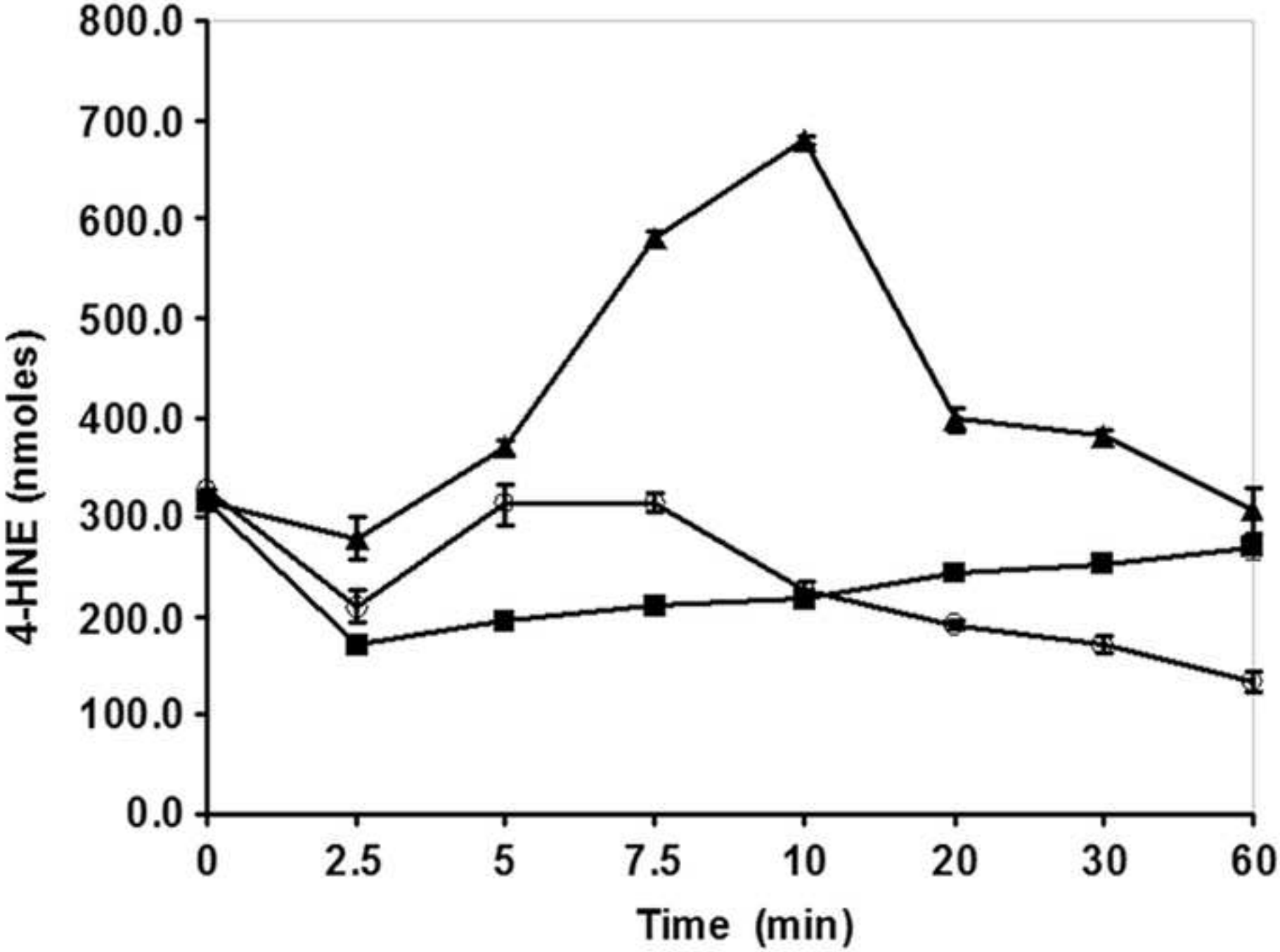

Figure S7

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