Inhibition of Eukaryotic Translation Elongation by Cycloheximide and Lactimidomycin

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Supplementary Methods

Cell Lines.

HeLa, HTB 1 and HEK 293T cells were grown in DMEM with 1 g glucose per liter and supplemented with 10% fetal bovine serum (FBS) at 37˚C and 5% CO_2. Jurkat, BT 474, HCC 1395, HCC 1937, HCC 2218 and MDA MB231 cells were grown in RPMI 1640 supplemented with 5% (MDA MB231) and 10% FBS respectively. MCF 10A were incubated in a 1:1 mixture of Ham's F-12 nutrient mixture and DMEM, supplemented with 5% equine serum, 1 μg/ml hydrocortisone, 5 μ/ml insulin and 100 ng/ml cholera toxin.

Cell Proliferation.

To test cell proliferation, 3000-5000 cells per well were plated in a 96-well plate and allowed to adhere overnight. Compounds dissolved in dimethyl sulfoxide (DMSO) at the indicated concentrations were then added and cells were incubated for a further 24 h. [^{3}H]-thymidine was added at 1 μCi per well and incubation was continued for an additional 7 h. Cells were washed twice with PBS and then trypsinized before they were collected with a Tomtec harvester and bound to GF/C filter mats. Thymidine uptake was then measured by scintillation counting.

Metabolic Labeling.

Cells were plated out as above. To measure RNA synthesis, cells were incubated with varying concentrations of compound in the presence of 1 μCi of [^{3}H]uridine for two hours and harvested as above.

In preparation for metabolic labeling of proteins, FBS was dialyzed against PBS for 24 h to reduce amino acid content. Cells in 96-well plates were washed twice with DMEM medium without cysteine and methionine and then incubated in the same medium for 20 min to deplete cellular amino acid pools. Methionine- and cysteine-free medium containing 10% dialyzed FBS was supplemented with [^{35}S]methionine and cysteine labeling pro mix (Perkin Elmer) to 15 µCi per well. Cells were incubated in presence of drug for 2 h before washing with 2x with cold PBS. An aliquot of 35 µl RIPA lysis buffer were added to each well and cells were lysed for 30 min at 4˚C. The plates with lysate were spun down at 1500 rpm in a tabletop centrifuge at 4˚C for 5 min. Millipore HV-HTS 96-well plates were washed with 100 µl PBS before application of 25 µl of 1 mg/ml bovine serum albumen carrier and 10 µl lysate. An aliquot of 150 µl of cold 10% trichloroacetic acid was added and reaction mixtures were gently shaken at 4˚C for 30 min for precipitation. The precipitate was collected by filtration under vacuum and washed 1x with each 100 µl of cold 10% TCA, 5% TCA and twice with 100% ethanol. Filter plates were then left to dry and counted by scintillation spectroscopy.

Yeast Growth.

Four isogenic pairs of yeast strains were grown in YPD medium. Saturated starter cultures were diluted out to 0.005 OD_{600} units and plated at 1 ml each into 24-well plates. Varying concentrations of CHX or LTM were added and the cells were incubated at 30˚C for 24 h. Growth was measured by optical density at 600 nm.

IRES dual luciferase reporter assay.

For IRES reporter assays, the reporter vectors were linearized with BamH1 and *in vitro* transcribed using the Promega Ribomax kit according to the manufacturer's instructions incorporating a ^{7}mGTP cap-analogue. Transcripts were isolated with the Qiagen RNeasy kit. The transcript (200 ng) was added to 25

µl rabbit reticulocyte lysate (RRL) *in vitro* translation reactions (Promega Flexi Rabbit Reticulocyte Lysate) and incubated at 30˚C for 30 min. Aliquots (5 µl) were assayed for expression of firefly and renilla luciferase according to Dyer et al[1]. Production of firefly and renilla luciferase was measured using a Wallac Microbeta luminometer.

Ribosome isolation.

Ribosomes were isolated from rabbit reticulocyte lysate similar to the methods utilized by Lorsch and Herschlag 1999[2]. For the peptidyl transfer assay, remaining mRNA was digested by incubation with micrococcal nuclease. $CaCl_2$ was added to 1 mM final concentration before 10 µg/ml of micrcocal nuclease was added. After a 20-min incubation at 20-25˚C, the reaction was quenched by addition of EGTA (3 mM). A total of 18 ml of lysate were centrifuged at 65,000 rpm in a TLA100.3 rotor at 4˚C for 2 h. The supernatant was discarded and the pellet resuspended in a high salt buffer (Buffer A: 20 mM HEPES-KOH pH 7.5, 100 mM KOAc, 2 mM $MgOAc_2$, 100 µM EDTA, 2 mM DTT. For high salt buffer, 400 mM KCl and 0.25M sucrose were added). Remaining debris was removed by centrifugation at 13,200 rpm and at 4˚C for 10 min. Each 1-ml aliquot of high salt resuspension was then applied to a 2 ml sucrose cushion (Buffer A plus 400 mM KCl, and 1M sucrose) and centrifuged in a TLA100.3 rotor at 85,000rpm and 4˚C for 2 h. For peptidyl transfer assays, the supernatant was saved and dialyzed against Buffer A and 10% glycerol. Aliquots of this "High Salt Wash" fraction were flash frozen and stored at -80˚C until use. For eEF1A- and eEF2-dependent assays and footprinting the micrococcal nuclease digestion was skipped and ribosomes were subjected to a second sucrose cushion before being resuspended in a low salt buffer (Buffer A with 0.25M Sucrose).

Polysome profiles.

In vivo polysome profiles were generated by growing 15-cm culture dishes of HEK 293T cells to confluency. Compound was added at the indicated concentrations and incubated with the cells for 30 min. Cells were lysed in 30 mM Tris-HCl, pH 7.4, 100 mM KCl, 5 mM Mg_2Cl, 2 mM DTT, and 1% Triton-X 100. After clearing the lysate of debris by centrifugation at 13,000 rpm in a TLA 100.3 rotor at 4˚C for 10 min, aliquots containing the same amount of RNA (measured by OD_{254}) were loaded onto 15-45% sucrose density gradients in 20 mM HEPES, pH 7.4, 100 mM KCl, 5 mM $MgCl_2$ and 2 mM DTT. Gradients were centrifuged at 40,000 rpm in a SW41Ti swinging bucket rotor for 90 min and then analyzed with an in-line spectrophotometer measuring optical density at 254 nm by displacing the gradient with Flurinert FC-40 solvent from the bottom of the tube.

For *in vitro* polysome profiles, β-globin RNA was reverse transcribed with T7 RNA polymerase in presence of 1 mM ATP, CTP, UTP, 0.5 mM GTP, 30µCi [α-^{32}P]-GTP and 2 mM ^{7}mGTP cap-analogue. 200 ng of radiolabed RNA was added to 25 µl RRL translation reactions supplemented with KCl, DTT and amino acids. After incubation at 30 ˚C for 15 min, samples were diluted to a total volume of 200 µl in a gradient buffer (20 mM HEPES, pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, and 2 mM DTT) and centrifuged through 15-35% sucrose gradients using an Sw55Ti rotor at 50,000rpm at 4 ˚C. 200 µl-fractions were collected by hand and radioactivity determined by scintillation spectroscopy.

Toeprinting.

Toeprinting was performed based on the method described by Anthony and Merrick[3]. Rabbit beta globin pβ-Hb plasmid DNA was digested with Hind III and transcribed as above. Briefly, a primer complimentary to the region 60 nucleotides 3' of the A of the AUG start codon (sequence 5'-TCACCACCAACTTCTTCCAC-3') of rabbit β-globin RNA was labeled with T4 polynucleotide kinase (NEB) according to the manufacturer's instructions in 20 µl reactions and purified using a sepharose G-50 spin column. An

aliquot of 6 µCi of labeled primer were hybridized to 4 µg of β-globin RNA in a 40 µl reaction buffered (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT) by heating to 95˚C for 2 min and then allowing the reaction to cool down to room temperature before placing it on ice. RRL (50 µl) *in vitro* translation reactions were set up according to the manufacturer's instructions but without mRNA. Reactions were pre-incubated with either 1 mM GDPNP, 2 mM CHX or 200 µM LTM for 5 min at room temperature before addition of 5 µl RNA:primer hybrid and further incubation at 30˚C for 5 min. Each 50µl reaction was brought up in volume by addition of 150 µl gradient buffer (20 mM HEPES, pH 7.4, 150 mM KOAc, 5 mM $MgCl_2$, and 2 mM DTT). The entire reaction was loaded onto a 10-35% sucrose gradient and spun for 1.5 h at 4˚C and 50,000 rpm in a SW55Ti swinging bucket rotor.

Fractions (200 µl) were collected by hand from the top of the tube and 20-µl aliquots of each fraction were scintillation counted to detect the 80S fraction for CHX and LTM or the 48S fraction for GDPNP. The fractions of interest received 1 mM dNTPs and 25 U avian myoblastoma virus (AMV) reverse transcriptase and were incubated at 37˚C for 30min. The transcript was isolated by extraction with buffer-saturated phenol and chloroform followed by ethanol precipitation. Pellets were redissolved in 8 µl formamide loading buffer (90% formamide, 10% 10xTBE buffer, xylene cyanol and bromophenol blue) and resolved on a 8% polyacrylamide gel containing 6M Urea. The gel was dried and exposed to a phosphoImager screen or X-ray film.

Polyphenylalanine Synthesis.

Polyphenylalanine synthesis was measured by the method developed by Merrick 1979[4]. Reaction mixtures (50 µl) containing 8 µg poly(U) RNA and 0.4 OD_{260} of ribosomes, 2 µg eEF1A, 0.5 µg eEF2, 10 pmol [^{14}C]Phe $tRNA^{Phe}$ in 30 mM Tris-HCl, pH 7.4, 100 mM KCl, 10 mM $MgCl_2$, 1mM DTT, 21 mM phosphoenol pyruvate and 0.3 U pyruvate kinase were incubated for 2 min at room temperature.

Reactions were quenched with 1 ml cold 10% trichloroacetic acid (TCA) and heated to 90˚C for 15 min. Boiled samples were passed through a nitrocellulose filter and washed 3x with 5% TCA before drying and scintillation counting.

Reconstituted translation elongation assays

These assays were carried out as described previously with the following modifications[30]. Purified initiation complexes were incubated with LTM or CHX (dissolved in DMSO) for three minutes before elongation factors were added. The final concentration of each drug was 200 µM. Control reactions included an equal volume of DMSO without any drug.

Mouse Xenograft Model.

2 X 10^6 MDA MB231 cells were embedded in matrigel and injected subcutaneously into the abdominal mammary fat pad of 6-8 week old female nu/nu mice (4 mice per group). With the detection of palpable tumors treatment commenced and mice received daily intraperitoneal injections of 0.6 mg/kg LTM in 5% DMSO in sterile 0.9% saline. Treatment continued for a month. Tumor volume was calculated from measured length and width every second day using the equation: volume=$\pi/6$*length*width^2.

Supplementary Results

Supplementary Table 1. IC_{50} values of different inhibitors on protein synthesis and RNA synthesis *in vivo*.

IC_{50} concentration (nM)		
Compound	**Protein**	**RNA**
Cycloheximide	532.5	2880.1
Actinomycin D	519.7	262.4
Lactimidomycin	37.82	>10,000
Isomigrastatin	624	>10,000
2H Lactimidomycin K1	98.47	>10,000
Didehydro Isomigrastatin	1909	>10,000

Supplementary Table 2. Cross-resistance of cyh2 Yeast Mutants to Cycloheximide and Lactimidomycin

Strain	Genotype	Cycloheximide		Lactimidomycin	
		IC_{50} (µM)	Fold resistance	IC_{50} (µM)	Fold resistance
J47α	*His3-01, Trp1-289*	0.088	**232.2**	0.0046	**241**
J407 (cyh2)		20.54		1.108	
JB503	*MATα ura3-52 lys2 his3D200 trp1D1 GAL+*	0.032	**232.4**	0.058	**10.3**
BY686 (cyh2)		7.42		0.596	
BY108	*MATα ura3-167 his3D200 leu2D1 trp1D1 GAL+*	0.675	**77**	0.488	**13.4**
BY724 (cyh2)		52.07		6.533	
JB281	*Mata leu2-3, 112 his4-519, ade1-100, ura3-52 GAL+*	0.2273	**76.3**	0.059	**20.5**
BY740 (cyh2)		17.34		1.209	

Supplementary Figure Legends

Supplementary Figure 1. Inhibition of cell proliferation by LTM, isomigrastatin and analogs. a. Structures of glutarimides used in this study. **b.** Anti-proliferative effects of glutarimides on HeLa cells. [^{3}H]thymidine uptake was measured after 24 h of incubation with 2.5 μM of each compound. **c.** Effects of glutarimides on translation. Compounds are graphed in same order as in **b**. Translation activity was measured by scintillation counting of TCA precipitated cellular protein on a PVDF membrane after a 2-h incubation with 2.5 μM of each compound in presence of [^{35}S]methionine and cysteine. Error bars denote standard deviation. **d.** The anti-proliferative activity of the glutarimide analogs on three tumor cell lines. Cells were treated as in Figure 1b, but in addition to HeLa cells the experiment was repeated with MDA MB231 breast cancer and Jurkat leukemia T-cell lines. **e.** The potency of protein synthesis inhibition mirrors cytotoxicity. Jurkat T-cells were treated with varying amounts of translation inhibitors before measuring their proliferative capabilities by [^{3}H]-thymidine incorporation.

Supplementary Figure 2. Ethoxycarbonylmethyl -LTM retains inhibitory activity on protein translation. **a.** Ethoxycarbonylmethyl conjugate of LTM (Ac-LTM). **b.** Effects of LTM and Ac-LTM on translation *in vitro*.

Supplementary Figure 3. Polysome profiles of cycloheximide (CHX) and LTM and their combination. Polysome profile of **a.** 50 μM Cycloheximide + 50 μM LTM (added together); **b.** 50 μM Cycloheximide prior to addition of LTM and **c.** 50 μM Cycloheximide + 1 μM LTM (added after cycloheximide.

Supplementary Figure 4. Titration of CHX and LTM. The toeprinting assay was repeated at three doses for each CHX and LTM. At the exceedingly high concentration of 10mM CHX arrests a larger fraction of ribosomes on the start codon. Yet even at low concentrations LTM efficiently prevents the ribosome from leaving the start codon.

Supplementary Figure 5. Effects of LTM on eEF1A-mediated tRNA binding and peptidyl transfer. a. LTM did not inhibit eEF1A-mediated tRNA binding. Ribosomes were incubated with eEF1A ternary complexes in presence of each inhibitor. tRNA binding was determined by filter binding. **b.** LTM did not inhibit peptidyl transfer. [^{35}S]-Met-tRNA$_i^{Met}$ charged initiation complexes were incubated with the indicated compound and a high-salt washed fraction containing initiation and elongation factors before addition of puromycin. Methionyl–puromycin formation was monitored on thin layer chromatography over time. Sparsomycin served as a positive control.

Supplementary Figure 6. Positions of primers used in footpring analysis in the ribosome.

a. Primer Summary. The figure shows the secondary structure of mouse 28S rRNA. Green sequences highlight positions against which primers were synthesized and their identification number (based on the mouse primers. Crossed out numbers indicate primers not hybridizing to rabbit rRNA. **b.** Visualization of primer coverage on a eukaryotic ribosome model[5], assuming a range of ~120 nucleotides upstream from primer site. All rRNA strands covered are colored in blue on the canine ribosome hybrid model. This model was displayed with the help of the UCSF Chimera software package [6].

Supplementary Figure 7. Selective inhibition of cancer cell proliferation in vitro and breast cancer xenograft *in vivo*.

a. LTM displays selectivity for transformed cell lines. Seven breast cancer cell lines and the non-tumorigenic cell line MCF 10A were treated with varying doses of LTM for 24 h and cell proliferation was determined by incorporation of [^{3}H]thymidine for an addititional 6-8 h. IC_{50} concentrations noted below. **b.** LTM inhibited tumor growth in nude mice. $2 \cdot 10^6$ MDA MB 231 cells were injected subcutaneously into female nude mice. Animals received a daily dose of 0.6 mg/kg LTM or solvent (5% DMSO in saline) alone. Error bars denote standard error.

Supplementary Figure 8. Model of the CHX and LTM binding site. The residues of ribosomal proteins L27a and L36a implicated in CHX resistance lie in close proximity to both the 3'-end of the de-acylated E-site tRNA and the cytidine residue protected by LTM and CHX. This structure is based on the previous ribosomal model[5,6]. Note that the terminal Adenosine residue is not modeled.

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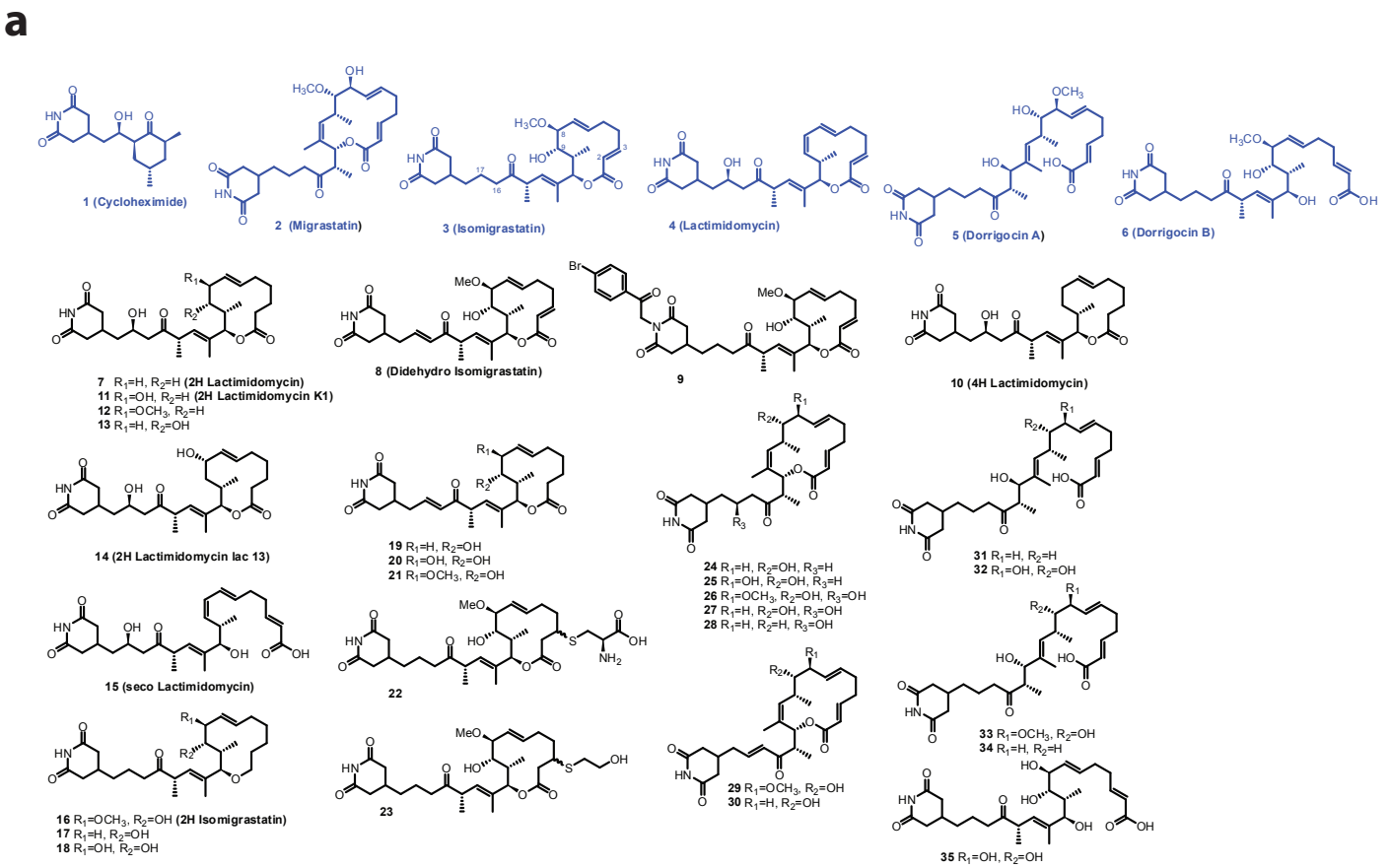
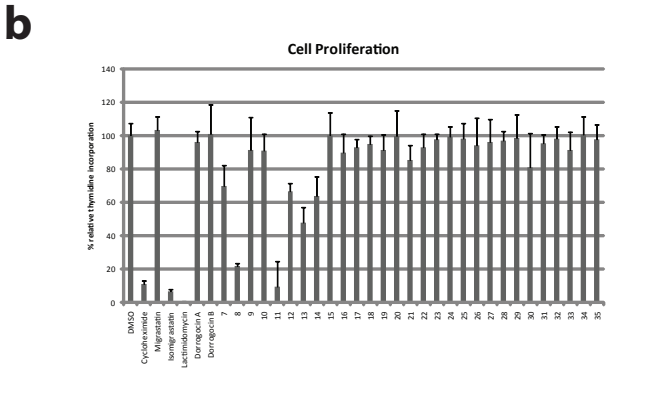
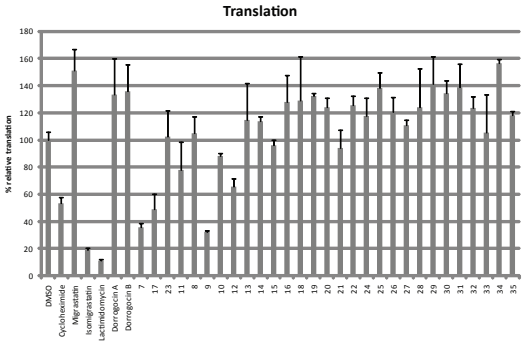
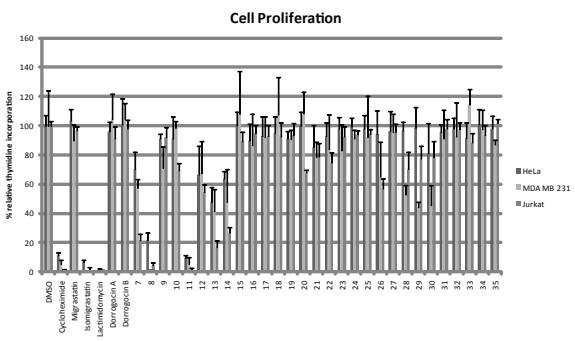
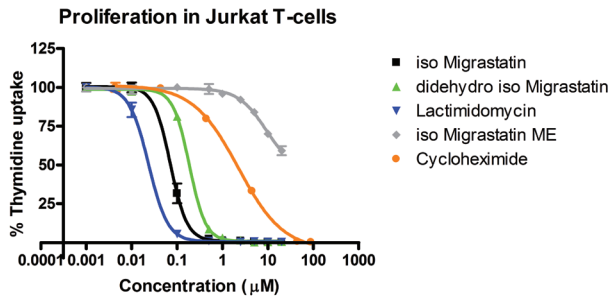
Supplementary Figure 1:





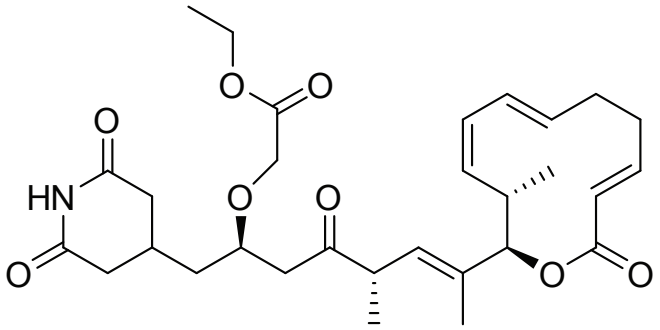






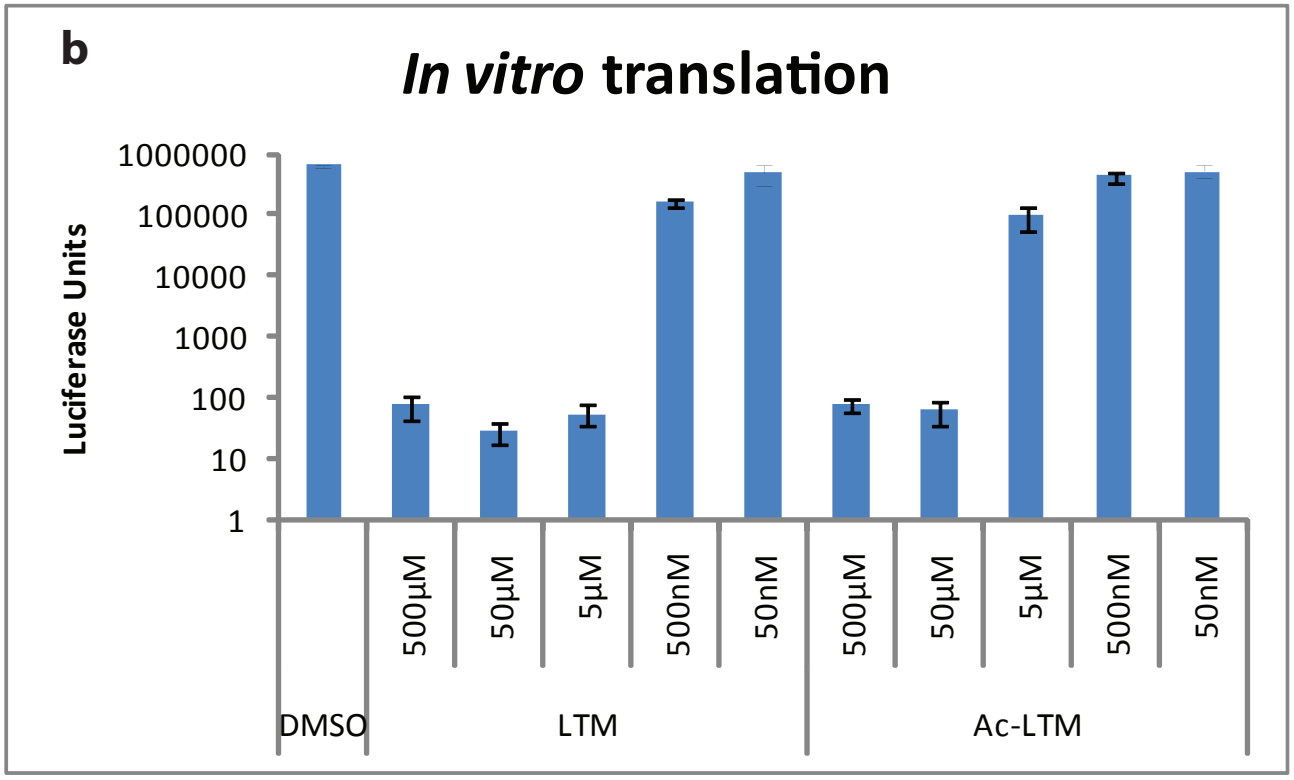
Supplementary Figure 2

a



Ethylacetyl-Lactimidomycin (Ac-LTM, **36**)

b



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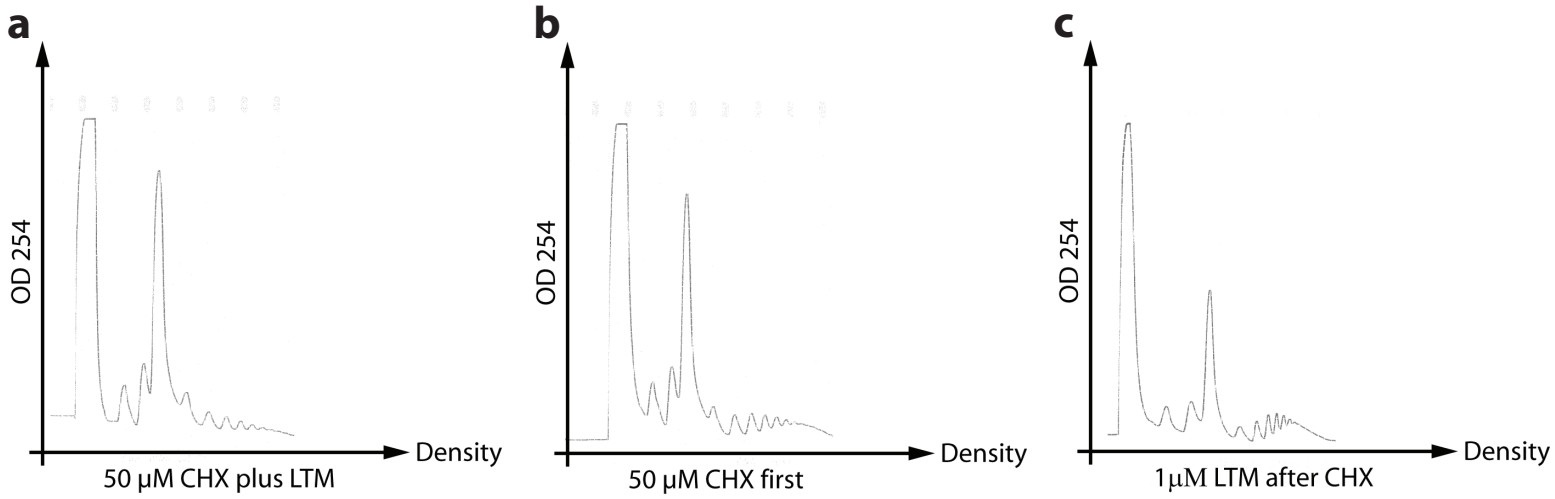

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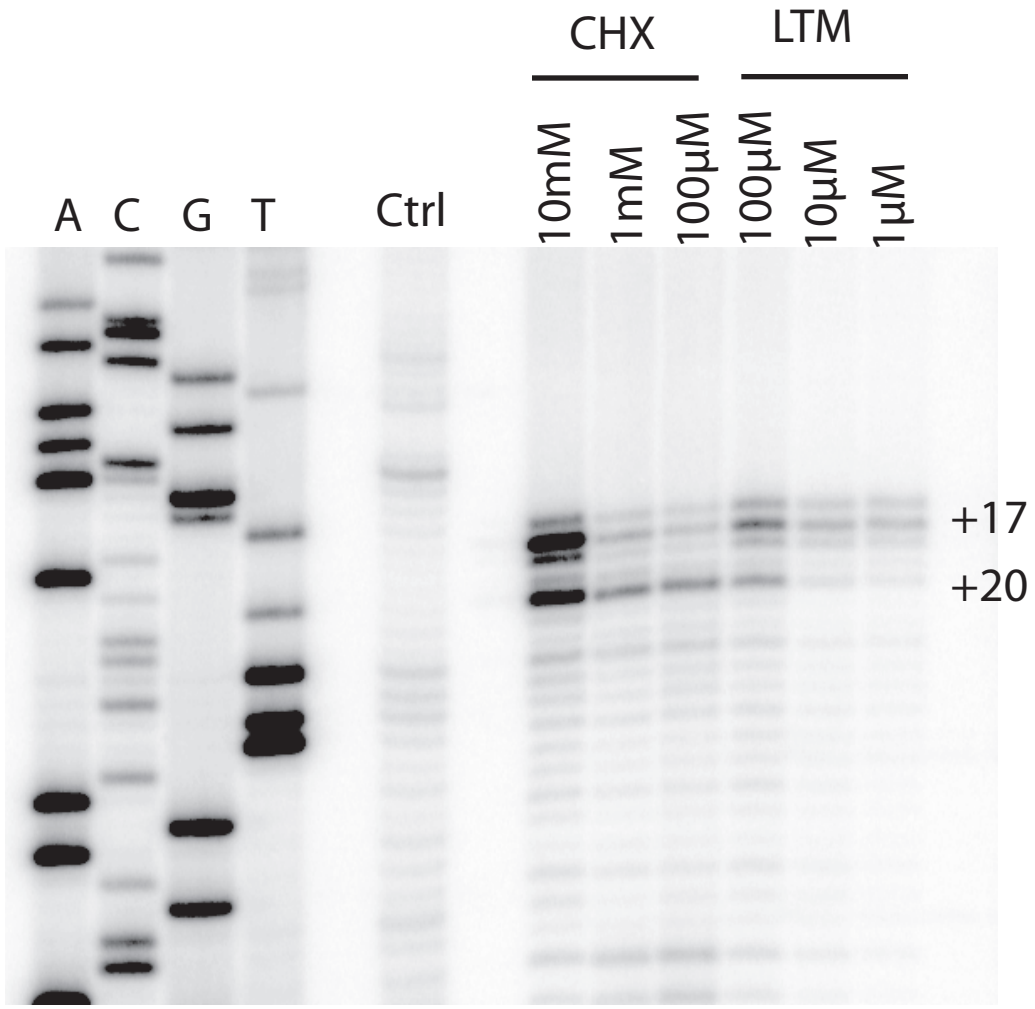
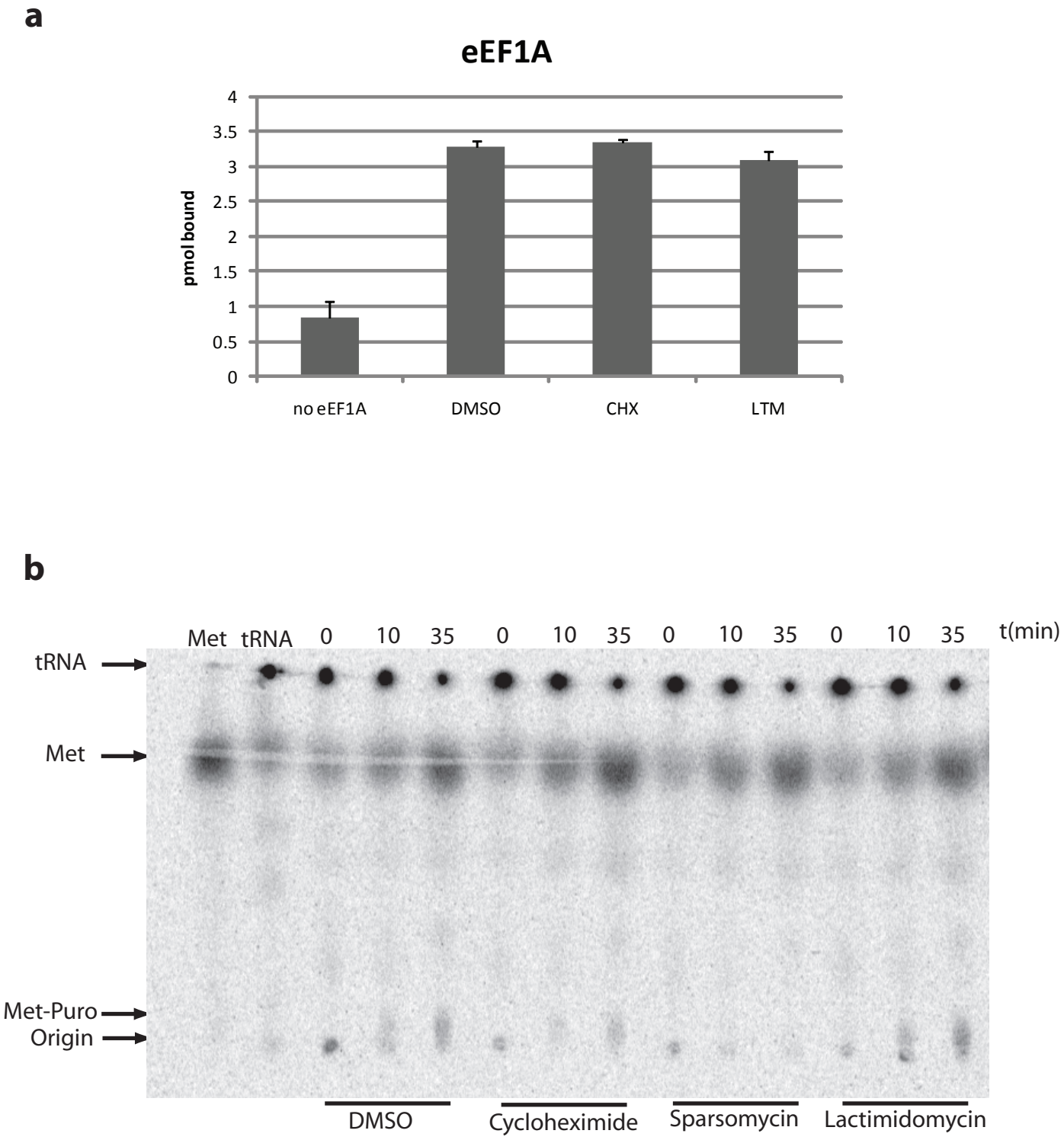
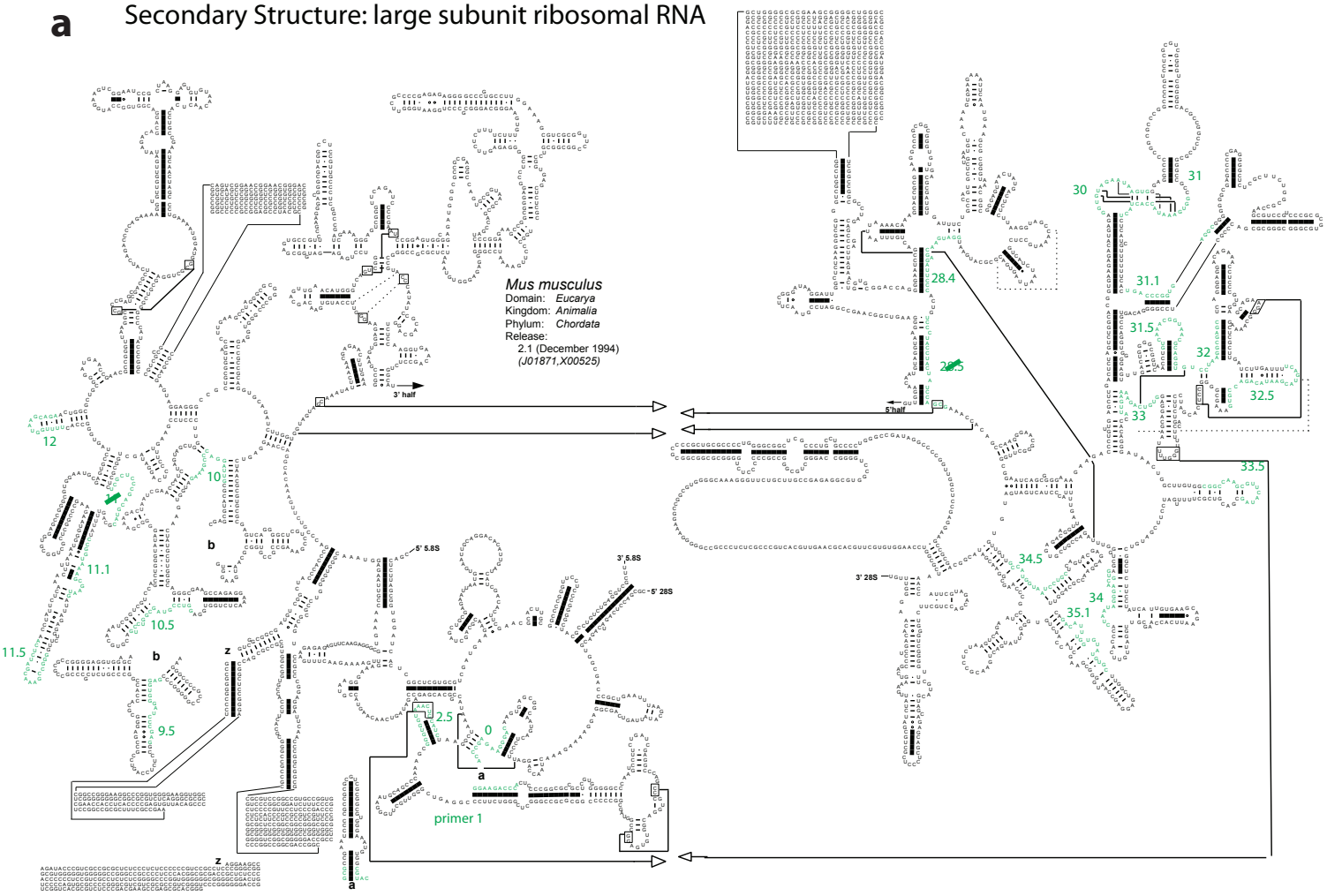


Supplementary Figure 5



Supplemental Figure 6

a Secondary Structure: large subunit ribosomal RNA



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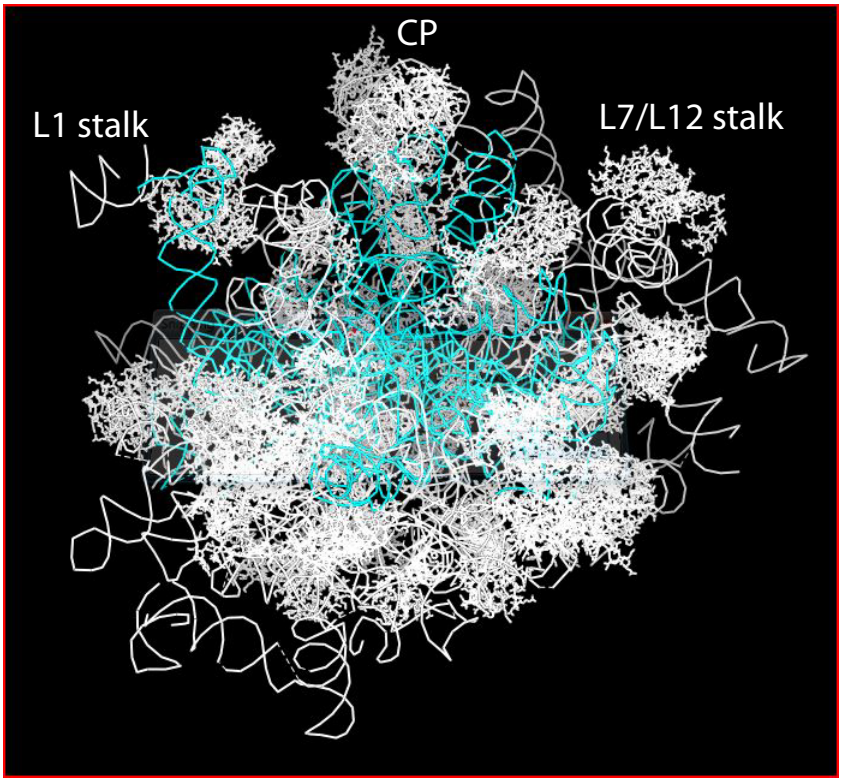
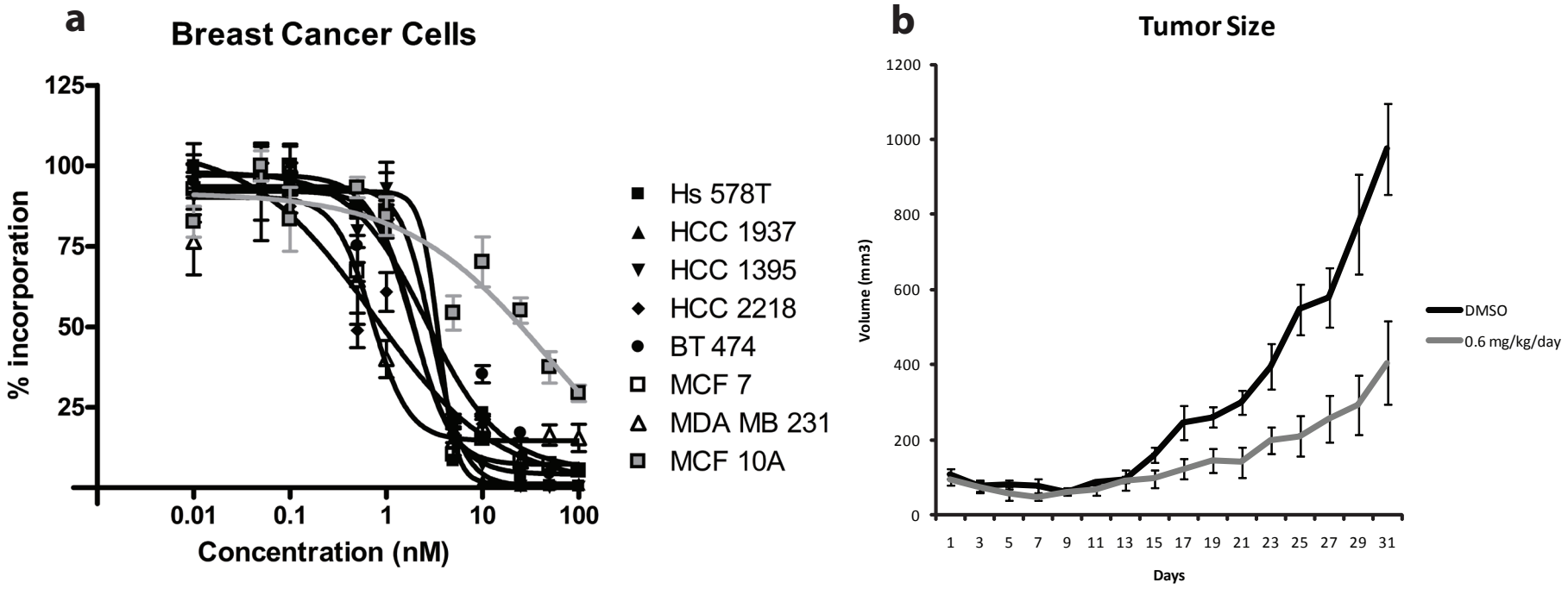


Supplementary Figure 7



Supplementary Figure 8: