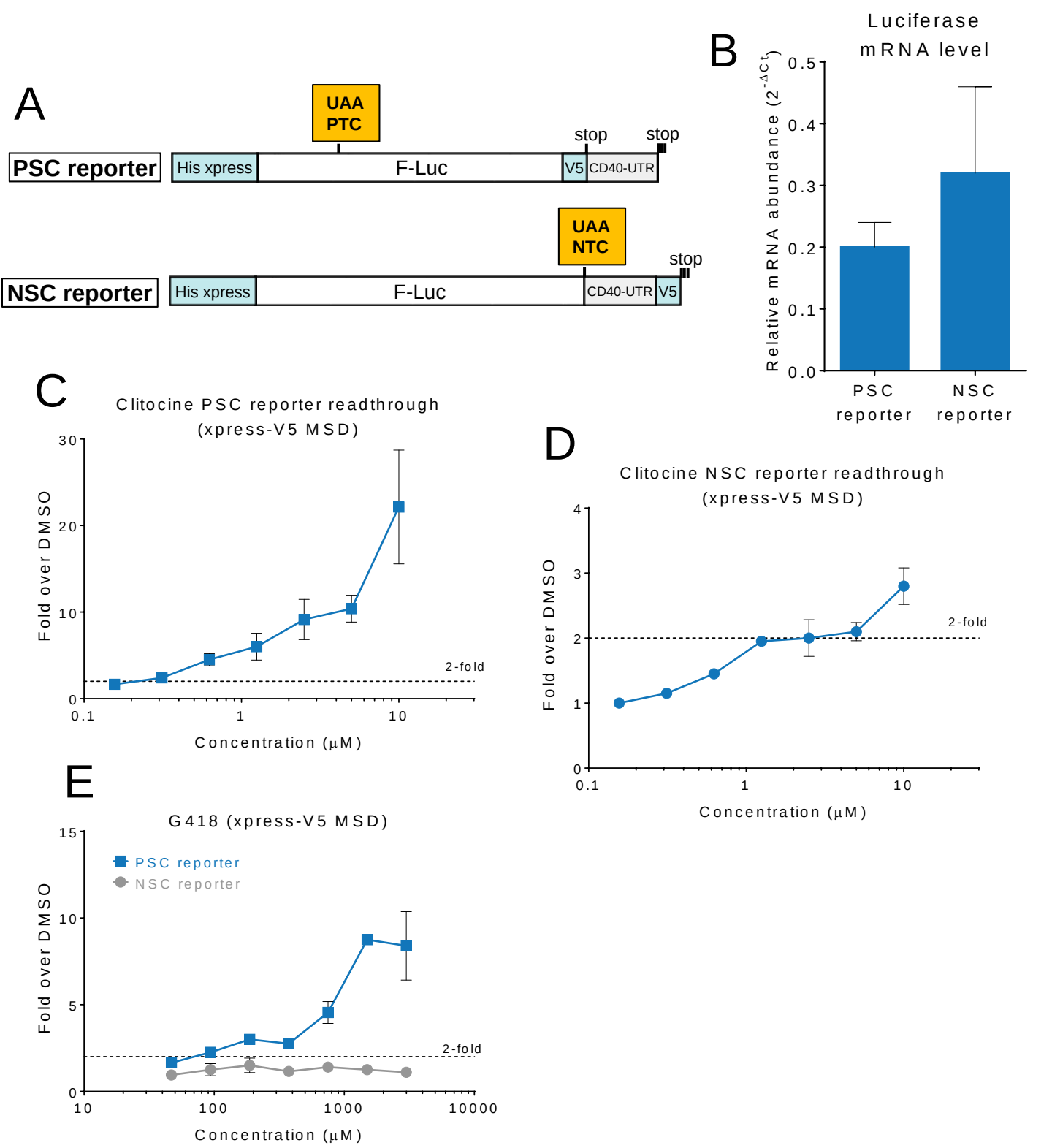
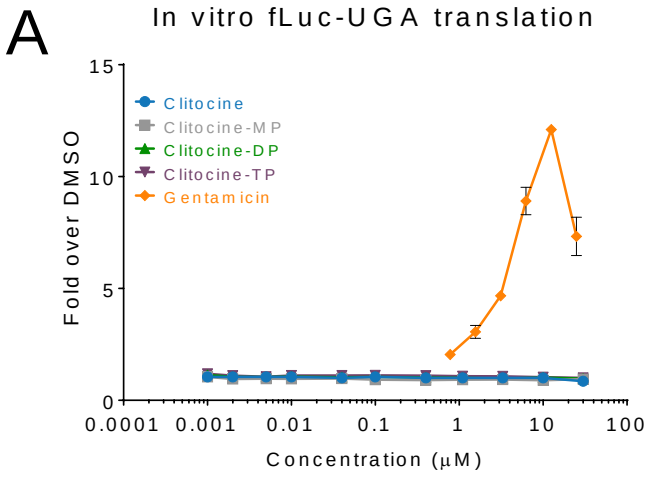
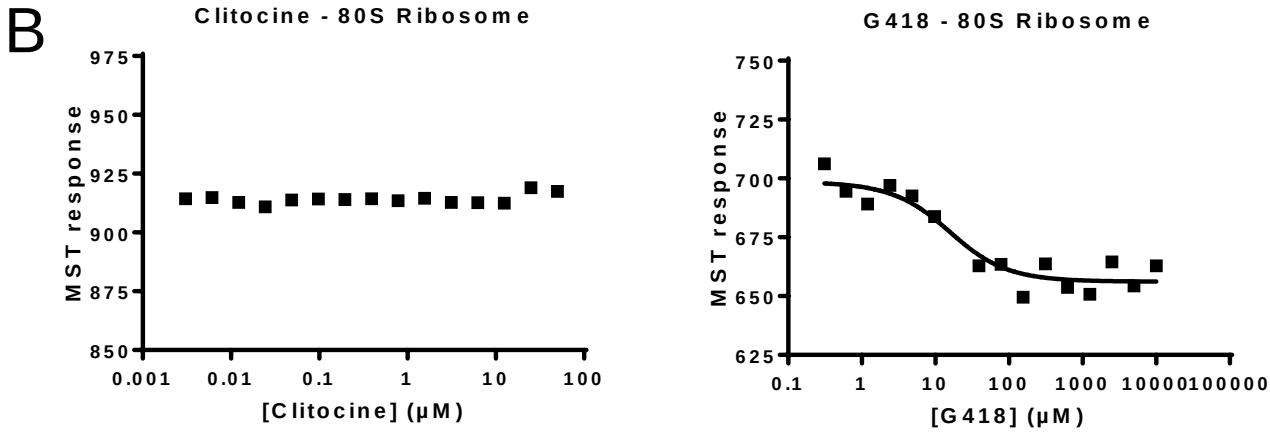
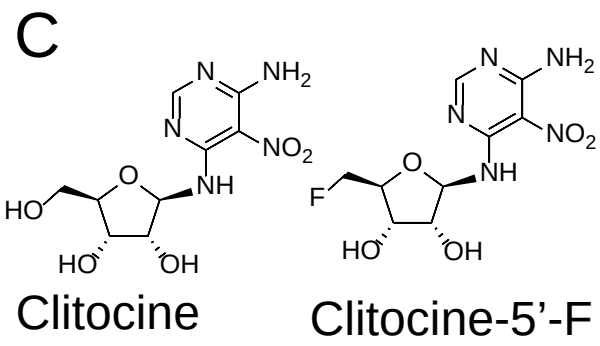


Figure S1: Clitocine preferentially induces readthrough of premature stop codons over normal stop codons (**A**) Schematic showing reporter constructs with a premature stop codon (PSC) (top) and a normal stop codon (NSC) (bottom). Antibody tags are indicated in blue (His, xpress and V5 tags) (**B**) mRNA expression levels of premature stop codon and normal stop codon reporters from stable cell lines as determined by RT-QPCR. (**C**) Clitocine readthrough of the premature stop codon reporter detected by xpress-V5 tag sandwich MSD ELISA. (**D**) Clitocine readthrough of the normal stop codon reporter detected by xpress-V5 tag sandwich MSD ELISA. (**E**) G418 readthrough of premature stop codon and normal stop codon reporters detected by xpress-V5 tag sandwich MSD ELISA.







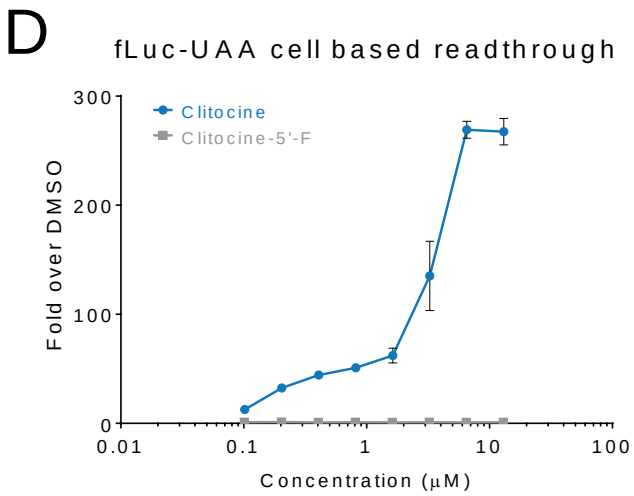


Figure S2: Clitocine is not active in in vitro readthrough, does not bind the ribosome and is not active in vivo without a 5' hydroxyl. (**A**) Clitocine and clitocine-5' phosphates (MP, monophosphate; DP, diphosphate; and TP, triphosphate) are not active when added exogenously to in vitro RRL translation reaction of the Luc-190-UGA mRNA. Gentamicin is included as a positive control. Y-axis, luciferase activity. X-axis, concentration of compounds. (**B**) Representative microscale thermophoresis (MST) measurement showing that clitocine does not bind to purified HeLa 80S ribosome. A Dissociation constant (K_D) of 5.6 µM +/- 3.2 was determined for G418-80S ribosome binding (control) based on three independent experiments. (**C**) Structure of clitocine (left) and an analog with the 5' OH substituted with fluorine (6-amino-5-nitro-4-(5-deoxy-5-fluoro-β-D-ribofuranosylamino)-pyrimidine) (right). (**D**) Substituting the 5' OH of clitocine with fluorine eliminates readthrough activity in the cell based Luc-190-UAA readthrough reporter assay. Y-axis,

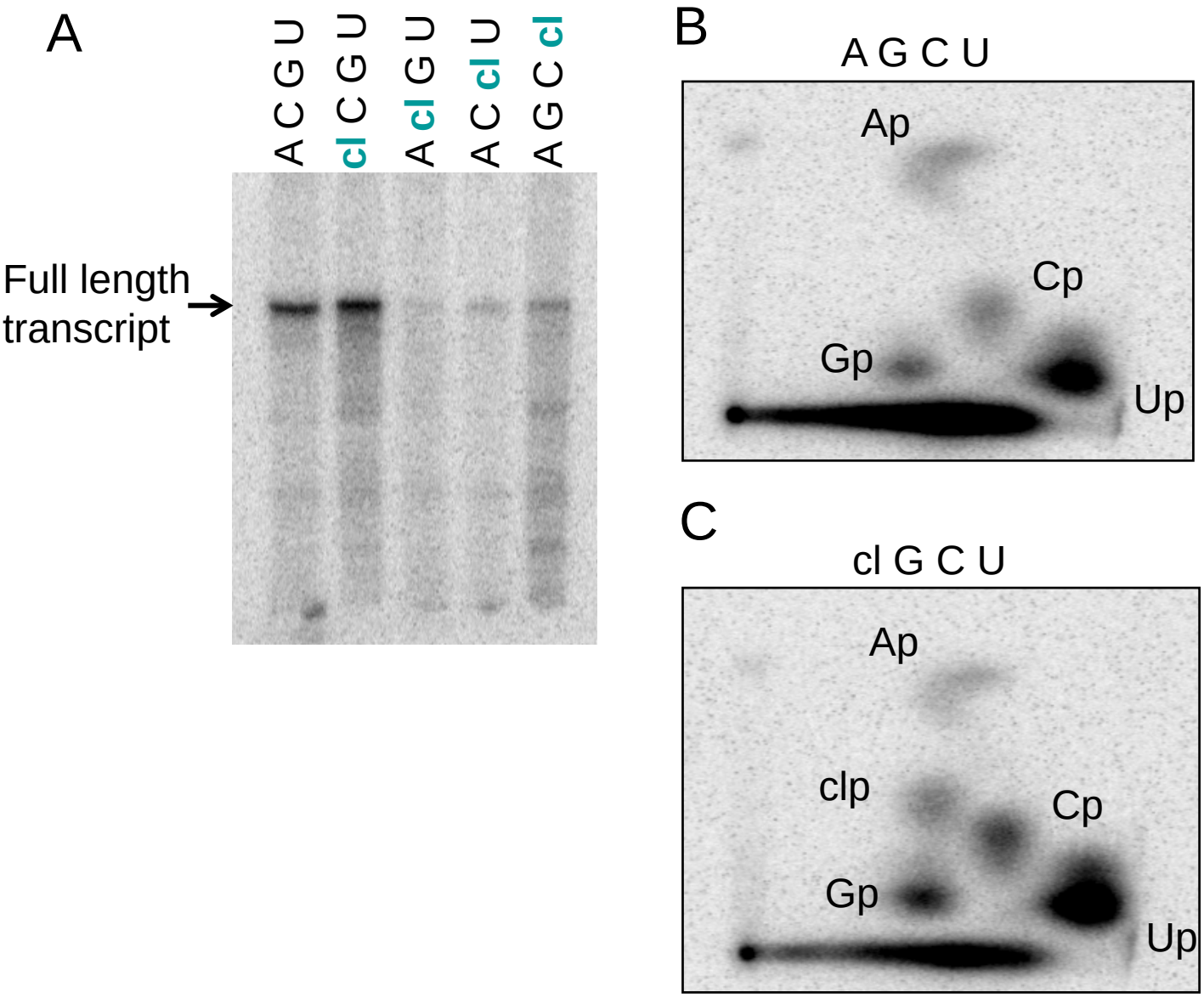


Figure S3: Clitocine is incorporated into RNA by mammalian RNA polymerase II in vitro. (**A**) Phosphor Imager analysis showing that RNA polymerase II from HeLa Scribe™ nuclear extract transcription of a pre-tRNA^{Tyr} cDNA labeled with UTP-α-^{32}P produces full length transcripts when all four cognate nucleotide triphosphates are present or when ATP is substituted with clitocine-triphosphate. (**B** and **C**) Phosphor Imager analysis of 2D TLC of T2 RNase digested products from **A** showing that clitocine is present in the in vitro transcribed RNAs. (**B**) TLC of nucleotides from RNA produced with the four canonical triphosphates. (**C**) Nucleotides from RNA produced with clitocine-TP without ATP. TLC spots corresponding to the monophosphate nucleotides, Ap (adenosine-3'-phosphate), Cp (cytosine-3'-phosphate), Gp (guanidine-3'-phosphate), Up (uridine-3'-phosphate) and clp (clitocine-3'-phosphate), are indicated. The presence of full length product in panel A (lanes 3-5) and clitocine-3'-phosphate in panel C are likely due to the presence of ATP in the HeLa Scribe™ nuclear extract.

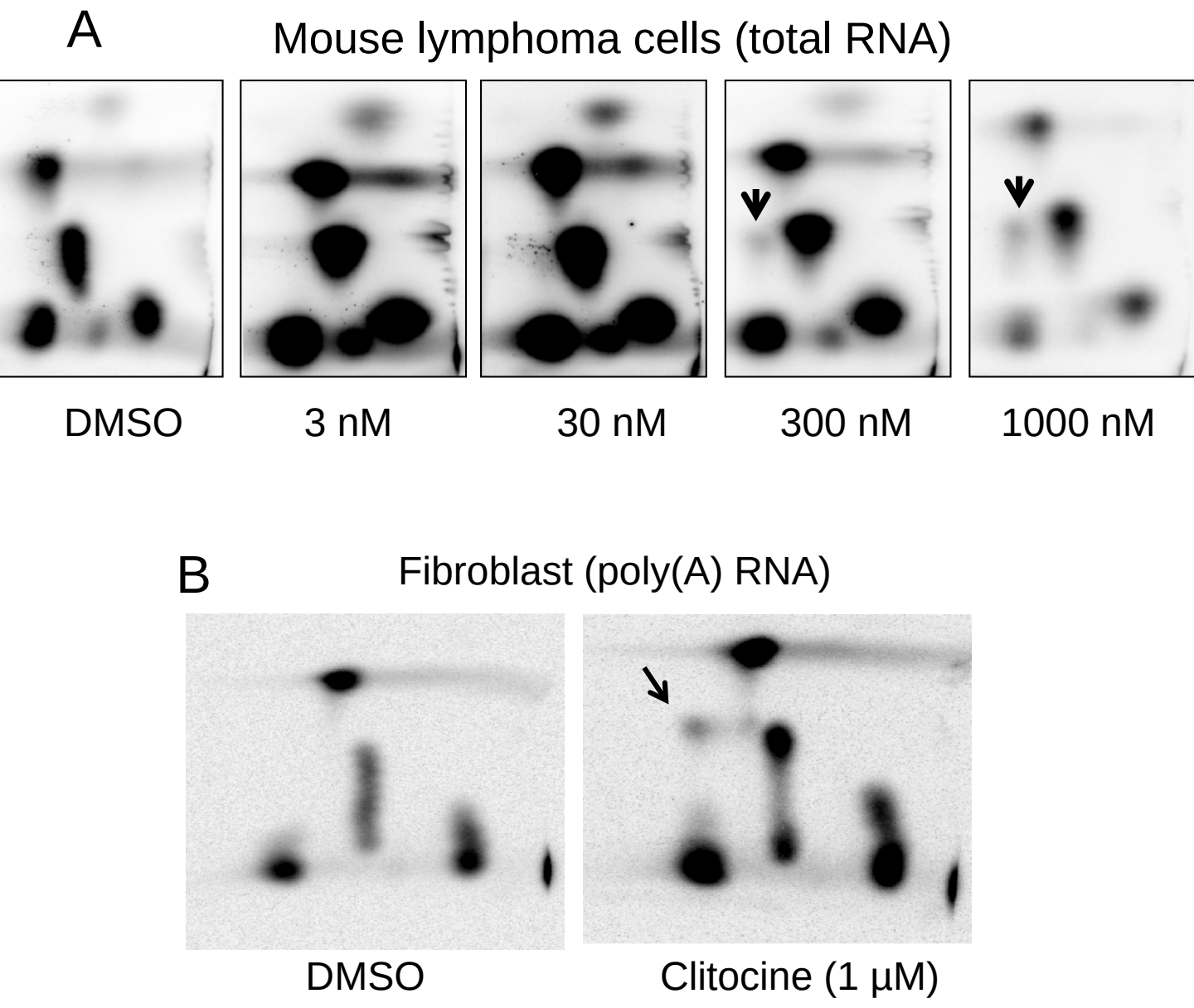


Figure S4: Clitocine is incorporated into RNA in cells. (**A**) Mouse lymphoma cells or (**B**) primary fibroblasts were labeled with ortho [^{32}P]phosphate and incubated with the indicated concentration of clitocine. After 48 hours, total RNA or poly(A) RNA was isolated and digested with nuclease P1. Nucleotides were purified, separated by 2D TLC and visualized by Phosphor Imager analysis. The arrows indicate clitocine-3'-phosphate. The separation pattern of the 3'-phosphate nucleotides is the same as in **Figure 3**.

A

…F Y P L E D G T A G…
…ttc tac cca ctc gaa gac ggg acc gcc ggc…

…S G S T G L…
…agt ggc agt acc gga ttg…

B

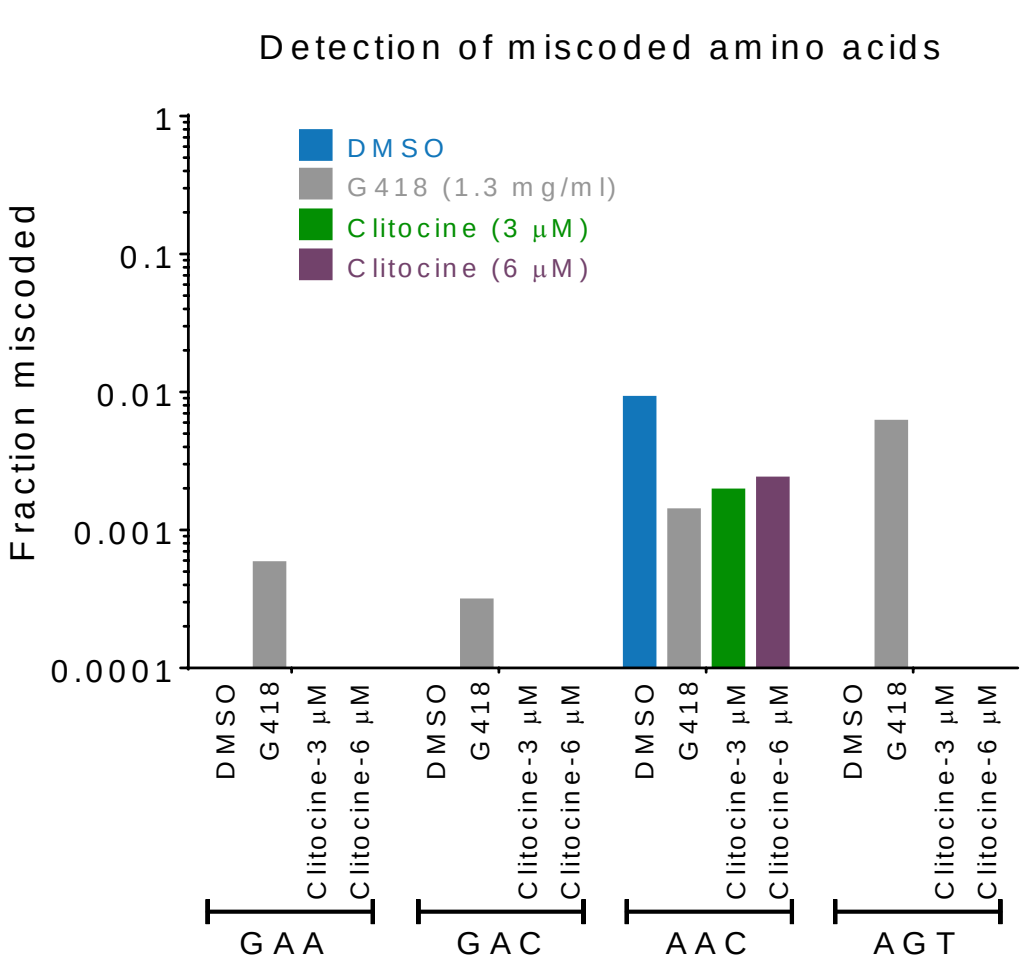
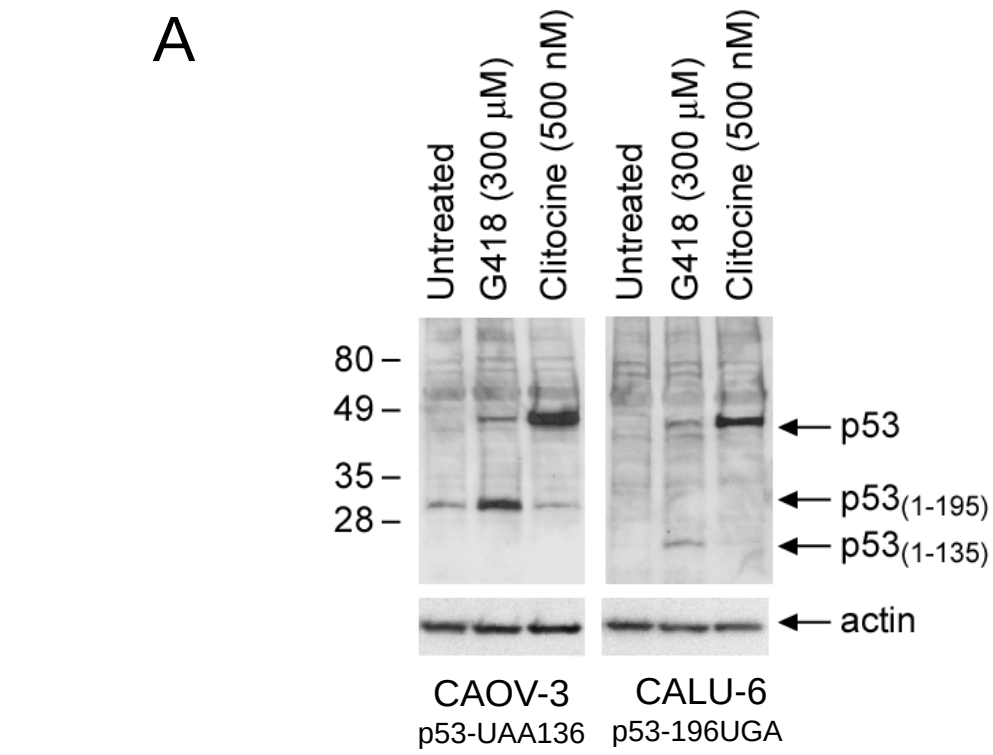
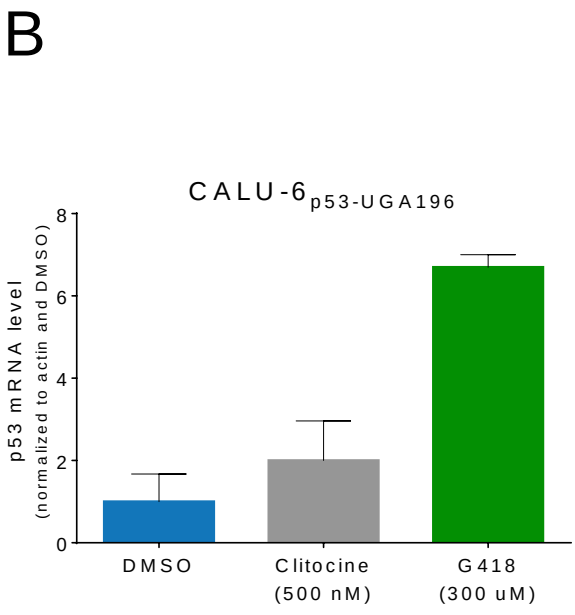


Figure S5: Clitocine does not affect sense codon decoding. (**A**) Context of luciferase codons and amino acids analyzed in B. Codons in red correspond to codon positions analyzed in B. (**B**) Graph showing the fraction of miscoding at different sense codons after treatment with DMSO (negative control), 1.3 mg/ml G418 (positive control) and 3 and 6 µM clitocine. HEK293 cells stably expressing wild type luciferase were treated as indicated and luciferase protein was purified and analyzed by LC-MS/MS as previously described {Roy, 2016 457 /id}.





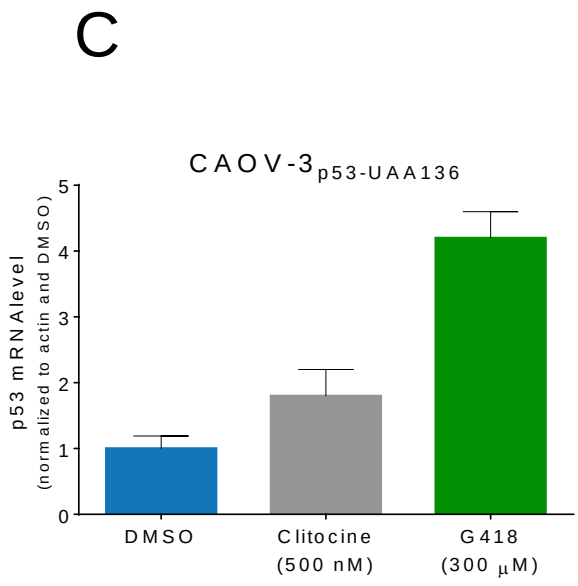


Figure S6: Clitocine readthrough has a minimal effect on NMD (**A**) p53 western blot (amino-terminal antibody) of extract from cells treated with G418 and clitocine. (**B** and **C**) p53 mRNA level (RT-qPCR) of extracts from cells treated with clitocine or G418.

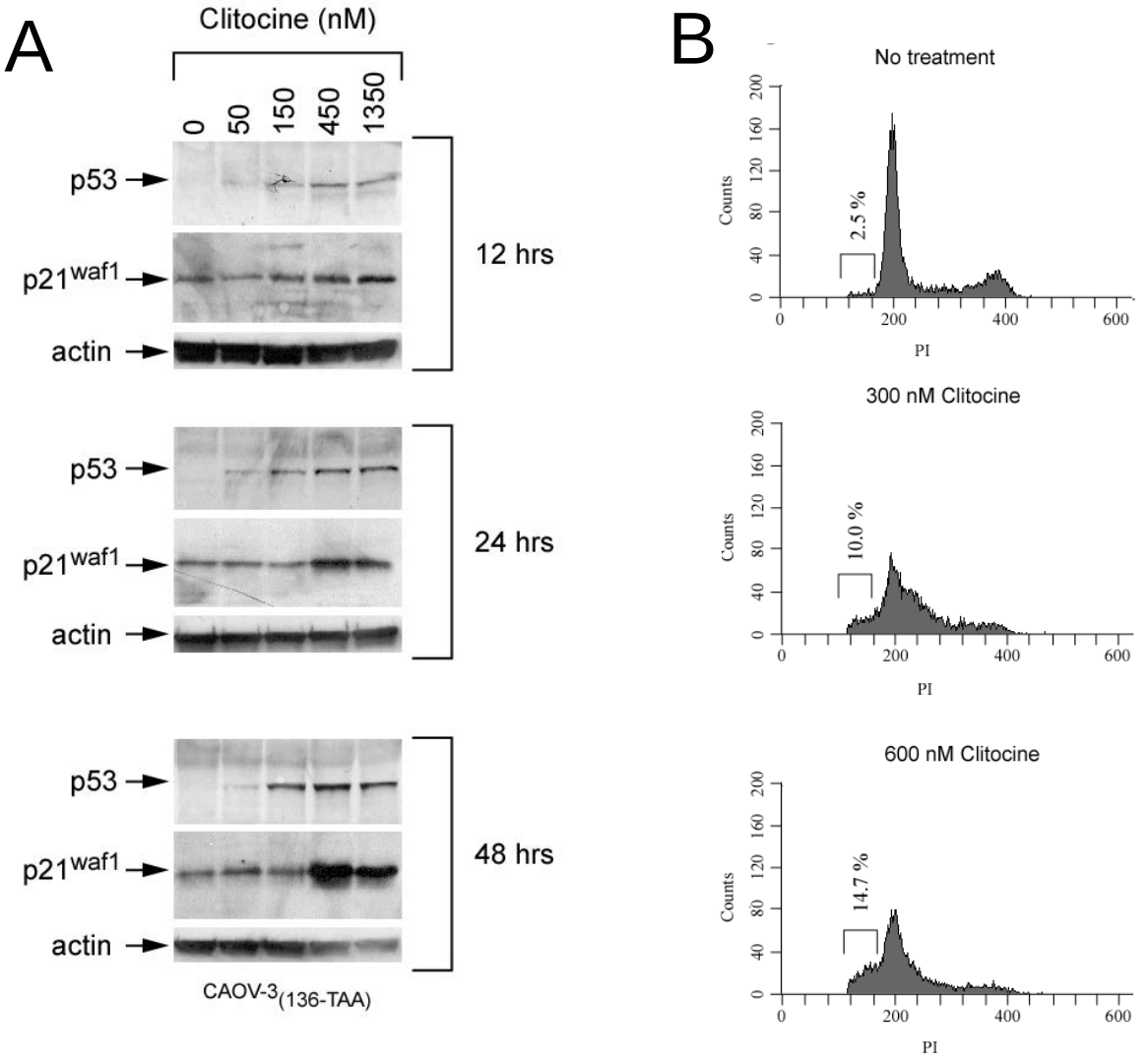


Figure S7: p53 induced by clitocine is active. (**A**) Western blot analysis showing that clitocine increases p53 and p21^{WAF1} in a dose- and time- dependent manner. (**B**) Flow cytometry experiment showing that clitocine treatment of CAOV-3$_{p53\text{-}UAA136}$ cells induces apoptosis. The DNA content was monitored by propidium iodide (PI) staining followed by cell sorting. The percentage of cells in subG_O/G_1 is indicated.

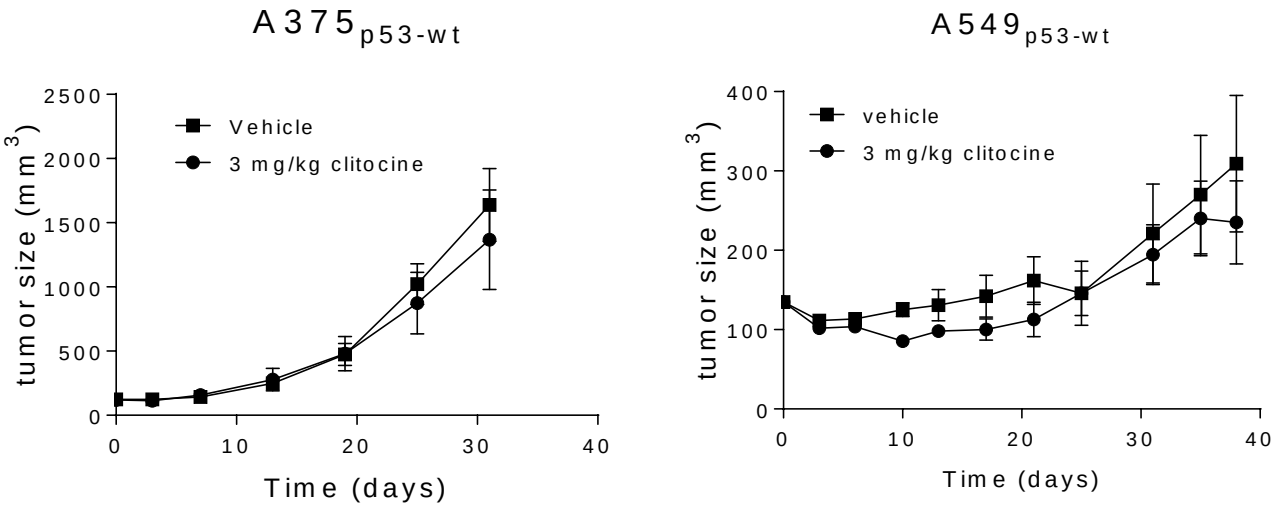


Figure S8: Clitocine is not active in p53-wt tumor xenografts. $A375_{p53\text{-}wt}$ and $A549_{p53\text{-}wt}$ tumors were established in the flanks of *scid* mice. After the average tumor volume reached 150 mm^3, mice were randomized and dosed subcutaneously once per day with clitocine (3 mg/kg) in PBS.

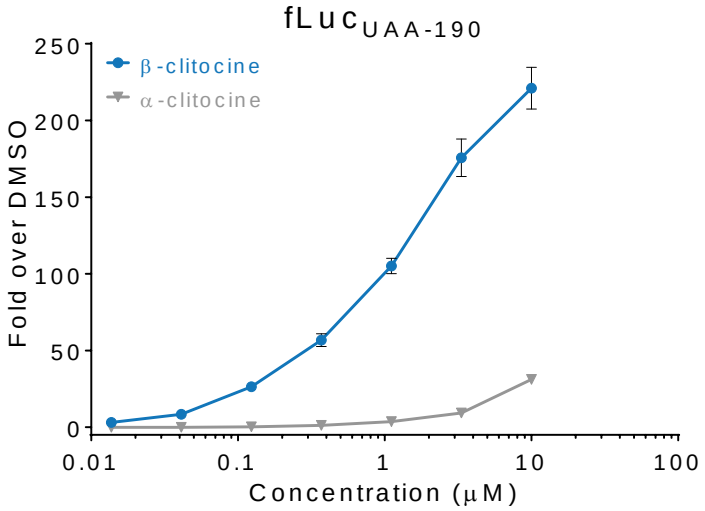


Figure S9: Premature stop codon readthrough of clitocine anomers. Premature stop codon readthrough activity of α- and β-clitocine anomers in the UAA luciferase reporter assay. Each point is the average of three determinations and error bars represent the standard deviation of the mean.