Supporting Document

HCVcc Infection Assay

The plasmid pJFH-1 was linearized at the 3' end of the full-genome JFH-1 cDNA, by XbaI digestion. Digested plasmid DNA was purified and used as a template for *in vitro* RNA synthesis with a MEGAscript T7 kit (Ambion, Austin, TX). Synthesized RNA was treated with DNase 1, followed by acid phenol extraction, to remove any remaining template DNA and transfected into Huh7.5.1 cells with DMRIE-C reagent (Invitrogen). A week after transfection, culture medium was harvested and stocked for further infection studies. Infectivity titer of the virus stock was determined by inoculation of serially 10-fold diluted culture medium to naïve Huh7.5.1 cells and detected by indirect immunofluorescence assay with anti-core C1 (provided by Harry Greenberg, Stanford Medical School) or anti-NS3 (George Luo, Univ of Kentucky) monoclonal antibodies. For infectivity assay, Huh7.5 cells were incubated with the virus inoculum together with the compounds and the numbers of HCV-positive foci were determined by immunofluorescence. Percentage of infection was calculated by dividing the number of HCV-positive foci in the presence of the compound by that in the absence of the compound. HCVcc was partially purified from 1 liter of culture medium of Huh7.5 cells infected with HCV JFH-1 by using iodixinol gradient.

To further assess HCV infection, HCV core antigen in the culture supernatant was quantified by a highly sensitive enzyme immunoassay (Ortho HCV antigen

ELISA Kit, Ortho Clinical Diagnostics, Tokyo, Japan). To determine the HCV RNA level in culture supernatant, RNA in the culture medium was extracted and purified from 100 μL of culture medium with a Viral RNA Extract Kit (QIAamp Viral RNA Mini Kit, Qiagen, Valencia, CA). Copy number of HCV RNA was determined by real-time quantitative RT-PCR (RT-qPCR).

HCVpp Infection Assay

To generate HCV pseudo-particles, the HCV envelope glycoprotein-expressing vector, the murine leukemia virus (MLV) gag-pol expression vector, and the MLV-derived transfer vector encoding the luciferase reporter protein, were transfected into 10^6 293T cells in 10 cm dish using Fugene 6 (Roche, Indianapolis, IN). The medium was replaced with DMEM + 10% FBS 16 h after transfection. Supernatants containing the pseudo-particles were harvested 48 h later, cleared by passage through 0.45-μM pore size filters, and used for infection assays. Target cells (Hep3B), were seeded in 12-well plates at a density of 1 X 10^5 cells per well and incubated overnight at 37°C. Viral supernatant containing the pseudo-particles were added to the Hep3B cells in the presence or absence of compounds. At 16 h post infection, medium was replaced with and cells were harvested 24 h later for luciferase activity determination. As a control, vesicular stomatitis virus (VSV) G protein-containing pseudovirus (VSVGpp) was produced with a G protein-expressing vector as above. HCVpp harboring E1E2 glycoproteins from genotypes 1a, 1b, 2a, 3a, 4a, 5a, and 6a were derived from

the plasmids pHCV7a, pHCV-E1E2.1b3, pCMV-HCV8.1, pCMV-UKN3A 1.28, pCMV-UKN4 21.16, pCMV-pUKN5 14.4 and pCMV-UKN6 5.340, respectively.

HCV Replicon Assay

Transient assay of genotype 1b (Con-1) and 2a (JFH-1) subgenomic reporter replicons have been reported previously. Subgenomic reporter replicon RNAs were synthesized by in vitro transcription with linearized template DNA plasmid, and 2 μg of RNA was transfected into Huh 7.5 cells seeded in 6-well plate at a density of $1x10^5$/ml 16 h before transfection. 4 h after transfection, cells in a portion of the plates were harvested as control for transfection efficiency, and a remaining portion of the cells in the plates received compounds. After compound administration, cells were harvested at 72 h after transfection for luciferase measurement. Replication efficiency was calculated as ratio of luciferase activities of 72 h over 4 h.

HCV Binding Assay

HCV-LPs were produced as described previously. The HCV-LP binding assay was performed at 4^0 C for 1 h in 100 μL of TNC (50 mM Tris pH 7.4, 100 mM NaCl, 1 mM $CaCl_2$) buffer containing 1% BSA. Both Hep3B and Huh7.5 cells were tested. Cells were incubated with 20 μg/mL of HCV-LPs in the presence of 100 nM of compounds. Chronic hepatitis C patient's serum containing high-level anti-HCV envelope antibodies was used as a control for blocking of HCV binding.

After HCV-LP binding, the cells were washed three times and incubated with anti-E2 monoclonal antibodies (ALP98, 10 μg/mL) for 30 min, followed by FITC labeled goat anti-mouse immunoglobulin G for 30 min. HCV-LP bound cells were analyzed by flow cytometry. Nonspecific fluorescence was measured by adding primary and secondary antibodies to cells in the absence of HCV-LP. The mean fluorescence intensity (MFI) of bound HCV-LP was determined after subtraction of the nonspecific fluorescence value. As an additional binding assay, Huh7.5 cells were incubated with HCVcc at 4^0C for 1 h in the presence of 100 nM of degenerate PS-ON or PO-ON. HCV serum described above was used as blocking control. After binding, cells were washed extensively with PBS and extracted for analysis of HCV RNA and core antigen levels.

Direct binding of PS-ON or PO-ON to HCV-LP was measured by a plate binding assay. HCV-LP or control preparation from insect cells infected with a recombinant baculovirus expressing β-glucuronidase was coated to a 96-well plate overnight at 4^0C (10 μg per well), washed, blocked with 5% skim milk, washed again, and then incubated with 100 μl of various concentrations of Cy3-labeled degenerate PS-ON or PO-ON (40-mer) for 2 h at 37^0C. The plate was then washed and analyzed by FluoStar Optima (BMG Labtech, Durham, NC). For HCVcc binding assay, HCVcc from culture supernatant of HCV infected cells was partially purified by iodixinol density gradient and incubated with 1 μM of Cy3-labeled PS-ON or PO-ON at 37^0C for 1 h. The mixture is subjected to iodixinol

density gradient again, and fractions are collected. Each fraction is then analyzed for HCV RNA, HCV core Ag, HCV infectious titer and fluorescence intensity. As control, culture supernatant from uninfected cells was subjected to the same procedure and analyzed for fluorescence intensity of each fraction. The fluorescence intensities of the fraction with detectable infectious HCV (fractions 7) were compared to those of the same fraction from the control preparation. A significant increase of fluorescence intensity in the HCV preparation over the control preparation would indicate binding of the oligonucleotide to HCVcc.

Viral Fusion Assay

HCVpp/liposome lipid mixing assays with rhodamine-labelled liposomes were performed as previously described. Liposomes were large unilamellar vesicles (100 nm) consisting of PC:cholesterol (egg yolk phosphatidylcholine and cholesterol, Sigma). Briefly, for lipid mixing R_{18}-labeled liposomes were obtained by mixing R_{18} (Octadecyl rhodamine B chloride, Molecular Probes, Invitrogen) and lipids in ethanol and chloroform solutions, respectively. Liposomes were prepared by extrusion through 100 nm-defined pore polycarbonate filters. Lipid mixing between HCVpp (or VSVGpp) and liposomes was monitored by quenching of R_{18}. R_{18}-labeled liposomes (final lipid concentration 15 mM) were added to a 37°C-thermostable cuvette containing pseudo-particles with or without compounds in PBS pH 7.4. After temperature equilibration and pH decrease to 5, fusion kinetics were recorded on a FluoroMax-4 spectrofluorimeter (HORIBA

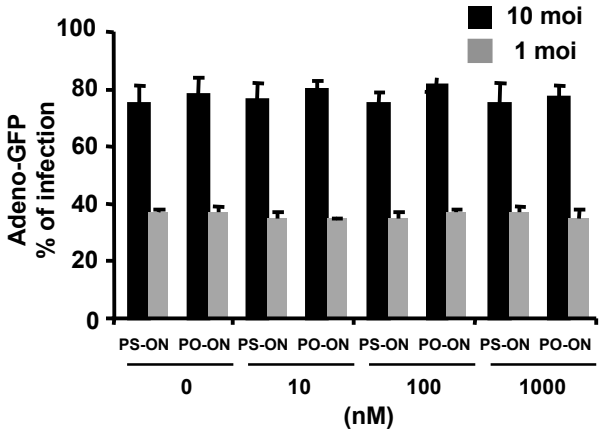
Jobin Yvon SAS) over a 20 min-time period, with λexc at 560 nm and λem at 590 nm. Maximal R_{18} quenching was measured after disruption of liposomes by addition of 0.1% Triton X-100 (final concentration, v:v).

Supplementary Figure 1: Effect of PS-ON on adenovirus infection. Huh 7 cells were infected with adenovirus expressing green fluorescent protein in various concentrations of 40mer PS-ON or PO-ON. GFP expression was detected by flow cytometry and percentage of infection was determined.

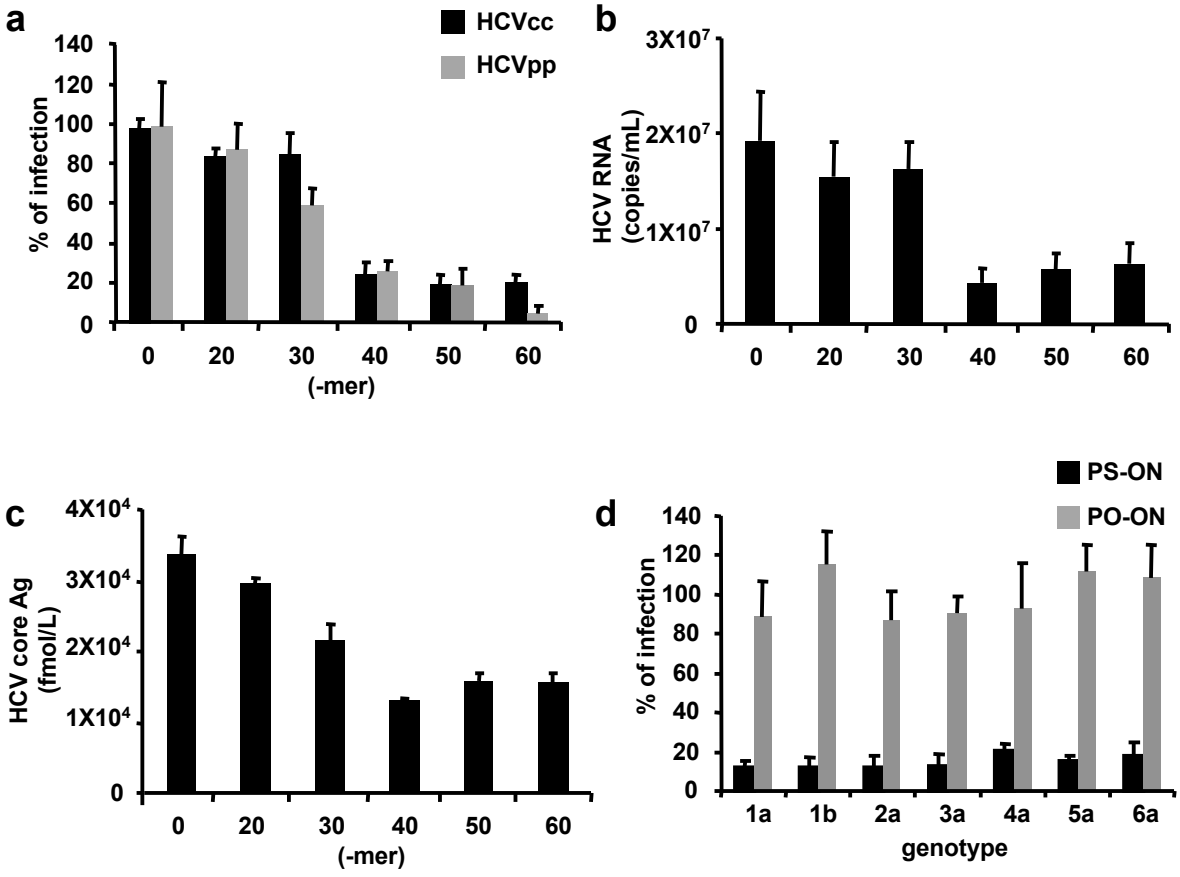
Supplementary Figure 2. Effect of poly C PS-ON on HCV infection. (A) Huh7.5 cells were infected with HCVcc in the presence of various concentrations of 40 mer poly C PS-ON or PO-ON. Two days after infection, Infected cells were detected by immunofluorescence assay using anti-core antibodies. Percentage of infection was determined by dividing the number of HCV expressing cells in treated over the untreated cells. Hep3B cells were infected with HCVpp genotype 1b and treated with various concentrations of poly C PS-ON and PO-ON, and luciferase activities were determined 2 days later. (B) HCV RNA levels (C) The HCV core Ag titers in the culture medium were determined. (D) HCVpp harboring E1E2 glycoproteins from genotypes 1a, 1b, 2a, 3a, 4a, 5a and 6a were inoculated into Hep3B cells and simultaneously treated with 100 nM of poly C PS-ONs and PO-ONs. All results are shown as percentages of infection $\pm$ SD.

Supplementary Figure 3. Structures of the oligonucleotide analogs. The various analogs of PS-ON and PO-ON used in this study are shown. The modifications are described in the text.

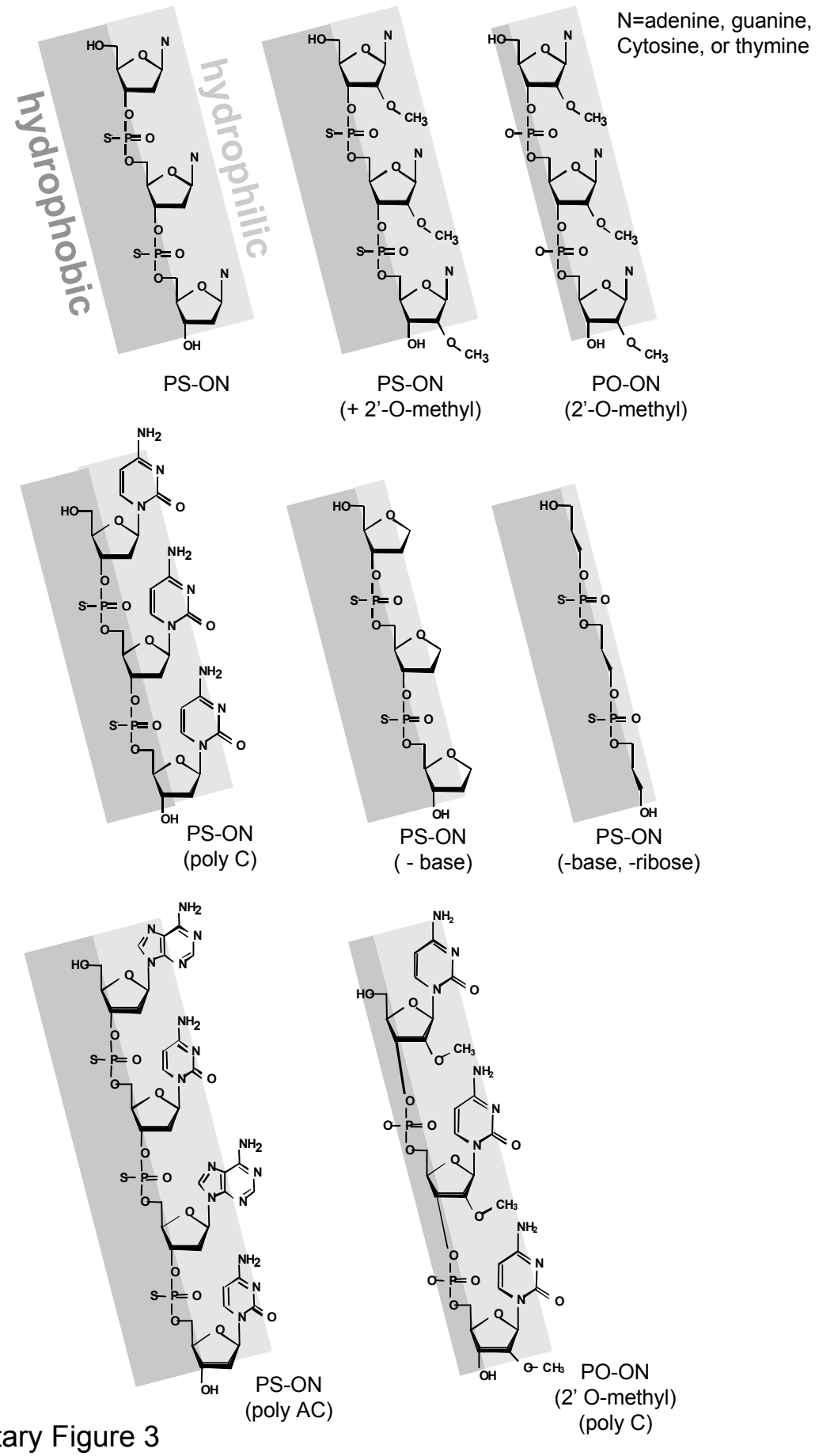
Supplementary Figure 4: Effects of PS-ON and PO-ON on HCVpp and VSVGpp fusion. HCV fusion assay was performed as described in Material & Methods. (A) HCVpp and (B) VSVGpp were tested in the presence of degenerate or poly C PS-ON or PO-ON at two different concentrations (1 and 10 nM). The kinetics of fusion reactions as monitored by fluorescence are shown for each compound and the results are representative of three independent experiments.



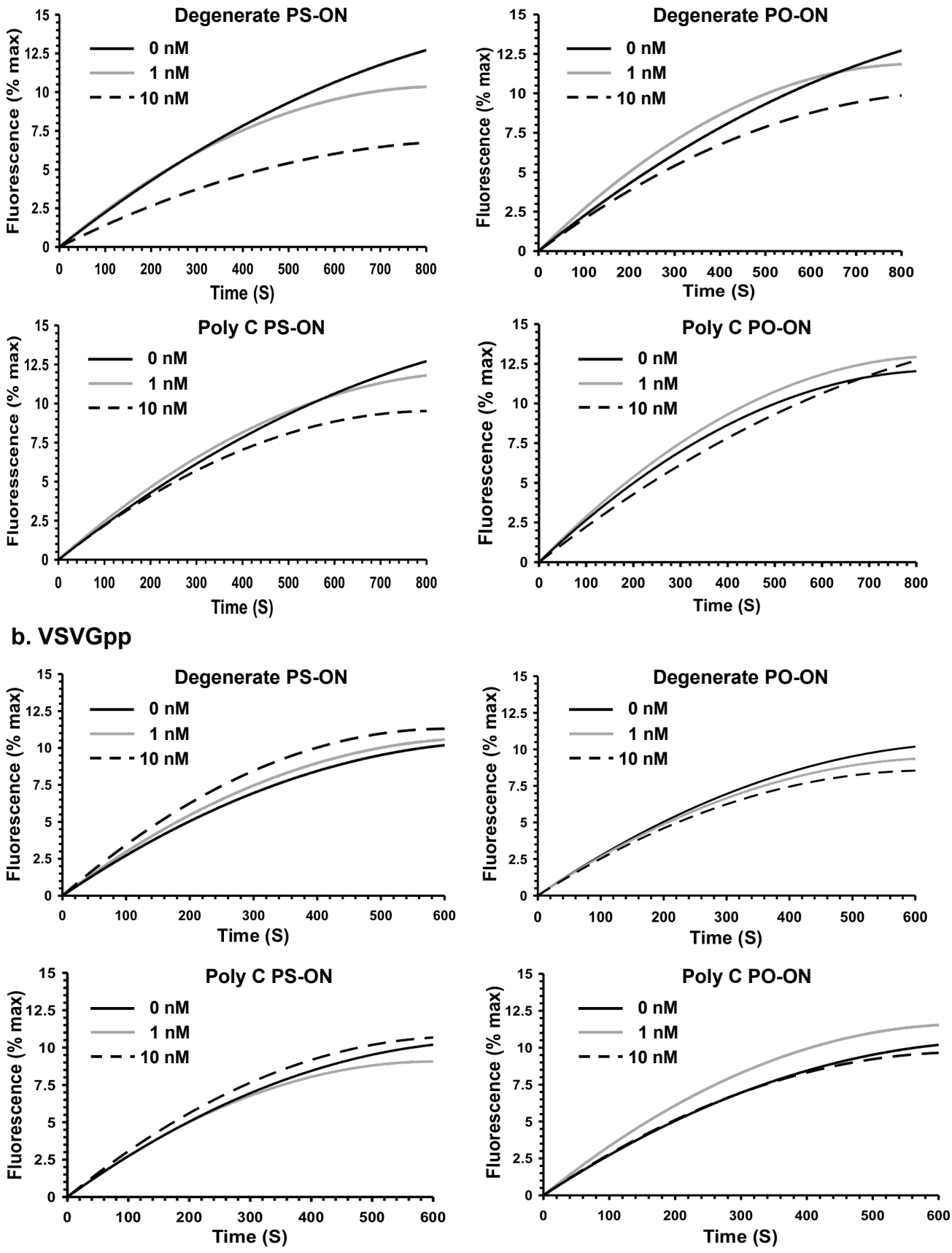
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4