Thiopurines activate an antiviral unfolded protein response that blocks influenza A virus glycoprotein accumulation

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Introduction

- Influenza A virus (IAV) requires cellular protein synthesis machinery to synthesize viral proteins
- We identified 6-Thioguanine (6-TG) and 6-Thioguanosine(6-TGo) in a small molecule screen as candidate antivirals that impair viral replication

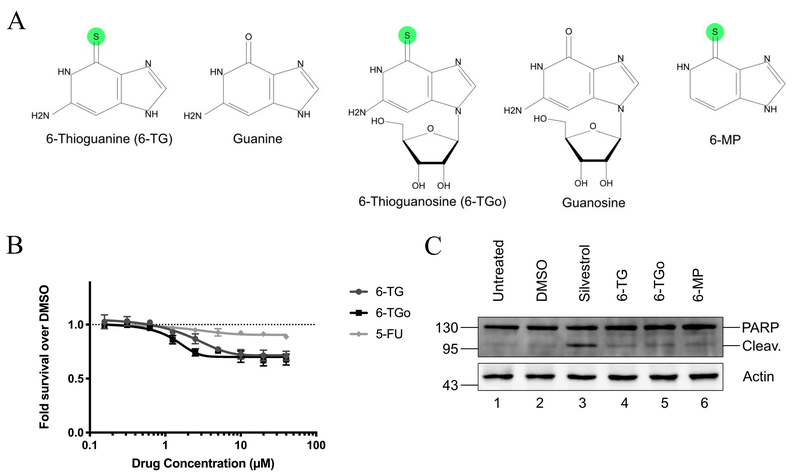

Figure 1. 6-Thioguanine and 6-Thioguanosine show cytostatic effects on treated cells. (A) Structures of 6-thioguanine (6-TG) and 6-thioguanosine (6-TGo) molecules identified in the screen and the indicated structurally-similar nucleobases and nucleosides. Thiol groups present in 6-TG, 6-TGo, and 6-mercaptopurine (6-MP) are highlighted in green. (B) A549 cells were treated with increasing doses of 6-TG, 6-TGo, 5-FU, or vehicle control (DMSO) for 23 hours and cell viability was measured using an alamarBlue assay. Relative fluorescence units were normalized to vehicle control. Error bars represent standard deviation (N=3). Horizontal dotted line represents the relative fluorescence of untreated cells. (C) Lysates of A549 cells treated with the Silvestrol (320 nM), thiopurines (10 μM), and vehicle DMSO control were analysed by western blotting for total PARP (full length and cleaved). B-actin antibody staining was used as loading control.

Thiopurines inhibit viral glycoprotein accumulation

- 6-TG and 6-TGo treatment impairs the accumulation of viral glycoproteins, while cytoplasmic proteins are mostly unaffected

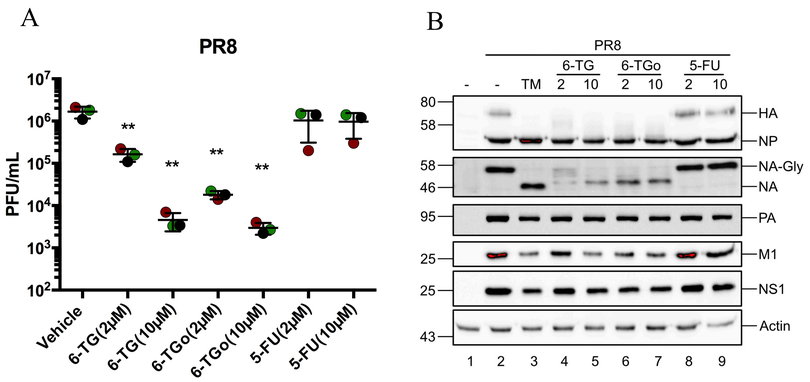

Figure 2. 6-TG and 6-TGo treatment impairs replication of IAV. A549 cells were infected with A/Porto Rico/08/34 (H1N1) (PR8) at a MOI of 0.1 for 1hpi. Cell monolayers were washed and treated with the compounds at the indicated concentrations for 23 hpi. Infectious progeny in the cellular supernatant was enumerated with a plaque assay. Error bars denote the standard deviation of three biological replicates (N=3). Cell lysates were collected and analyzed by western blot, using a polyclonal IAV antibody that detects HA, NP and M1 as well as antibodies that detect IAV PA, NA, NS1, or cellular actin. The representative of 3 independent experiments is shown.

6-TG and 6-TGo induce the UPR

- 6-TG and 6-TGo treatment induces the UPR and its down stream targets in uninfected cells

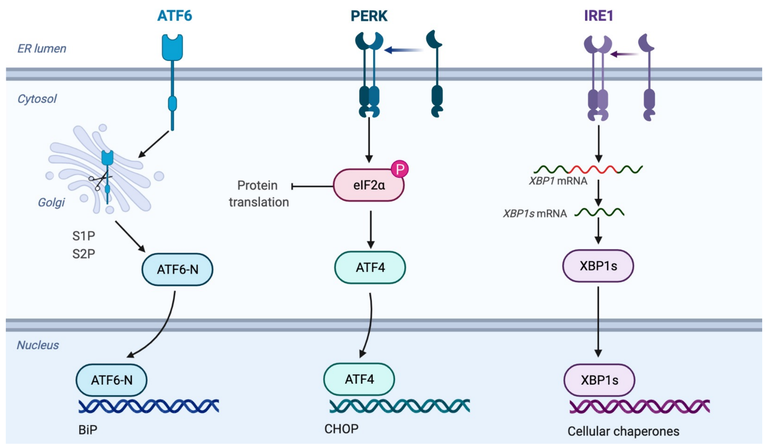

Figure 3. Illustration of the unfolded protein response (UPR). The three arms of the UPR are simplified in this illustration. The first arm is Activating transcription factor 6 (ATF 6) is cleaved by a Golgi resident protease that releases the N-terminal fragment, that in turn upregulates cellular factors such as BiP. PKR-like-ER resident kinase (PERK) dimerizes and phosphorylates eIF2 alpha to reduce global translation and preferentially translate stress response proteins such as ATF 4, which in turn promotes the synthesis of CHOP. Inositol-requiring enzyme 1 (IRE1) dimerizes and splices out a 26-nucleotide sequence of XBP1, creating XBP1s. Orange boxes highlight proteins that were analyzed via western blot in figure 4. This figure was generated in BioRender.

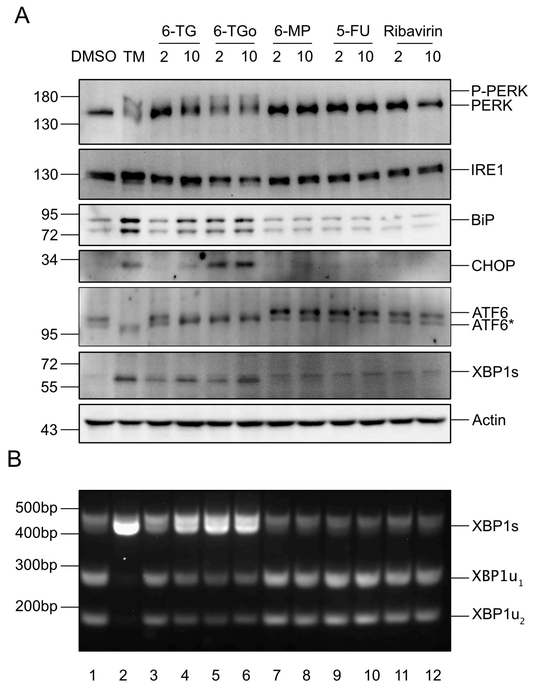

Figure 4. 6-TG and 6-TGo activate the UPR. (A) A549 cells were treated with 6-thioguanine (6-TG), 6-thioguanosine (6-TGo), 6-mercaptopurine (6-MP), 5-fluorouracil (5-FU) or ribavirin at the indicated concentrations for 6 hours prior to harvesting lysates for immunoblotting for the indicated cellular proteins. 5 μg/ml tunicamycin (TM) served as positive control for UPR activation. ATF6* indicates a lower molecular weight species that is presumably not glycosylated and not cleaved to its active form. (B) cDNA was generated from total RNA that was isolated from treated cells. XBP1 mRNA splicing was determined by the semi-quantitative RT-PCR splicing assay. XBP1u1 and XBP1u2 indicate the cleaved produces from digesting the unspliced XBP1 cDNA with PstI-HF.

Modulating the UPR restores protein synthesis

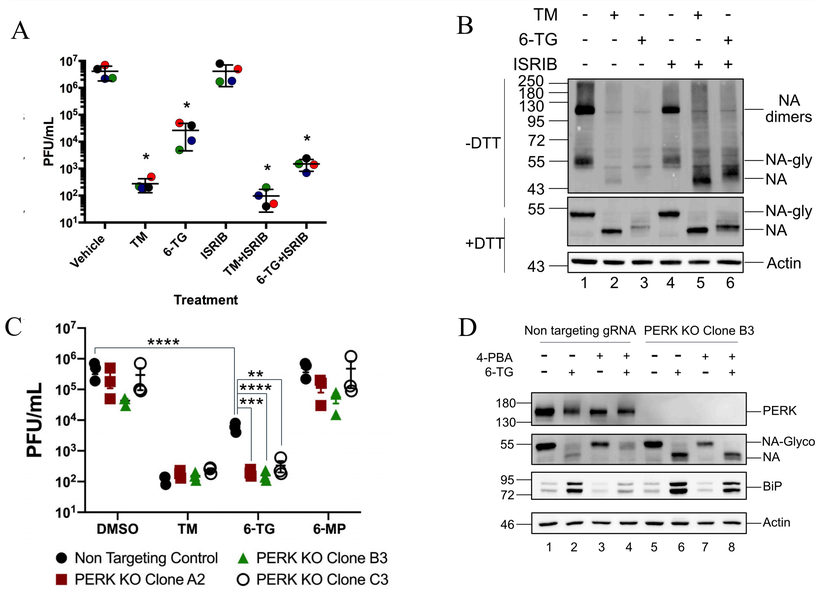

Figure 5. ISR inhibition restores NA synthesis in the presence of 6-TG but NA processing and virion production remain impaired. A549 cells were infected with PR8 at a MOI of 1. After 1 h, cells were washed and treated with tunicamycin (TM, 5 μg/ml), 6-thioguanine (6-TG, 10 μM) and/or 500 ng/ml integrated stress response inhibitor (ISRIB). (A) At 24 hpi, cell supernatants were collected and infectious IAV-PR8 virions were enumerated by plaque assay. Error bars represent the standard deviation between biological replicates (N=4); circles represent biological replicates. (B) Cell lysates were collected and processed for native SDS-PAGE and immunoblotting using an anti-NA antibody. (C) A549 cells were lenti-transduced with the pLentiCRISPRv2 system with the associated guide RNA to knock out PERK. A549 PERK KO cells (Clone A2, B3, and C3) and the non-targeting control cell line were infected with PR8 at a MOI of 0.1 and were treated with the indicated compound for 23 hours. Supernatant was collected at 24 hpi and viral titre was quantified with plaque assay. (D) Cell lysates were collected and processed for native SDS-PAGE and immunoblotting using an anti-NA antibody. Cells were co-treated with a chemical chaperone 4-PBA (10mM).

Conclusion

- 6-TG and 6-TGo treatment impairs IAV replication
- 6-TG and 6-TGo induce the UPR impairing viral glycoprotein synthesis
- The UPR can be modulated to improve viral glycoprotein synthesis without rescuing viral replication

Future Directions

- Determine the molecular target of 6-TG to further elucidate the mechanism of action
- Determine if IAV can develop resistance to host targeted antivirals
- Test 6-TG and 6-TGo on other viruses, such as SARS COV-2

Acknowledgments

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