



ANTI-HBV AND ANTI-HCV MECHANISMS OF TIZOXANIDE

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NTZ and Viral Hepatitis

- **Anti-HBV activity discovered at GUMC in 1994 in cell culture in collaboration with Romark Laboratories, LC (CATG program)**
- **Anti-HCV activity discovered during long-term treatment of HIV infected individuals for opportunistic infections (2000-2003)**
 - **Improvement of serologic markers of chronic liver disease**
 - **Mixture of HBV and HCV infected individuals**
- **Subsequent evaluations in cell culture at GUMC beginning in 2004 at request of Romark**
 - **confirmed direct anti-HBV activity**
 - **proved direct anti-HCV activity**
- **Several clinical trials with IFN/RBV demonstrated increased efficacy¹**



NTZ is effective against HBV replication in cell culture

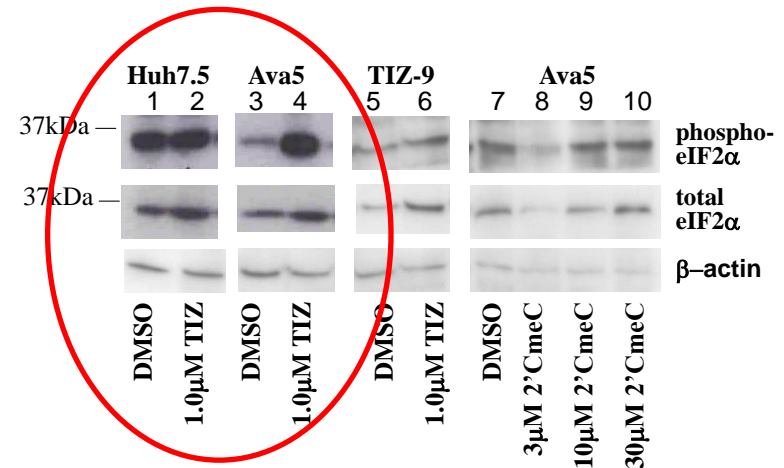
- **Model system: 2.2.15 cell line; EC₅₀ 0.2uM, EC₉₀ 1.0uM**
- **NTZ inhibits intracellular HBV replication and extracellular virion production**
- **TIZ is equally effective against clinically-relevant LMV and ADV resistant mutants**
- **TIZ is synergistic with licensed nucleosides against HBV replication (LMV, ADV, ETV, TDF)**
- **NTZ induces late reductions in HBV protein levels without affecting HBV RNA levels (post-transcription, post-translation target)**

NTZ is effective against HCV replication in cell culture

- **Model system: HCV replicon; EC₅₀ 0.2uM, EC₉₀ 1.0uM¹**
- **NTZ inhibits genotypes 1a¹, 1b^{1,4}, 2a (infection)^{1,4}**
- **TIZ is equally effective against clinically relevant DAA-resistant mutants (NS5a, NS5b, NS3), prevents resistance to TPV^{1,5}**
- **TIZ is synergistic¹ with IFN/RBV, nucleosides (2'CmeC), NNRTI (HCV-796)⁵**
 - **Highly synergistic⁵ with PIs (TPV, BOC, DPV, BILN2061), DCV**
- **High barrier to resistance, resistance is property of host, not virus^{2,3}**
- **Given the broad spectrum of antiviral activity and HCV resistance patterns, host processes appear to be the primary drug target**

Enhancement of eIF2 α ¹ phosphorylation is not the primary antiviral mechanism of TIZ

- HCV significantly down-regulates eIF2 α phosphorylation²
 - TIZ/NTZ restore basal Huh7.5 levels¹
- Inducers of UPR and ER/cellular stress responses known to increase eIF2 α -P have **no effect** on HCV replication
 - Appears to be a downstream effect of other pathways directly modulated by TIZ (required for HCV inhibition?)
- Loss of HCV (2'CmeC) not sufficient
- Effect occurs only in presence of HCV
 - NTZ does not induce eIF2 α -P in influenza or rotavirus infected cultures¹ or in 2.2.15 cells
 - No effect in Huh7.5



| | CC50 | EC50 |
|----------------------------|-------|--------|
| Tunicamycin (μ M) | 2.5 | >2.5 |
| Geldanamycin (μ M) | 2.9 | >2.9 |
| Sialic acid (mM) | 43 | >43 |
| Thapsigargin (μ g/ml) | 0.01 | >0.01 |
| Brefeldin A (μ g/ml) | 0.01 | >0.01 |
| Calyculin A (μ g/ml) | 0.13 | >0.13 |
| Salubrinal (μ g/ml) | 10 | >10 |
| Sodium arsenite (μ M) | 0.002 | >0.002 |
| Cyclohexamide (μ M) | 1.2 | >1.2 |
| DTT (mM) | 2.1 | >2.1 |

TIZ does not directly inhibit HCV enzymatic activities

| Compound | Conc. (μ M) | Relative HCV Enzyme Activity (% control \pm S.D.) | | |
|--------------|---------------------|---|--------------------|--------------------|
| | | NS3/4A protease | NS3/4A helicase | NS5B polymerase |
| none | 10 | 100 \pm 7 | 100 \pm 2 | 100 \pm 5 |
| Tizoxanide | 0.1 | 98 \pm 7 | 99 \pm 5 | 84 \pm 2 |
| | 1.0 | 96 \pm 6 | 105 \pm 8 | 94 \pm 10 |
| VX-950 | 0.1 | 19 \pm 5 | --- | --- |
| Thioflavin-S | 50 | --- | 7.0 \pm 0.9 | --- |
| HCV-796 | 1.0 | --- | --- | 8.0 \pm 1.0 |

TIZ reduces HCV NS protein levels consistent overall with loss of viral RNA

| | TIZ (1.0 μ M, EC90) | | | | |
|-------------------------------|-------------------------|--------------|--------------|-------------|---------------|
| | DMSO (control) | 24hr | 36hr | 48hr | 72hr |
| HCV RNA (copies/ cell) | 100 \pm 12 | 91 \pm 9 | n.d. | 9 \pm 1.5 | 5.5 \pm 0.6 |
| Total lysate | | | | | |
| NS3 | 100 \pm 5 | 103 \pm 28 | 78 \pm 13 | 84 \pm 10 | 49 \pm 17* |
| NS5A | 100 \pm 13 | 56 \pm 13 | 33 \pm 7* | 38 \pm 6* | 34 \pm 15* |
| NS5B | 100 \pm 5 | 71 \pm 8 | 134 \pm 21 | 68 \pm 19 | 47 \pm 17* |
| Membrane preps | | | | | |
| NS3 | 100 \pm 28 | 70 \pm 11 | 98 \pm 9 | 88 \pm 11 | 55 \pm 4 |
| NS5A | 100 \pm 16 | 71 \pm 29 | 125 \pm 22 | 68 \pm 12 | 92 \pm 6 |
| NS5B | 100 \pm 15 | 115 \pm 46 | 59 \pm 16 | 89 \pm 19 | 28 \pm 16* |

* P <0.02

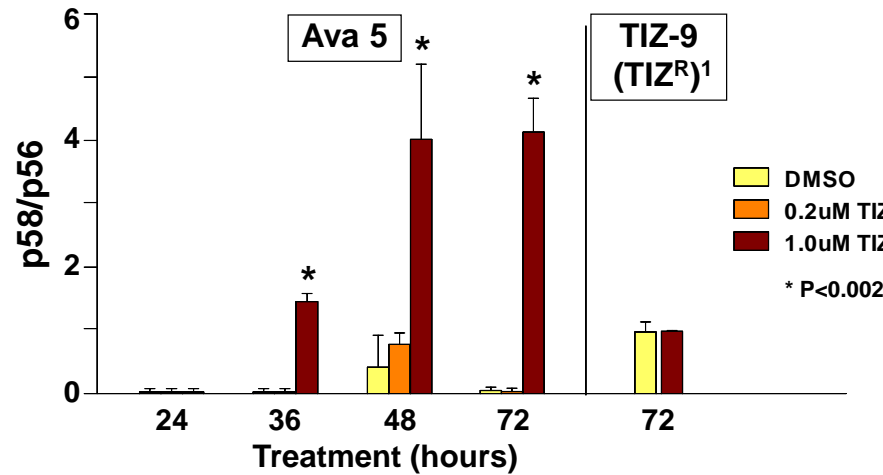
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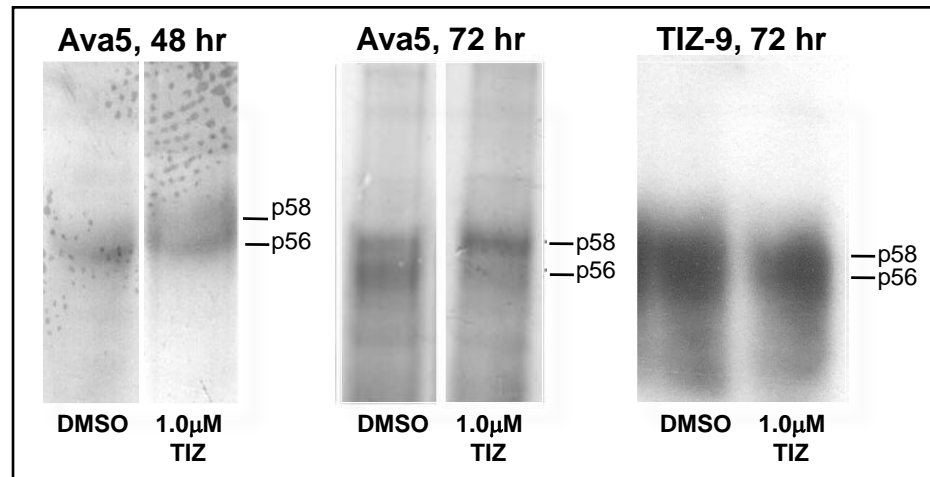
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Is early reduction of NS5A responsible for restoration of eIF2 α -P via reduction of NS5A interference with PKR?

NTZ induces hyper-phosphorylation of HCV NS5A in cell membrane preparations

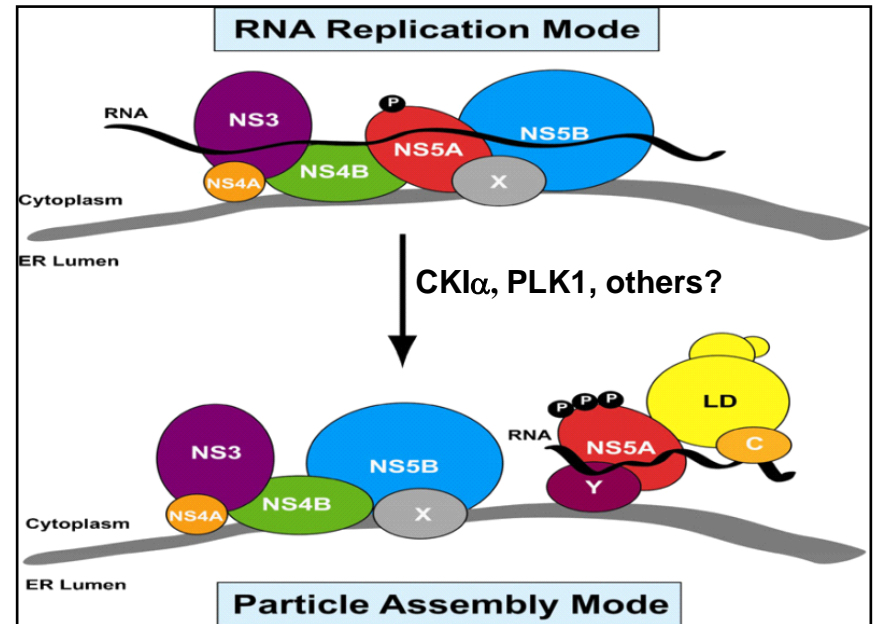


Induction of p58 coincident with timing of initial reduction in HCV RNA



HCV NS5A protein

- Phosphorylation state of NS5A regulates the switch from active HCV replication to virion assembly
- Overproduction of p58 decreases HCV replication

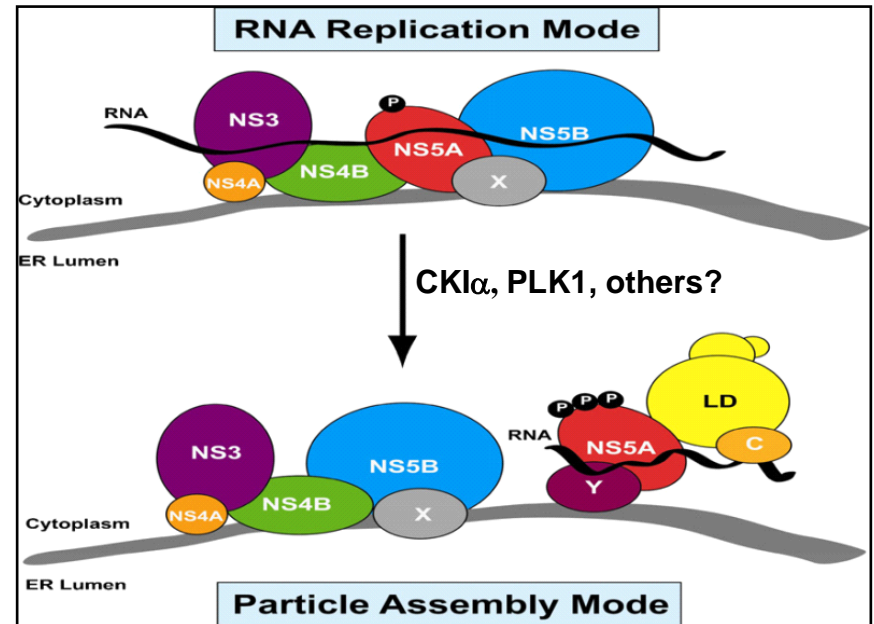


Quintavalle, *et al.* 2007 JBC 282:5536
Benga, *et al.* 2010 Hepatol.51:43
Masaki, *et al.* 2008 J.Virol.82:7964
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Quintavalle, *et al.* 2006 J. Virol.80:11305
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HCV NS5A protein

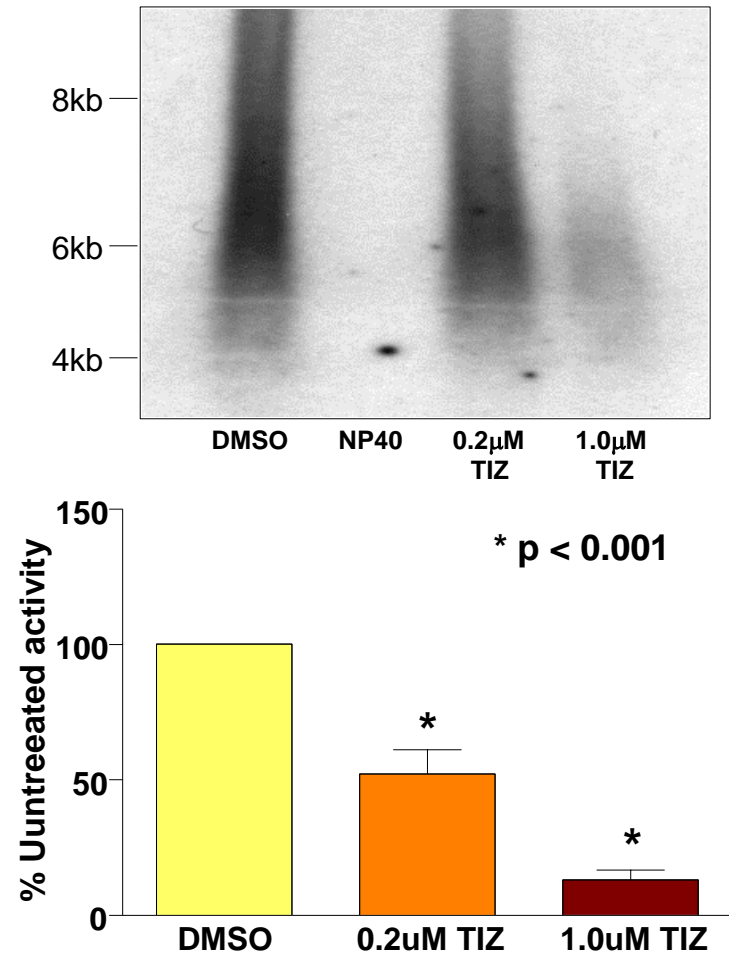
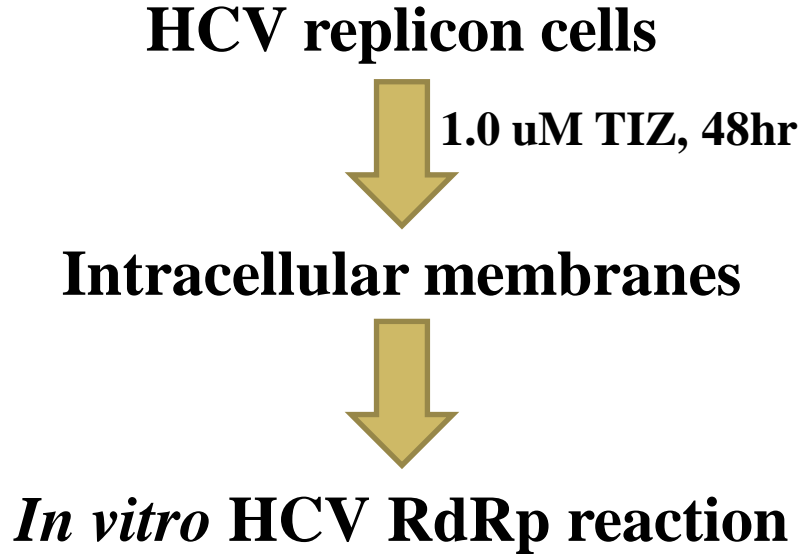
- Phosphorylation state of NS5A regulates the switch from active HCV replication to virion assembly
- Overproduction of p58 decreases HCV replication
- Due to disassociation of HCV genome from the replicase complex, overproduction of p58 predicts a reduction of NS5B RdRp activity on resident HCV genomes



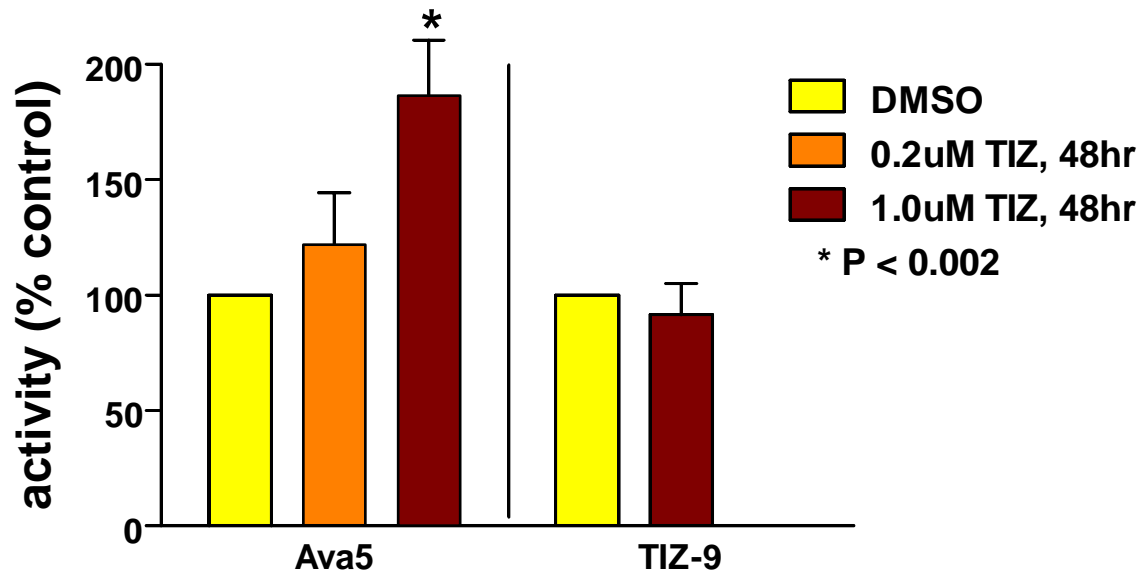
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RdRP activity on nascent viral replicon RNAs is down-regulated by TIZ

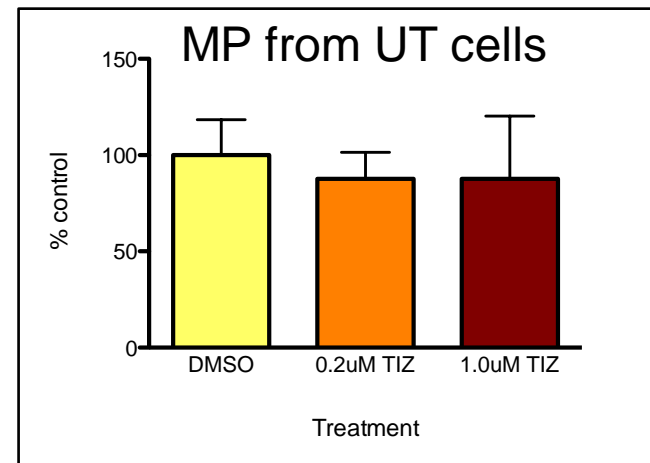
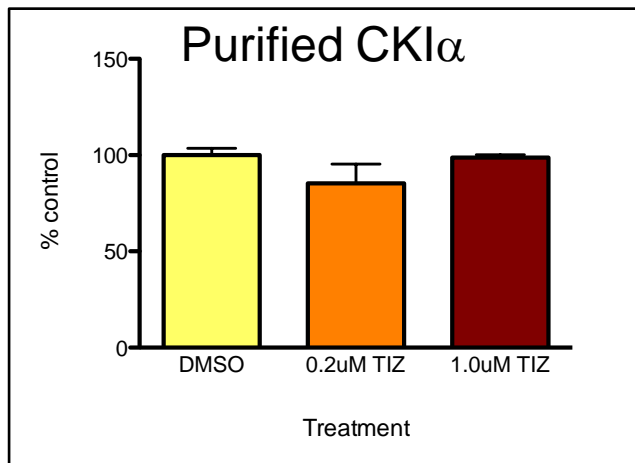


NTZ Treatment enhances CKI activity in cell membrane preparations



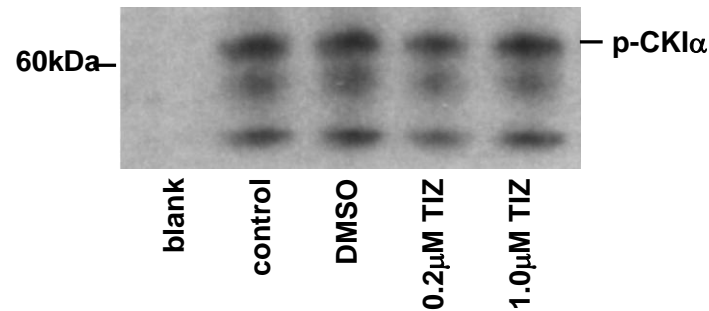
TIZ does not affect PLK1 (NS5A p58¹) or PI4KIII α (replication²)

TIZ does not directly affect CKI α activity *in vitro*

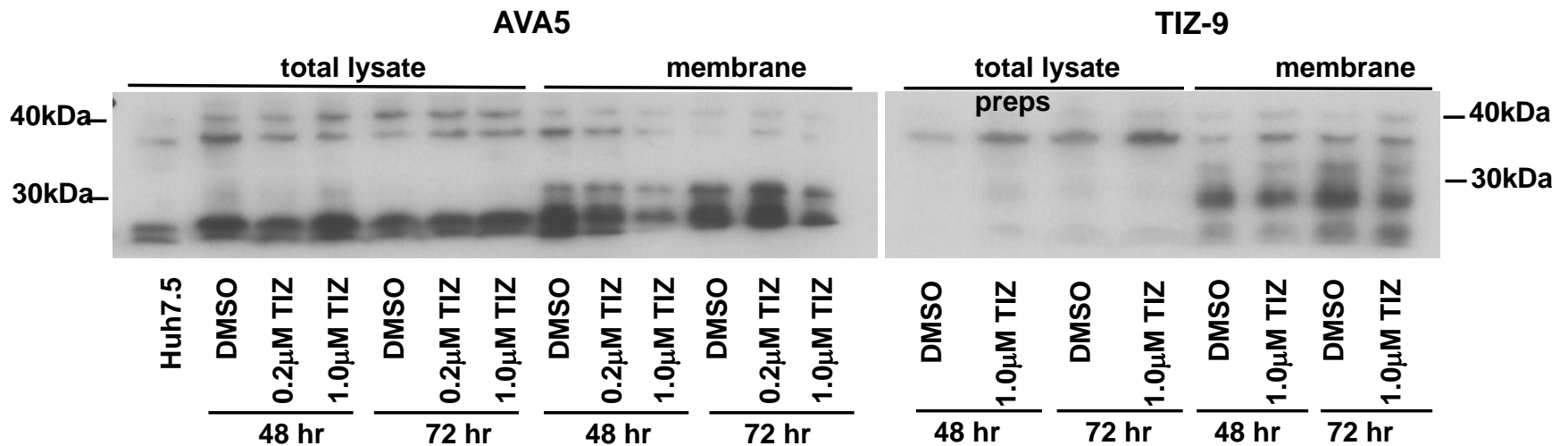


TIZ does not alter CKI α auto-phosphorylation or distribution of splice variants

In vitro auto-phosphorylation:



Intracellular splice variants:

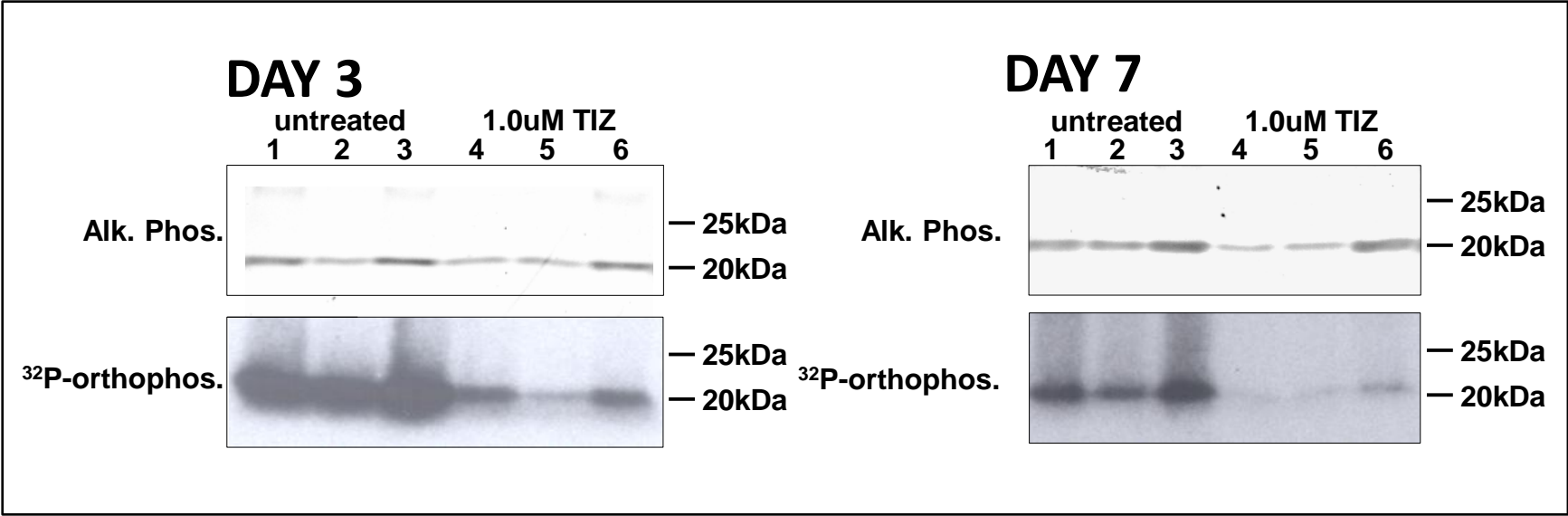


HBV Mechanisms

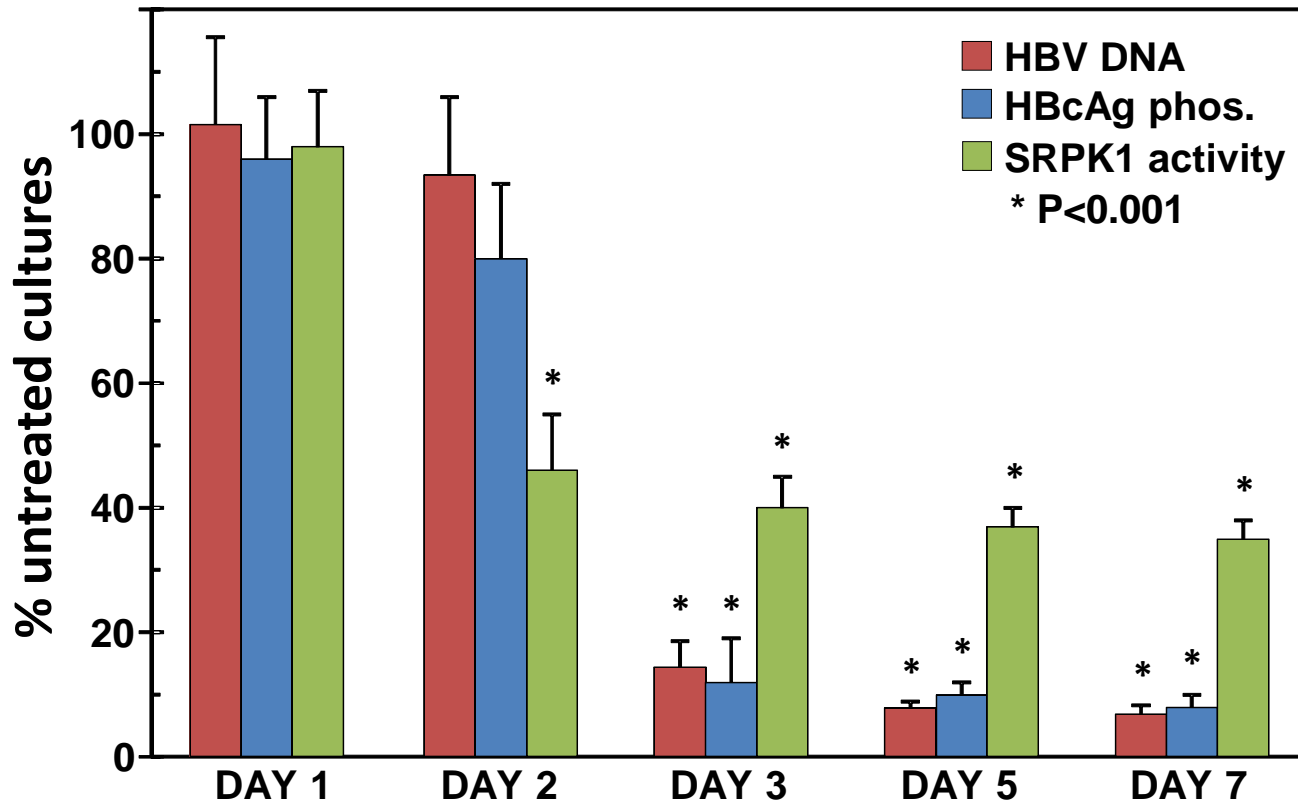
- **Phosphorylation of HBcAg is essential for HBV replication**
- **Inhibition of HBcAg phosphorylation interferes with encapsidation of pregenomic RNA producing an excess of ‘empty capsids’**
- **Primary kinases reported to be responsible**
 - **PKC - now believed to be involved in primarily in maturation and not early events in viral genome replication**
 - **SRPK1, SRPK2 – shown to phosphorylate key serines (in consensus sequence) necessary for efficient encapsidation of pregenome and capsid stability**
 - **others**

Roosnick & Siddiqui 1987 J Virol 61:955; Schlicht, *et al.* 1989 J Virol 63:2995; Daub, *et al.* 2002 J Virol 76:8124; Li, *et al.* 2004 Methods Mol Med 95:227; Kang *et al.* 2008 Biochem J 416:47; Weigand, *et al.* 2010 J Gen Virol 91:59; Zlotnick & Mukhopadhyay 2011 Trends in Microbiol. 19:14.

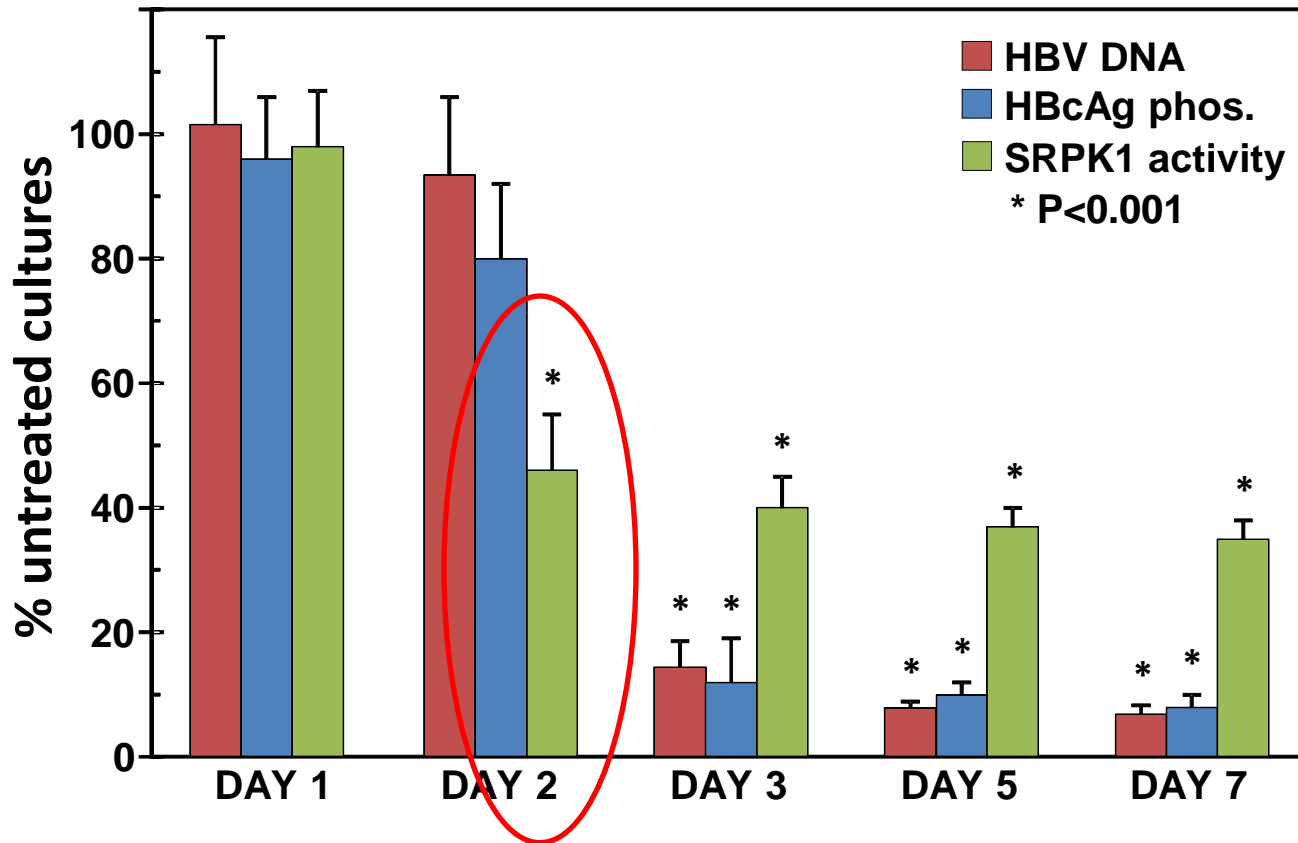
TIZ inhibits HBcAg Phosphorylation in 2.2.15 cells



Loss of HBV replication correlates with loss of HBcAg phosphorylation

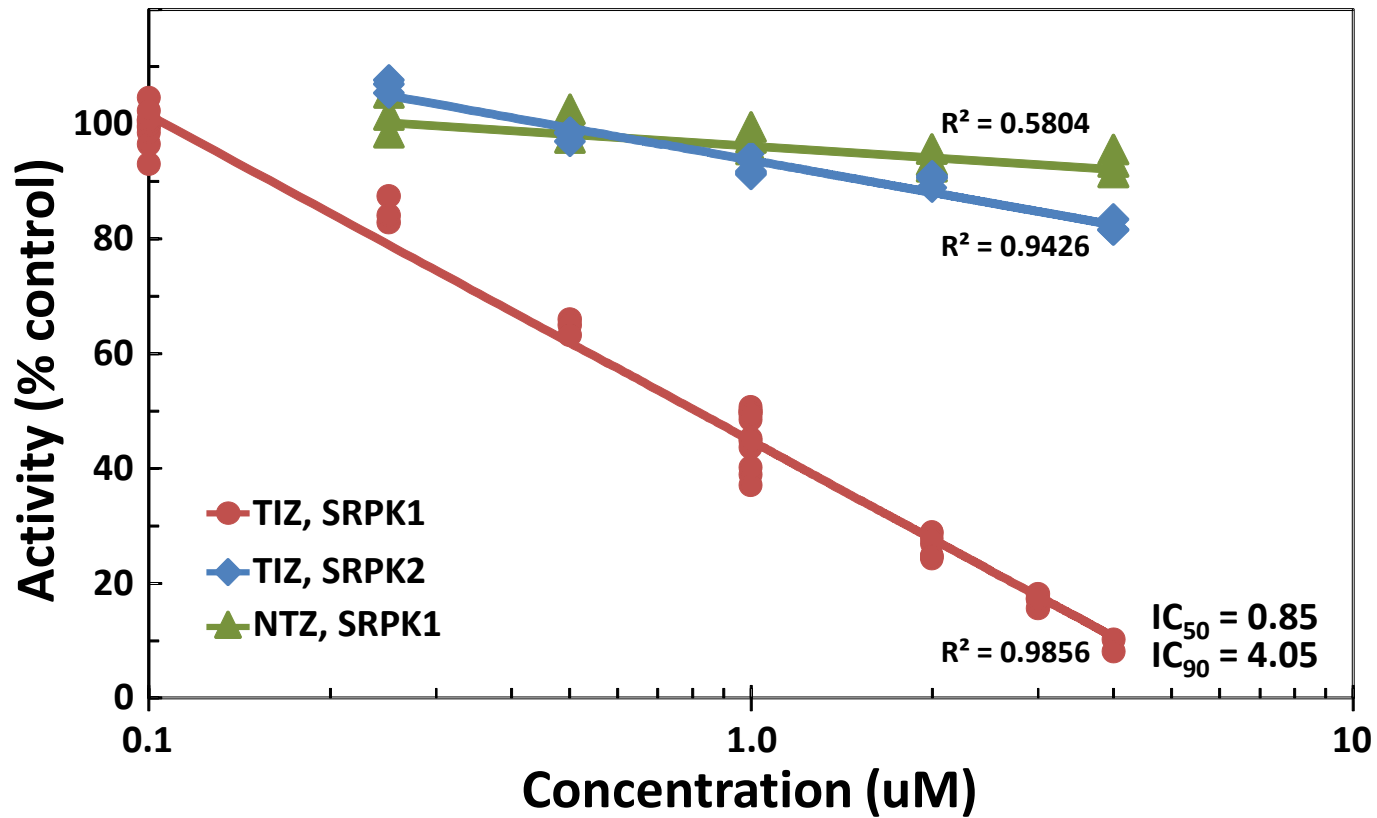


Loss of HBV replication and HBcAg-P correlate with reduction of SRPK1 activity



Need to confirm presence of 'empty capsids' in TIZ-treated cells

TIZ directly inhibits SRPK1 kinase activity *in vitro*



Summary

- **Two distinct antiviral effects are induced by TIZ upon HBV and HCV**
- **Both mechanisms involve TIZ mediation of cellular kinases**
- **Unclear how these activities are related**
 - **kinetics of anti-SRPK1 activity are more protracted than anti-CKI α**
 - ***in vitro* activity of TIZ against SRPK1 correlates with anti-HCV effect**
 - **anti-SRPK1 compounds inhibit HCV (unknown mechanism)¹**
- **TIZ interaction with a single (or limited) number of host targets likely results in pleiotropic downstream effects on interrelated cellular pathways**
 - **interactions likely to manifest in different effects due to divergence in intracellular environments, especially those related to viral infections**

Acknowledgements

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M Elazar

J Glenn

Romark Laboratories

M Ayers

J-F Rossignol

E Semple

wtHCV replicons, Huh7.5 supplied by Apath, Inc.

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