

Nitazoxanide: A Potent Antiviral Agent against HCV and HBV

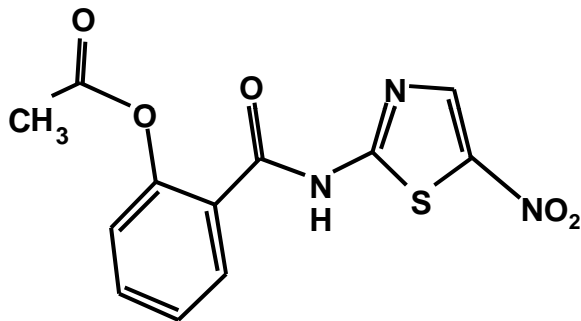
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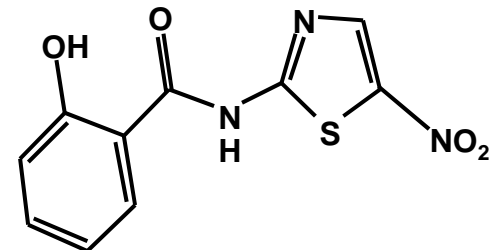
3 - The Romark Institute for Medical Research, Tampa, FL, USA

Background



- Nitazoxanide (NTZ) is a thiazolide anti-infective with broad spectrum activity against anaerobic bacteria, protozoa and viruses
- Active circulating compound is tizoxanide (TIZ)

- NTZ is marketed in the USA for treating diarrhea and enteritis caused by *Cryptosporidium spp* or *Giardia lamblia* in adults and children to 12 months of age (Romark Laboratories, Tampa, Florida USA)
- Phase II clinical trials completed against *Clostridium difficile*, rotavirus and norovirus infection
- Estimated 12 million prescriptions since 2000



Background

- Anti-HBV properties in cell culture first revealed in 1995, confirmed in 2005 along with initial culture activity against HCV (GUMC)
- Phase IIb trials against HCV in Egypt in progress (preliminary report at AASLD 2007)
- Phase IIb trials against HCV in the USA initiated in July 2007 (combination with interferon/ribavirin)
- Most clinical experience with NTZ is 3 to 14 days
- No significant drug-related adverse events in:
 - continual use for as long as 4 years in HIV patients (AIDS-related cryptosporidiosis)
 - 48 week clinical trials in HBV and HCV patients
- Mechanism(s) of antiviral activity are unknown



NTZ is effective against HCV replication in replicon cell cultures

| COMPOUND | CC50 (uM) | Intracellular HCV RNA (uM) | | Selectivity Index (CC50/EC50) |
|---------------------------|--------------|-------------------------------|-------------|-------------------------------------|
| | | EC50 | EC90 | |
| <i>Genotype 1b (CON1)</i> | | | | |
| IFN α | >10000* | 1.9* | 8.9* | >5263 |
| 2'CmeC | >300 | 1.6 | 8.3 | >188 |
| NTZ | 38 | 0.21 | 0.93 | 181 |
| TIZ | 15 | 0.15 | 0.81 | 100 |
| <i>Genotype 1a (H77)</i> | | | | |
| IFN α | >10000* | 2.1* | 9.4* | >4762 |
| 2'CmeC | >300 | 1.8 | 8.1 | >167 |
| NTZ | 49 | 0.33 | 1.1 | 149 |
| TIZ | 14 | 0.25 | 1.0 | 56 |

* Interferon expressed in 'IU/ml'

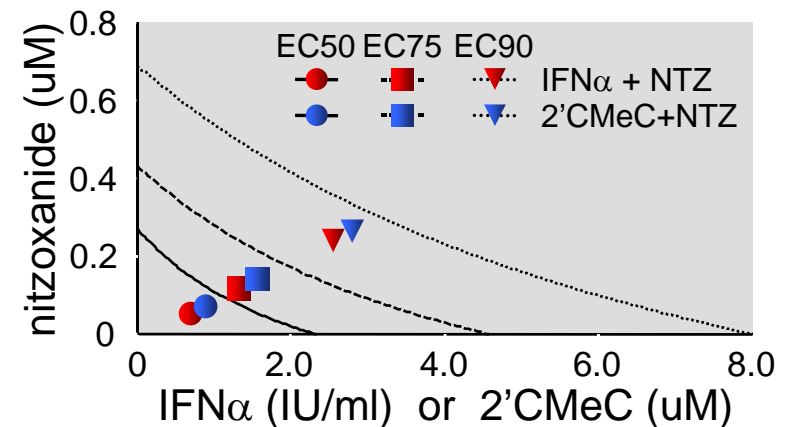
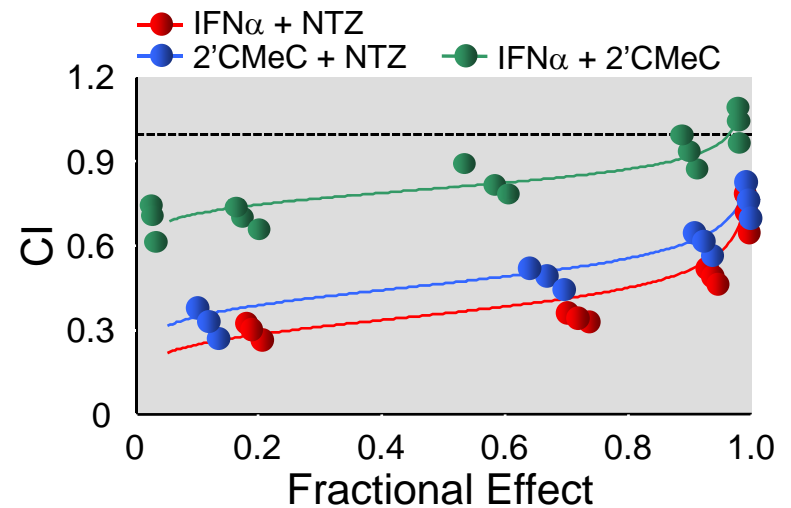
Activity against genotype 2a (replicon, JFH-1 infection) in progress

NTZ is synergistic in combination therapies

| COMPOUND | CC50 (uM) | Intracellular HCV RNA (uM) | |
|---|-----------|----------------------------|-------------|
| | | EC50 | EC90 |
| IFN α | >10000* | 1.9* | 8.9* |
| 2'CmeC | >300 | 1.6 | 8.3 |
| TIZ | 15 | 0.15 | 0.81 |
| TIZ+IFNα, 1:10 | 17 | 0.07 | 0.22 |
| TIZ+2'CmeC, 1:10 | 18 | 0.06 | 0.19 |

Interferon expressed in 'IU/ml'

Studies with VX950 in progress





NTZ pretreatment enhances subsequent interferon combination therapy

| Treatment (6 days total duration) | EC ₅₀ | EC ₉₀ |
|--|--|---|
| IFN α | 1.7 \pm 0.2 | 7.8 \pm 0.8 |
| 2'CmeC | 1.3 \pm 0.2 | 5.8 \pm 0.9 |
| NTZ | 0.20 \pm 0.02 | 0.92 \pm 0.10 |
| NTZ + IFN α , 1:10 | 0.09 \pm 0.010 | 0.24 \pm 0.04 |
| NTZ monotherapy (3 days), then NTZ+IFNα (3days) | 0.03 \pm 0.004[#] | 0.09 \pm 0.011^{\$} |
| NTZ +2'CmeC, 1:10 | 0.05 \pm 0.007 | 0.17 \pm 0.03 |
| NTZ monotherapy (3 days), then NTZ+2'CmeC (3 days) | 0.06 \pm 0.005 | 0.15 \pm 0.02 |

p = 0.0018 vs. EC₅₀ of 6 days treatment with NTZ + IFN α simultaneous combination

\$ p = 0.0026 vs. EC₉₀ of 6 days treatment with NTZ + IFN α simultaneous combination.

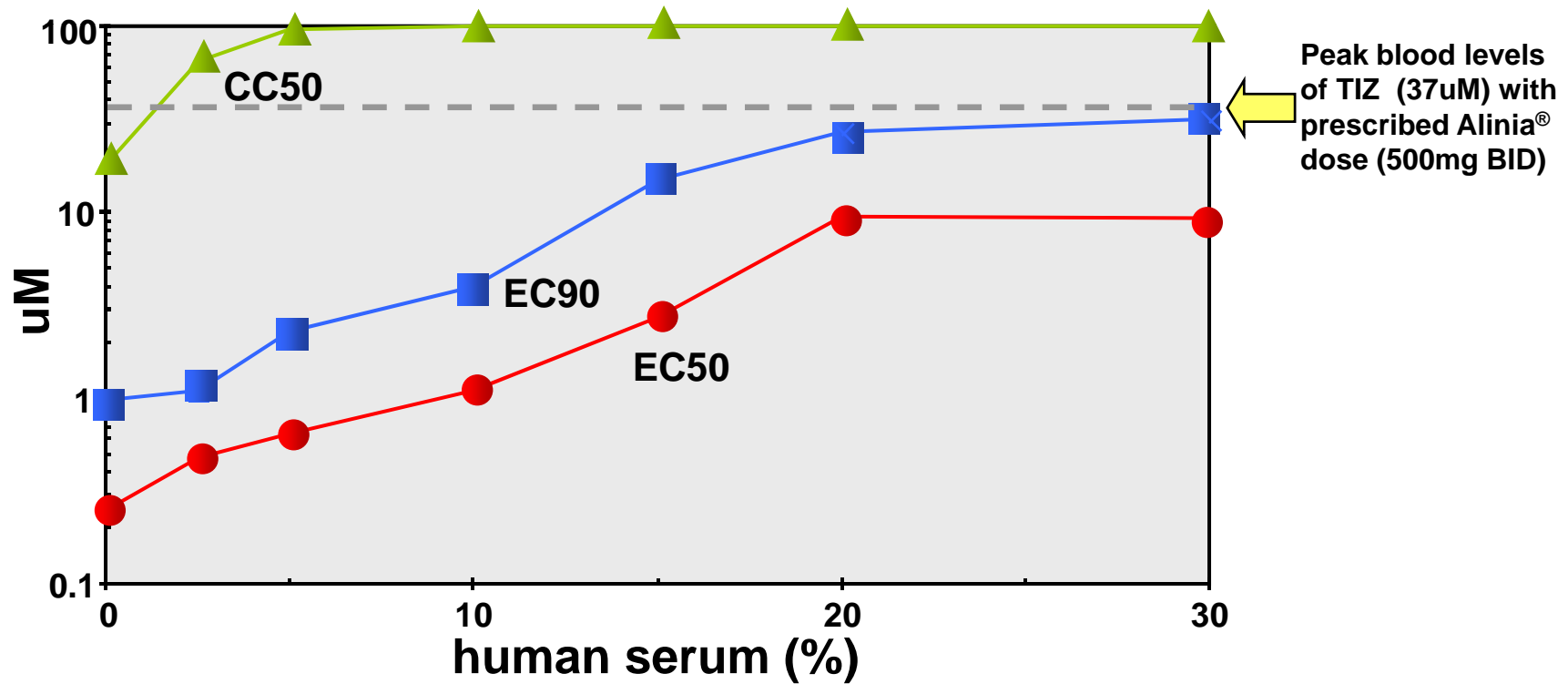


NTZ is effective against drug-resistant HCV mutants in replicon cell cultures

| | NTZ (uM) | | IFN α (IU/ml) | | 2'CmeC (uM) | | VX-950(uM) | |
|------------|----------|------|----------------------|------|-------------|------|------------|------|
| | EC50 | EC90 | EC50 | EC90 | EC50 | EC90 | EC50 | EC90 |
| Wild-type | 0.25 | 0.97 | 1.8 | 9.0 | 1.7 | 7.0 | 0.20 | 0.83 |
| NS5B S282T | 0.30 | 1.1 | 1.6 | 8.7 | 40 | >100 | 0.25 | 0.90 |
| NS3 A156T | 0.28 | 1.0 | 1.7 | 8.5 | 1.5 | 6.5 | >10 | >10 |
| NS3 A156S | 0.23 | 0.90 | 2.0 | 9.2 | 1.8 | 7.5 | >10 | >10 |

Activity against A516V (1b), R155K (1a) in progress

Effect of human serum on the anti-HCV potency of TIZ





NTZ is effective against HBV replication in 2.2.15 cells

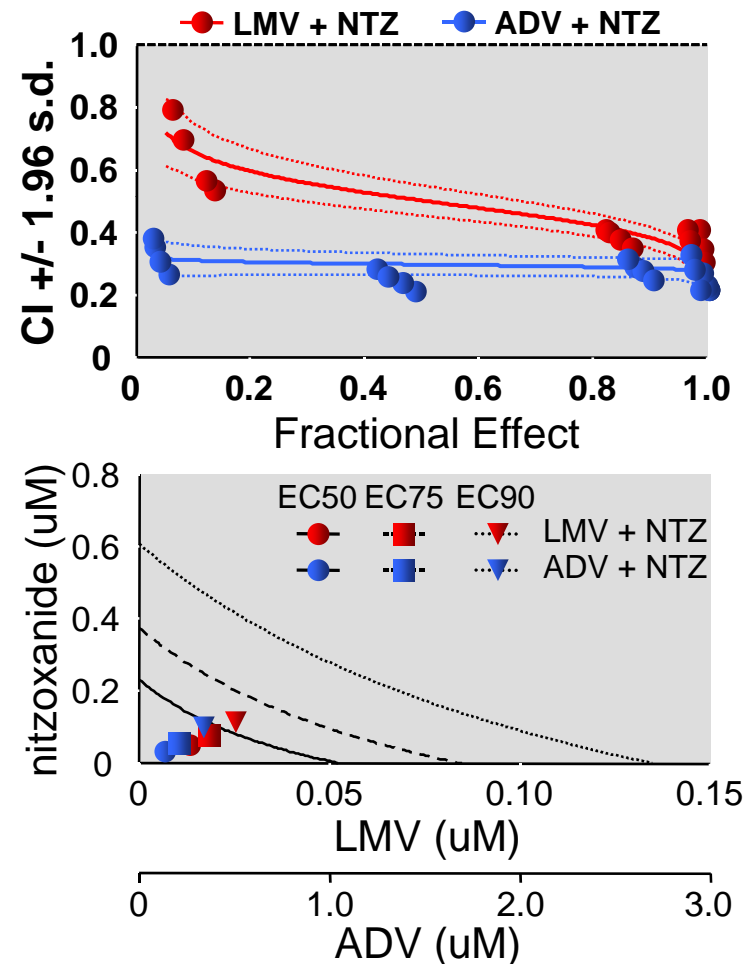
| COMPOUND | CC50 (uM) | Extracellular VIRION (uM) | | Intracellular DNA R.I. (uM) | | Selectivity Index (CC50/EC90) | |
|------------|-----------------|------------------------------|-------------|--------------------------------|------------|----------------------------------|---------------|
| | | EC50 | EC90 | EC50 | EC90 | Virion | RI |
| LMV | 2347 | 0.05 | 0.15 | 0.16 | 0.55 | 15646 | 4267 |
| ADV | >300 | 1.0 | 3.0 | | | >100 | |
| NTZ | >100* | 0.12 | 0.83 | 0.59 | 2.1 | >121 | >48 |
| TIZ | >100* | 0.15 | 0.58 | 0.46 | 1.2 | >172 | >83 |

* CC50 in dividing cultures, 20-30uM

NTZ is synergistic in combination therapies

| COMPOUND | CC50 (uM) | Extracellular VIRION (uM) | |
|---------------------|----------------|---------------------------|-------------|
| | | EC50 | EC90 |
| LMV | 2347 | 0.05 | 0.15 |
| ADV | >300 | 1.0 | 3.0 |
| TIZ | >100 | 0.15 | 0.58 |
| NTZ+LMV, 5:1 | >100 | 0.06 | 0.16 |
| NTZ+ADV, 1:3 | >100 | 0.03 | 0.11 |

Combination studies with ETV and TNF are in progress



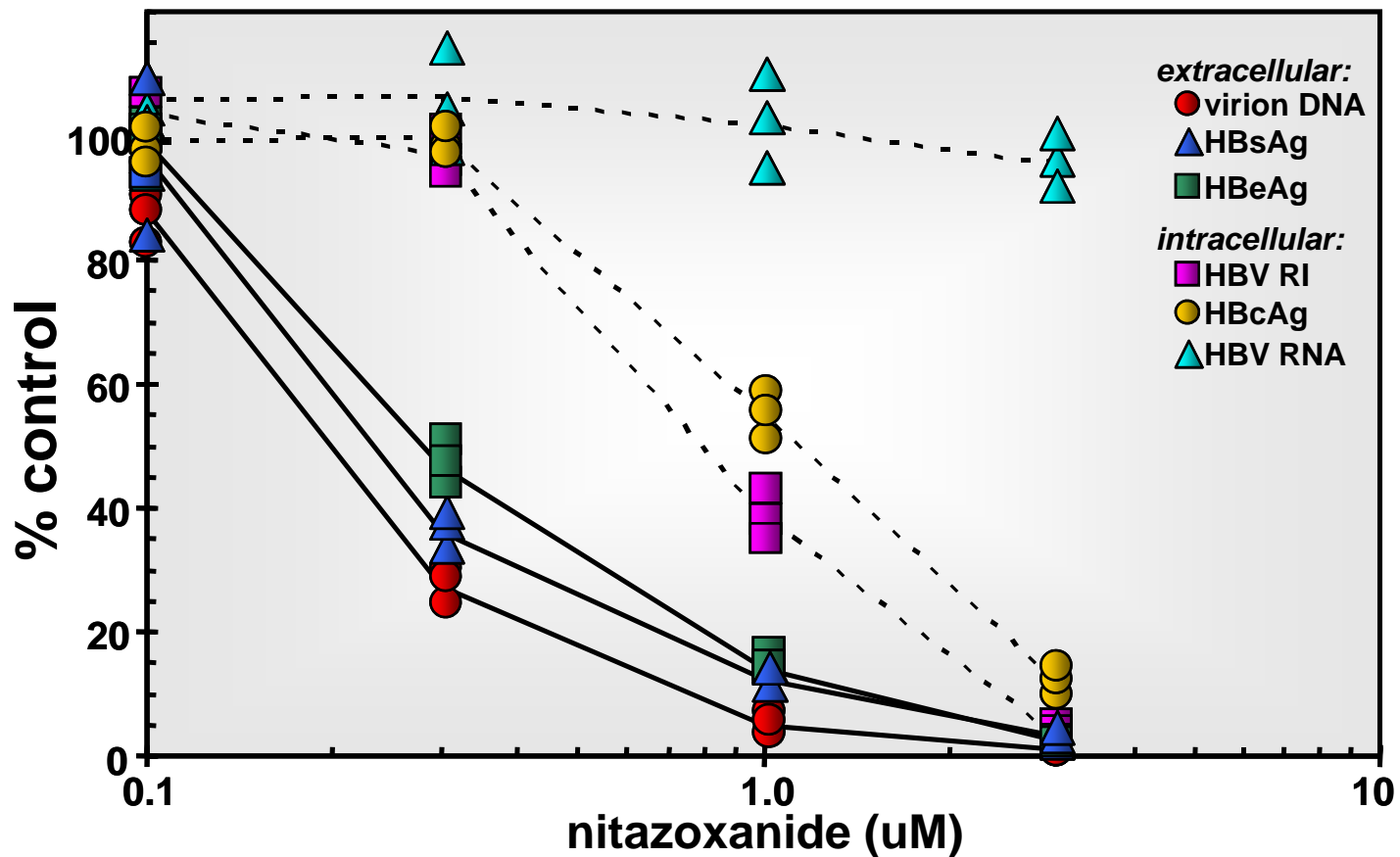


NTZ is effective against drug-resistant HBV mutants in cell culture

| | NTZ (uM) | | LMV (uM) | | ADV (uM) | |
|------------------|----------|------|----------|------|----------|------|
| | EC50 | EC90 | EC50 | EC90 | EC50 | EC90 |
| Wild-type | 0.21 | 0.77 | 0.21 | 0.93 | 2.0 | 7.0 |
| <i>pol</i> M204V | 0.15 | 0.70 | >100 | >100 | 1.5 | 7.2 |
| <i>pol</i> M204I | 0.31 | 1.0 | >100 | >100 | 2.5 | 8.5 |
| <i>pol</i> L180M | 0.23 | 0.80 | 16 | 46 | 2.6 | 7.3 |
| <i>pol</i> LM+MV | 0.18 | 0.72 | >100 | >100 | 2.5 | 7.6 |
| <i>pol</i> N236T | 0.28 | 0.85 | 0.31 | 1.2 | 11 | 32 |

Analysis performed using intracellular HBV R.I. following transient transfection of Huh7 cells

NTZ induces reductions in HBV proteins without affecting HBV RNA transcription



Analysis by semi-quantitative EIA and Northern Blot hybridization



HDV?

- Can the NTZ-induced loss of HBsAg be a future therapy for HDV infection?
- Clevudine-induced loss of WHsAg >2.0 logs was correlated with a loss of HDV viremia in chronically-infected woodchucks (Casey, *et al.*, AAC 49:4396-4399)



NTZ resistant cell lines can be selected

- HCV replicon cell lines that will grow in the presence of G418 and levels of NTZ or TIZ which are approximately 10X EC90 values (c.a. 10uM)(J. Glenn, unpublished observations)
- Cell lines selected with difficulty
- Cell lines grow poorly (slowly, difficult to confluence)
- Morphology changed significantly (more elongated, 'fibroblast-like')



Selected cell lines are less sensitive to both antiviral and cytotoxic effects of TIZ

| CELL LINE | CC50 (uM) | EC50 (uM) | EC90 (uM) | SELECTIVITY INDEX (CC50/EC50) |
|--|------------------|--------------------|--------------------|-------------------------------------|
| tizoxanide | | | | |
| RP7 | 19 ± 0.1 | 0.11 ± 0.02 | 1.0 ± 0.1 | 173 |
| NTZ-11 | 94 ± 4.5 | 0.94 ± 0.11 | 23 ± 3.3 | 100 |
| TIZ-9 | 88 ± 2.6 | 1.6 ± 0.2 | 32 ± 3.3 | 55 |
| 2'C-methyl cytosine | | | | |
| RP7 | >300 | 2.0 ± 0.1 | 7.0 ± 0.5 | >150 |
| NTZ-11 | >300 | 1.6 ± 0.1 | 7.7 ± 0.9 | >188 |
| TIZ-9 | >300 | 2.0 ± 0.1 | 8.0 ± 1.0 | >150 |
| IFNα 2b (values in 'IU/ml') | | | | |
| RP7 | >10000 | 1.0 ± 0.1 | 4.4 ± 0.5 | >10000 |
| NTZ-11 | >10000 | 0.11 ± 0.01 | 0.47 ± 0.04 | >90090 |
| TIZ-9 | >10000 | 0.16 ± 0.02 | 0.42 ± 0.04 | >62500 |

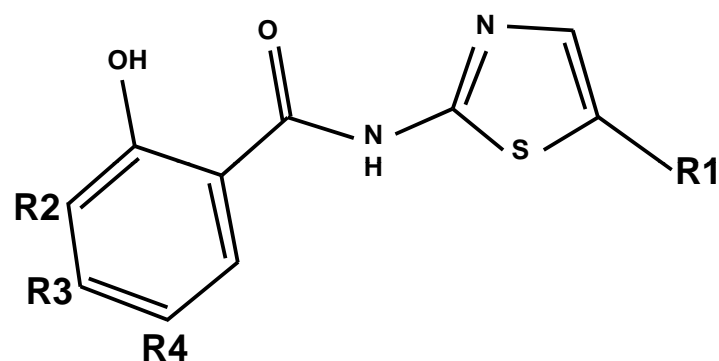
HCV replicon levels are approximately equal in all cell lines

TIZ-resistance phenotype is not transferred by HCV

| Treatment | Cell line source RNA (% mean untreated \pm S.D.) | | |
|--------------------|---|-----------------------------|-----------------------------|
| | TIZ-9 | NTZ-11 | RP7 |
| None | 100 \pm 7 | 100 \pm 14 | 100 \pm 16 |
| IFN @ 10IU/ml | 7 \pm 1 | 6 \pm 4 | 6 \pm 2 |
| IFN @ 100U/ml | nd | 0 | 0 |
| TIZ @ 1.0uM | 11 \pm 4 | 9 \pm 2 | 7 \pm 4 |
| TIZ @ 10uM | nd | 0 | 0 |
| 2'CmeC @ 10uM | 9 \pm 2 | 12 \pm 5 | 9 \pm 2 |

Based on G418^R colony formation following transient transfection of Huh7.5 cells (up to 5 expts. per treatment)

Other thiazolides have varying activities against HBV and HCV



| COMPOUND | HBV (uM) | | | HCV (uM) | | | R1 | R2 | R3 | R4 |
|---------------|----------------|---------------|---------------|-----------|---------------|---------------|-----------------------|-----------------|-----------------|-----------------------|
| | CC50 | EC50 | EC90 | CC50 | EC50 | EC90 | | | | |
| TIZ | >100 | 0.15 | 0.58 | 15 | 0.15 | 0.81 | NO ₂ | -- | -- | -- |
| RM4832 | >100 | 1.2 | 4.0 | 98 | 4.9 | 20 | Br | -- | -- | -- |
| RM4848 | >100 | 0.37 | 1.7 | 15 | >20 | >20 | Cl | -- | -- | -- |
| RM4850 | >100 | 0.33 | 0.83 | 12 | >20 | >20 | Cl | -- | -- | CH₃ |
| RM4851 | >100 | >10 | >10 | 5.6 | >20 | >20 | Cl | CH ₃ | -- | -- |
| RM4852 | >100 | 1.0 | 3.3 | 6.7 | >20 | >20 | Cl | -- | CH ₃ | -- |
| RM4863 | >100 | >10 | >10 | 13 | 0.04 | 0.59 | SO₂ | -- | -- | -- |

Panel of 71 analogues currently under study



Mechanisms = ?

- Antiviral mechanism(s) under investigation
- Broad spectrum activity indicates cellular, not viral target
- multiple (related?) cellular proteins/pathways most likely involved
 - divergent effects on HBV proteins, virion production, and replication suggest more than one target
- similar TIZ-induced alterations of cellular pathways most likely equates to different consequences for each virus
 - Intracellular environment induced by each virus produces differential responses of cellular factors to TIZ
- Activity against rotavirus and influenza A correlated with induction of *grp78* following infection of cultured cells (G. Santoro, *et al.*, ISAR, 2007)
 - Leads to inhibition of glycosylation of selected viral envelope proteins



Summary: *in vitro* studies

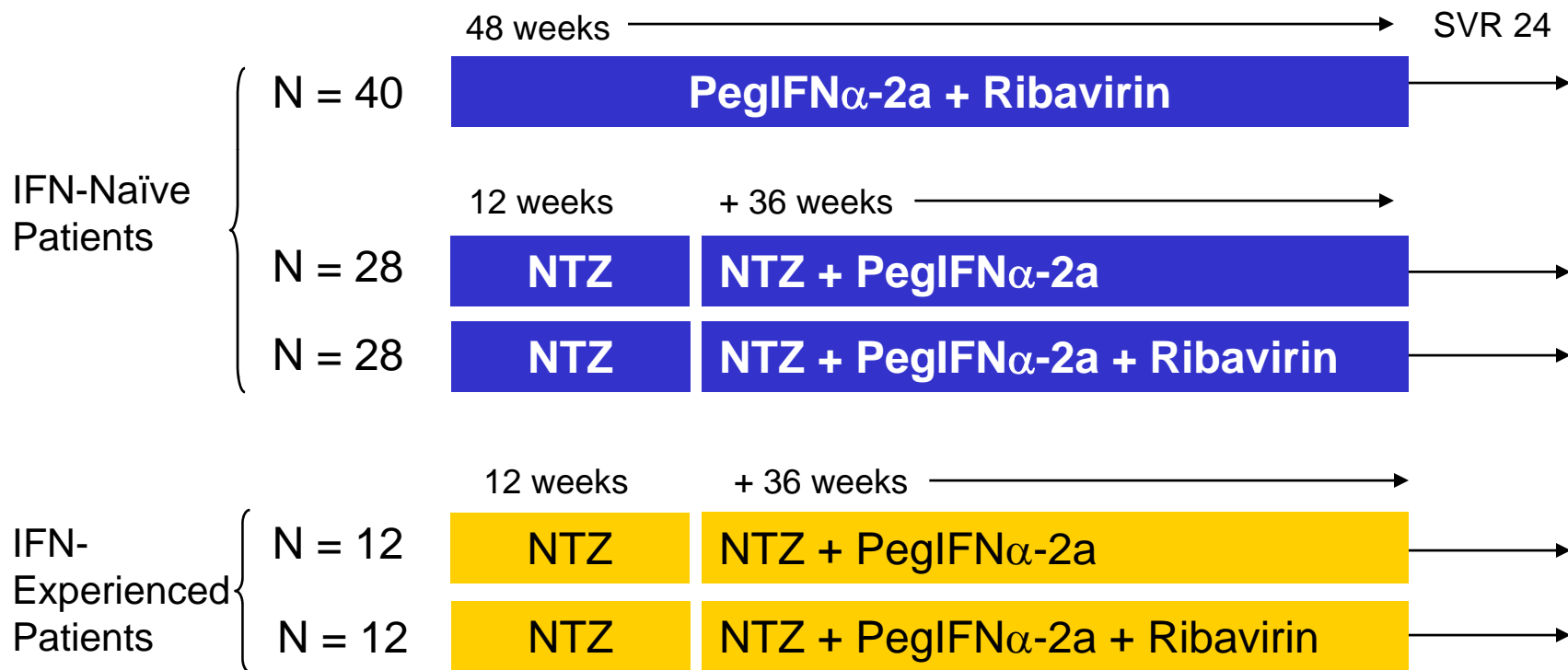
- NTZ exhibits selective inhibition of both HBV and HCV replication
- NTZ is effective against drug-resistant HBV and HCV mutants
- Viral resistance to NTZ not yet observed
 - Cellular resistance (HCV replicons) possible but extremely difficult
- NTZ displayed synergistic interactions with LMV or ADV against HBV, and with IFN α or 2'CmeC against HCV
 - Pre-treatment with NTZ further potentiated the effect of NTZ plus IFN α , but not NTZ plus 2'CmeC
- Novel target(s) and/or mechanism(s) and action make NTZ a candidate for use in combination treatments with current and future licensed and drugs
- antiviral mechanism(s) of action unknown and under active investigation
 - Indications for cellular target(s)



STEALTH C-1

- Reported at AALSD, November 2007
- Interim report (SVR12)
- Phase II randomized controlled trial
 - Patients with chronic hepatitis C genotype 4
 - Two sites in Alexandria and Tanta, Egypt
- 3 treatment groups
 - NTZ in dual therapy with PegIFN
 - NTZ in triple therapy with PegIFN-RBV
 - Standard of care control (PegIFN-RBV)
- Objectives: antiviral activity, safety

STEALTH C-1 Trial Design



NTZ (Alinia, Romark USA) : 500mg BID

PegIFN (Pegasys, Roche Switzerland): 180 μ g/week

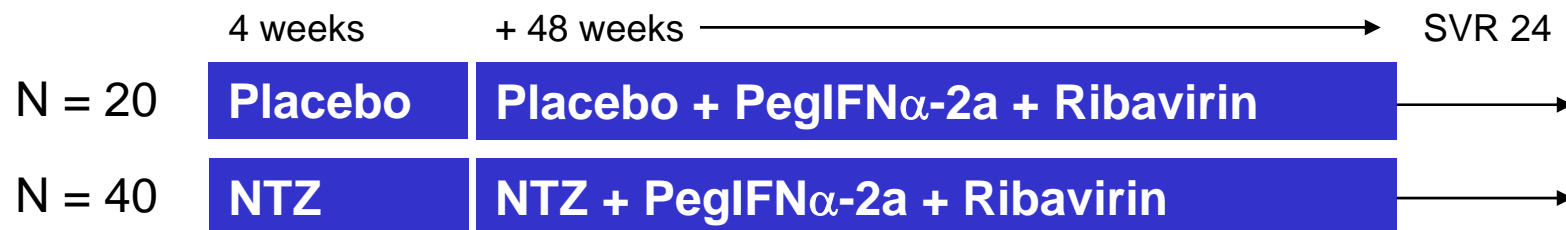
Ribavirin (Viracure, October Pharma, Egypt): weight based: 1000-1200 mg/day



US Trial in Nonresponders

STEALTH C-2 (ongoing, July 2007):

Patients with chronic hepatitis C genotype 1 that have failed to respond to pegylated interferon and ribavirin



NTZ (Alinia, Romark USA) : 500mg BID

PegIFN (Pegasys, Roche Switzerland): 180 μ g/week

Ribavirin (Copegus, Roche Switzerland): weight based: 1000-1200 mg/day



STEALTH C-1 Summary

In IFN-naïve patients with CHC genotype 4:

- Triple regimen
 - Higher SVR12 than standard of care
79% vs. 45% (P=0.007)
- Dual regimen
 - SVR12 not inferior to standard of care
68% vs. 45% (+19%; 95% CI: -7%, +42%)
- No increases or changes in side effects
- *Reduced duration of PegIFN + RBV by 12 weeks*



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