A Real-Time Cytotoxicity Assay That Delivers More Relevant Data

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Biological Outcomes in Cell Culture

Treatment

Small molecule

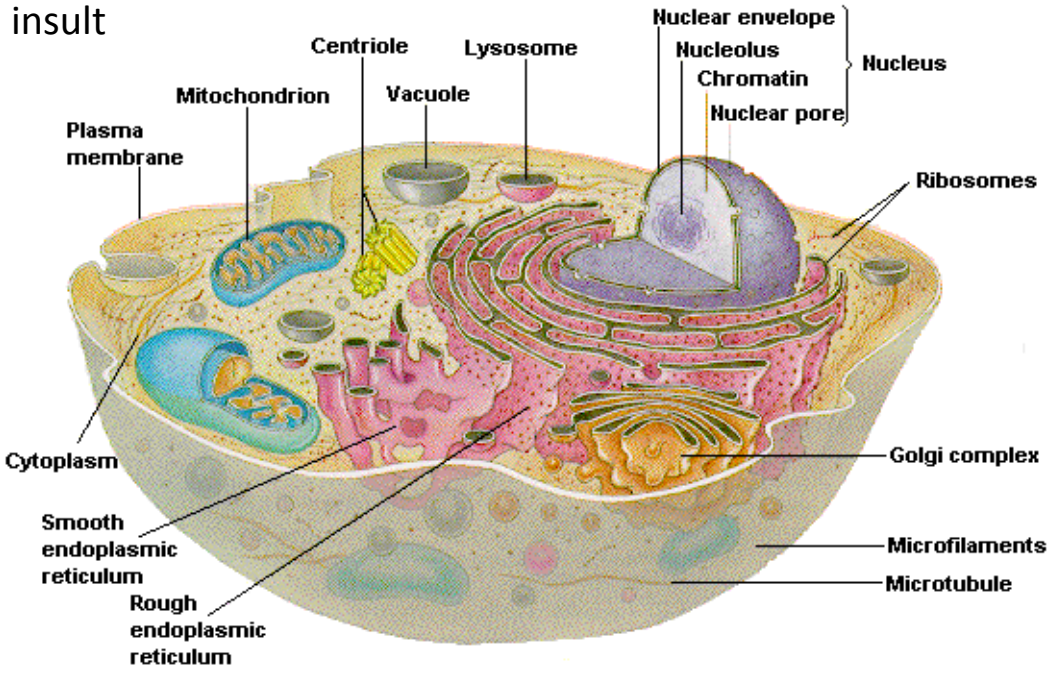
Biological

Industrial chemical

Transgene

Physical insult

Cause → **Effect**



Normal Proliferation

Enhanced Proliferation

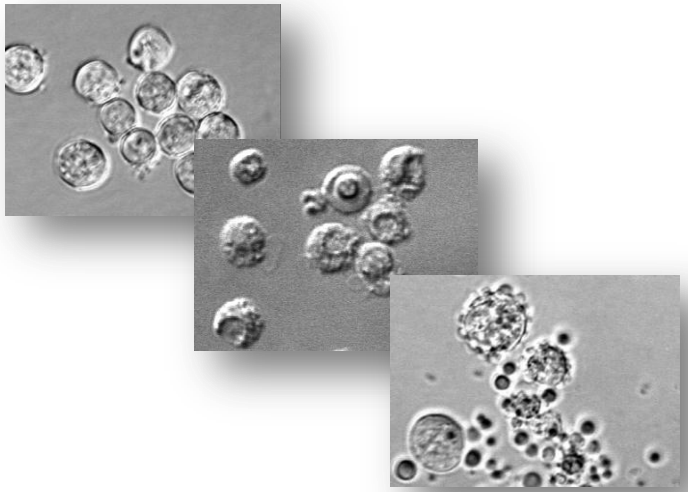
Cell-cycle Arrest
Senescence/Quiescence
Autophagy
Primary Necrosis
Apoptosis
Necroptosis
Secondary Necrosis

"Cytotoxicity Phenotype"

Is My Compound or Treatment Toxic?

Rarely a simple answer

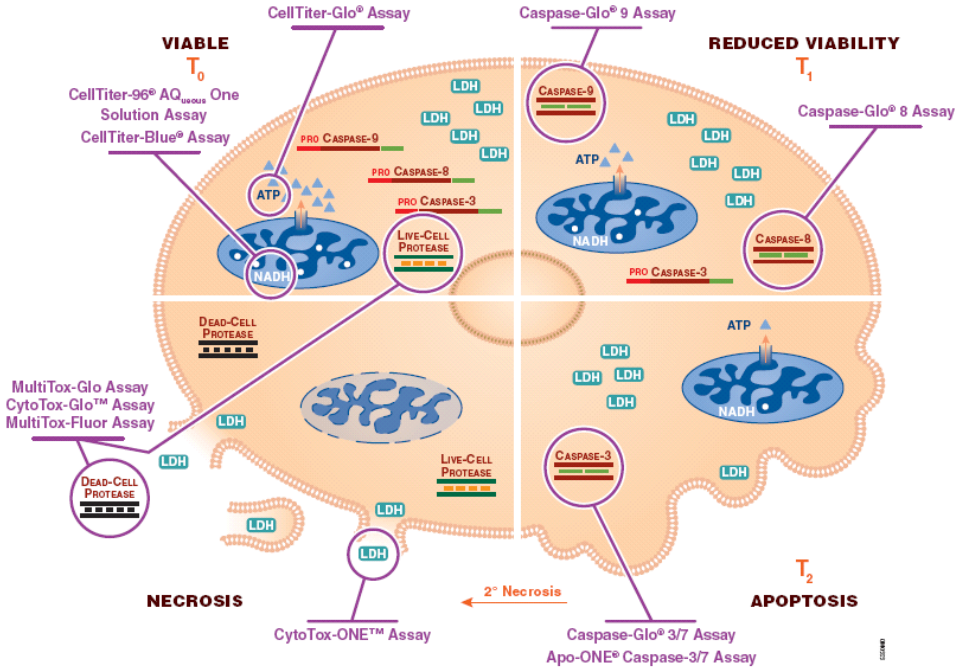
Often subject to experimental qualification.



The biological “profile” of any treatment is dependent upon:

1. **Dosage**
   - Addressed through serial dilution series
2. **Exposure time** (cells with compound contact)
3. **Mechanism of action** of the test compound
4. **Cell type**
   - Specific Target
   - Off Target

Biomarkers of a cytotoxic response



As cells lose vitality and health,

Viability biomarkers

- Metabolism biomarkers (ATP, reductase)
- Constitutive enzymatic biomarkers

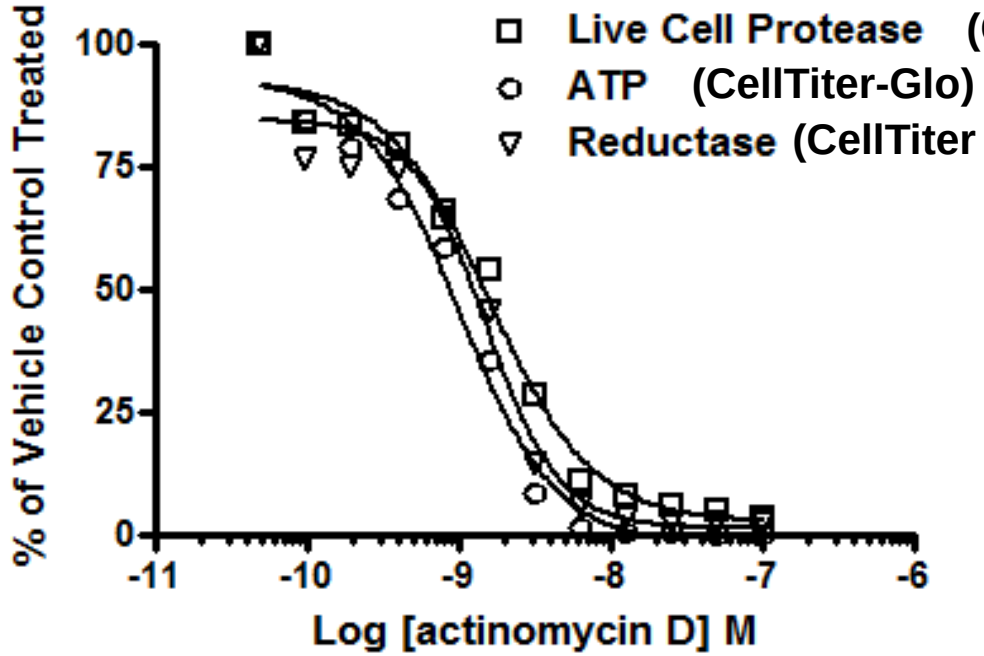
Cytotoxicity biomarkers

- Constitutive enzymatic biomarkers
- Inducible biomarkers

Viable “Stress” biomarkers anti-proliferative effects MOA death **Non-Viable**

Elapsed Time

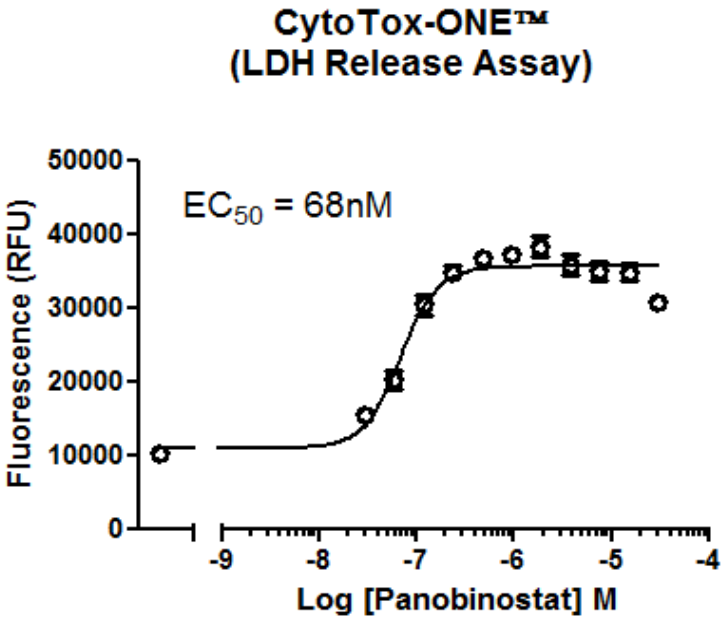
Viability Assays (in general) show good concordance with loss of membrane integrity

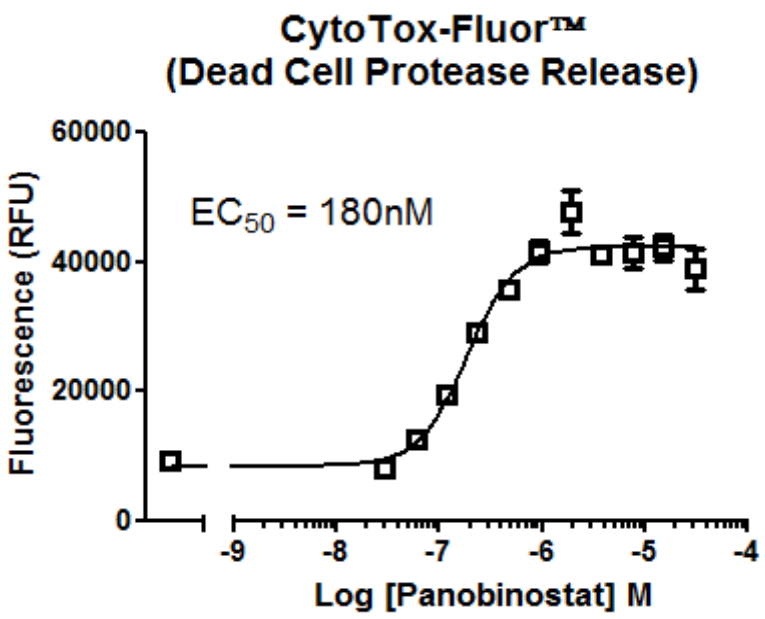


	Live Cell Protease	ATP	Reductase
EC50	1.589e-009	1.005e-009	1.499e-009

Reliably report the relative number of viable cells… but don't differentiate between cytotoxicity and cytostasis

Conventional Cytotoxicity Assays

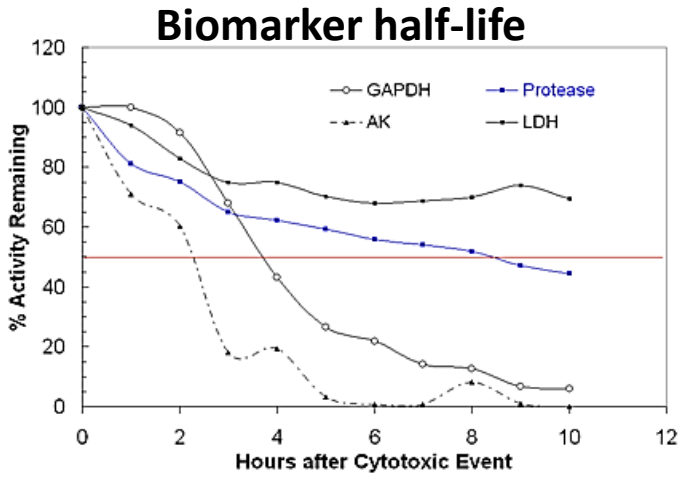




iCell™ Hepatocytes dosed for 72hr with panobinostat, endpoint chemistries added.

Definitive for cell death. Utility dependent upon kinetics of cytotoxic event…

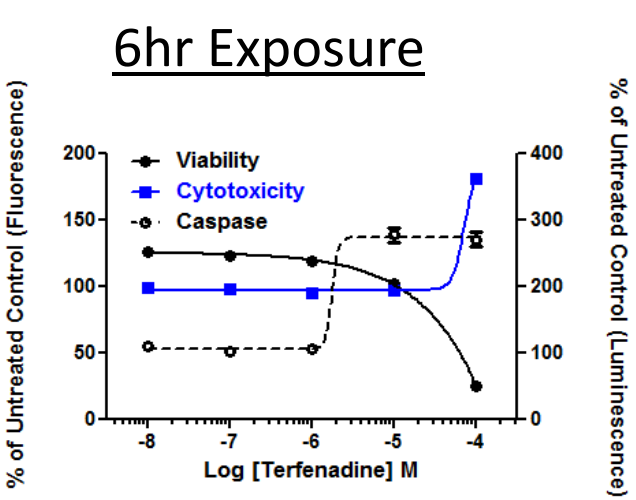
Current Cytotoxicity Assay Limitations

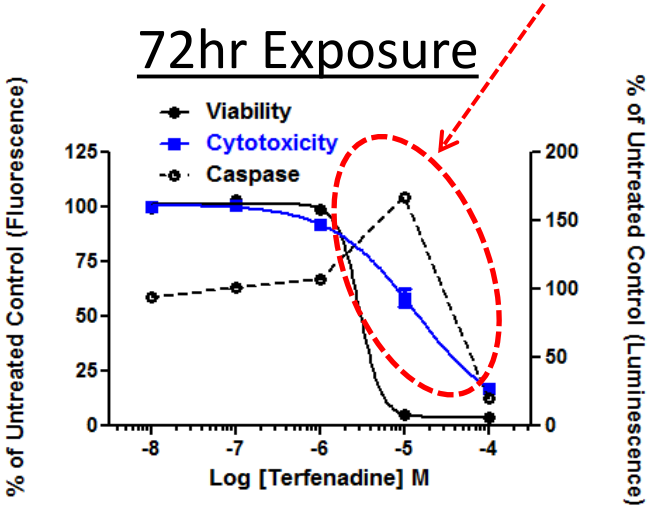


Activity-based cytotoxicity measures:

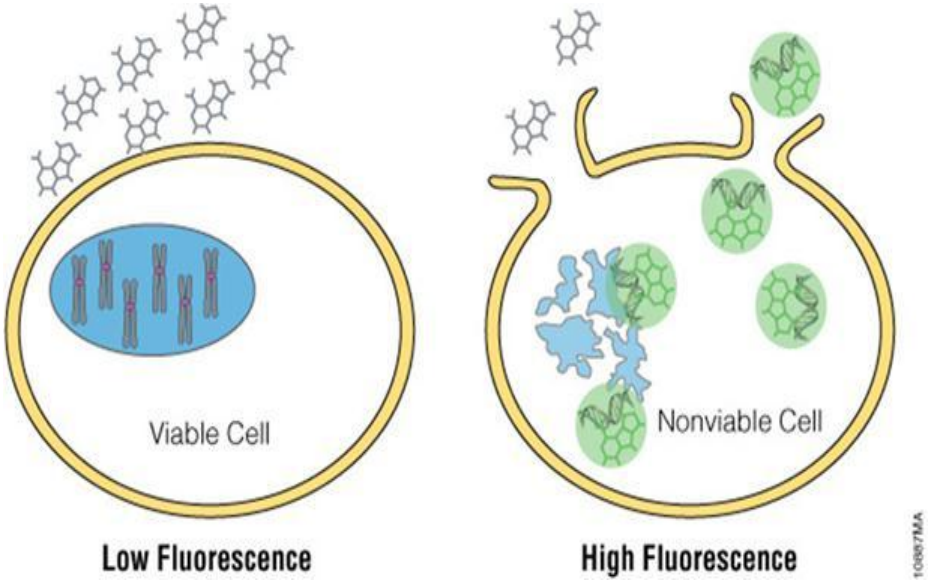
1. are susceptible to biomarker degradation
2. can underestimate cytotoxicity in long-term exposure models.

Caspases have a half-life, too.

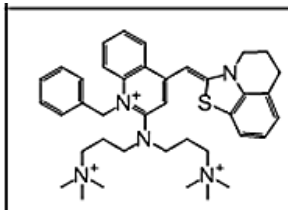




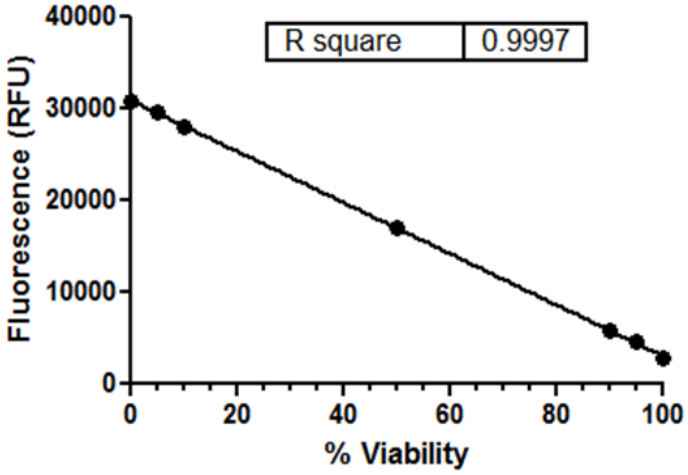
What About Dye Excludability?



CellTox™ Green Dye is a cell impermeant asymetric cyanine dye with affinity for genomic DNA.

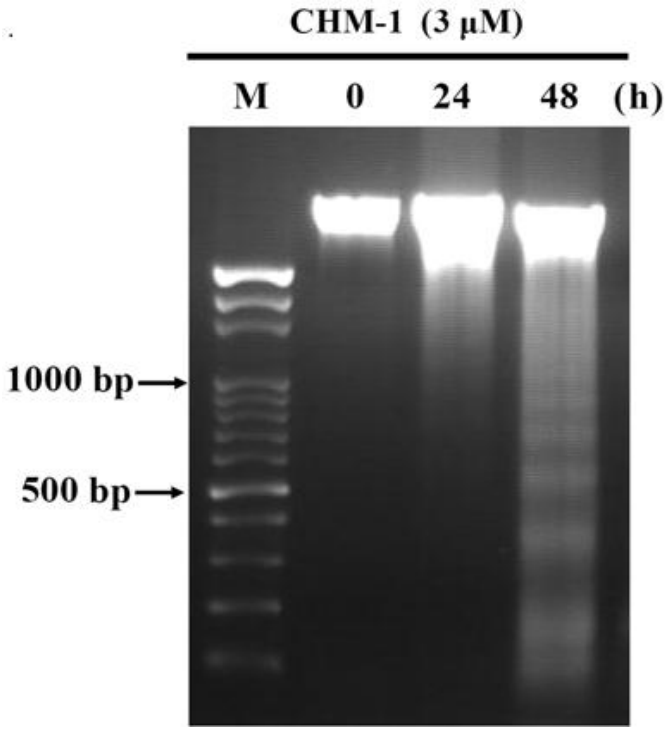


	4174	Ex = 512 nm Em = 532 nm ε = 88550 Φ_{dsDNA} = 0.28 Φ_{oligo} = 0.54

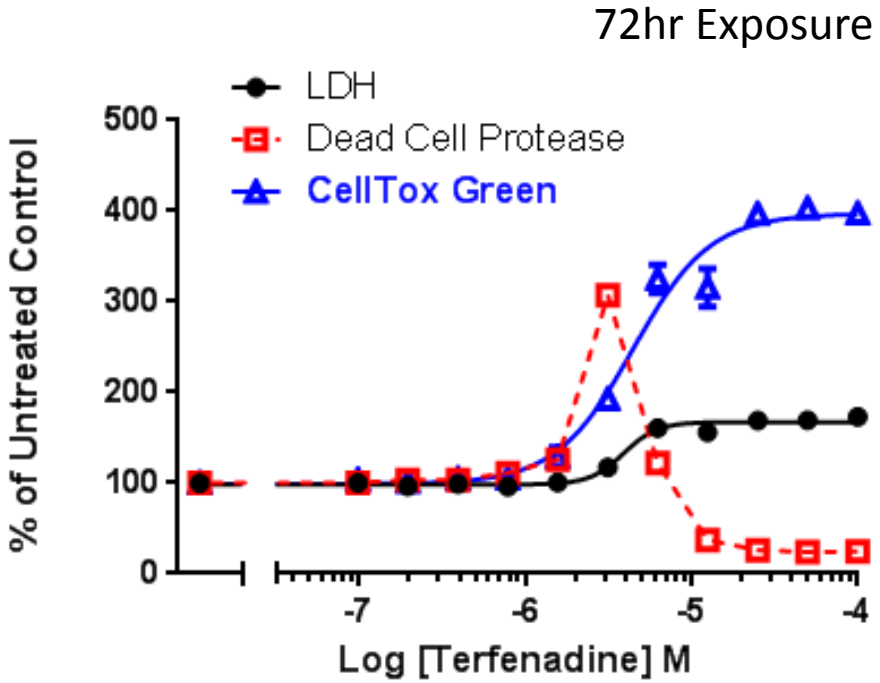


Signal intensity is proportional to cell death

DNA as a Biomarker of Cytotoxicity

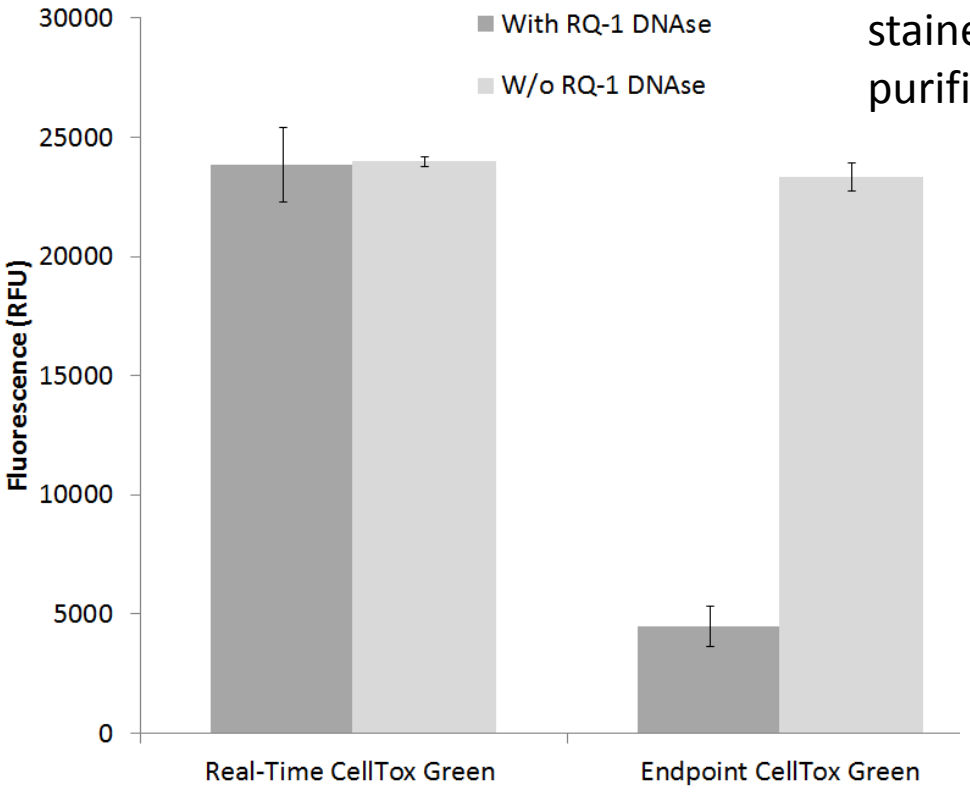


Chen et al (2010) Anti-Cancer Research 30:4187

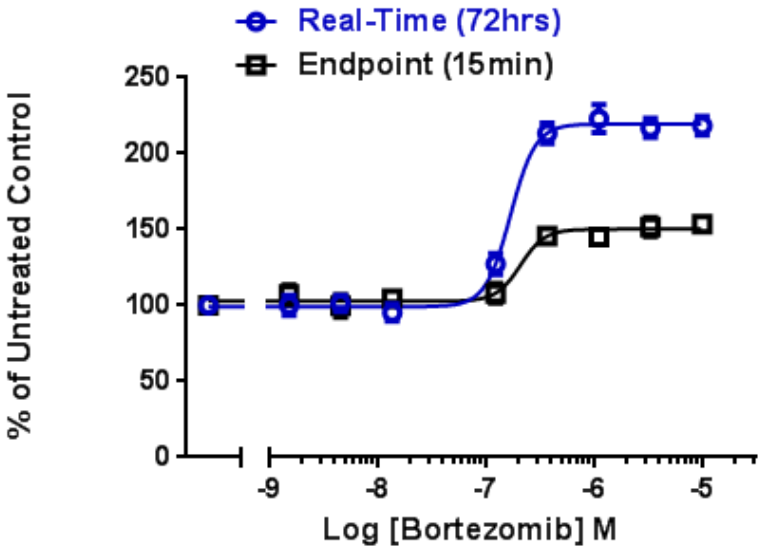


DNA degrades too, but retains sufficient base-pair pairing to facilitate staining

CellTox™ Green Staining Actually Protects DNA from Nuclease Activity

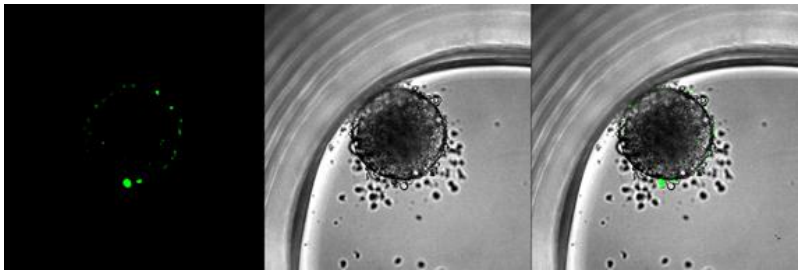


Purified genomic K562 DNA stained before or after addition of purified nucleases



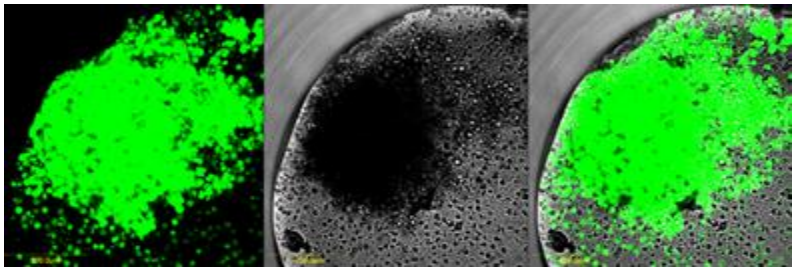
Non-Toxic, Allowing Real-Time Format

Untreated HCT-116 spheroid at 72hrs

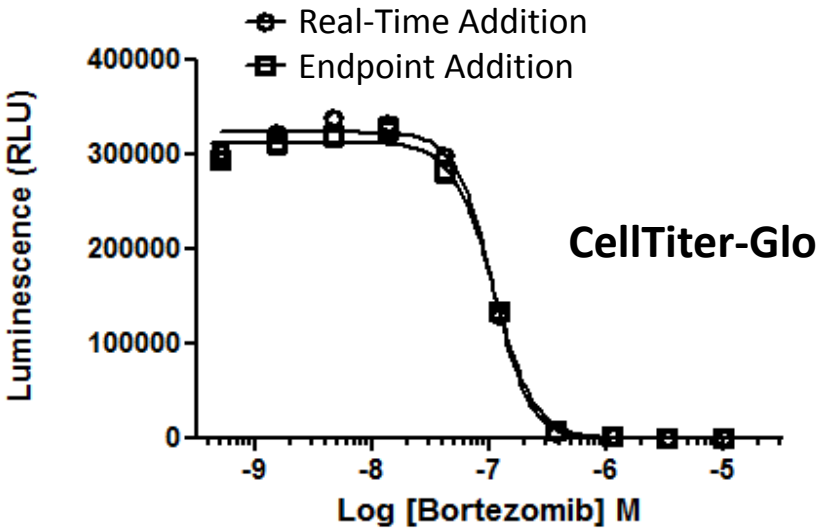


Fluorescence Bright Field Merge

Panobinostat-treated HCT-116 at 72hr



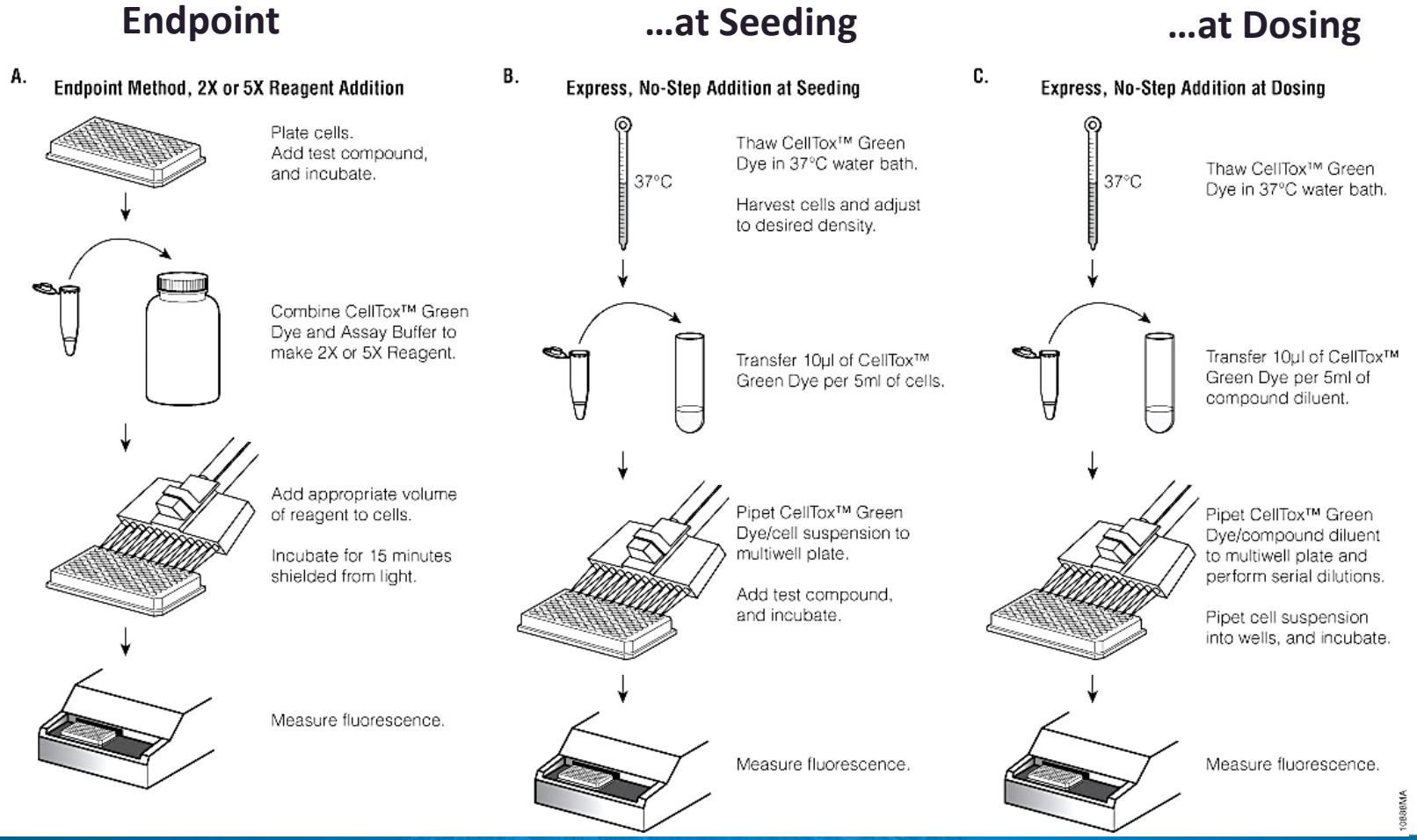
Fluorescence Bright Field Merge



Spheroids were formed using "hanging drop method" courtesy of InSphero

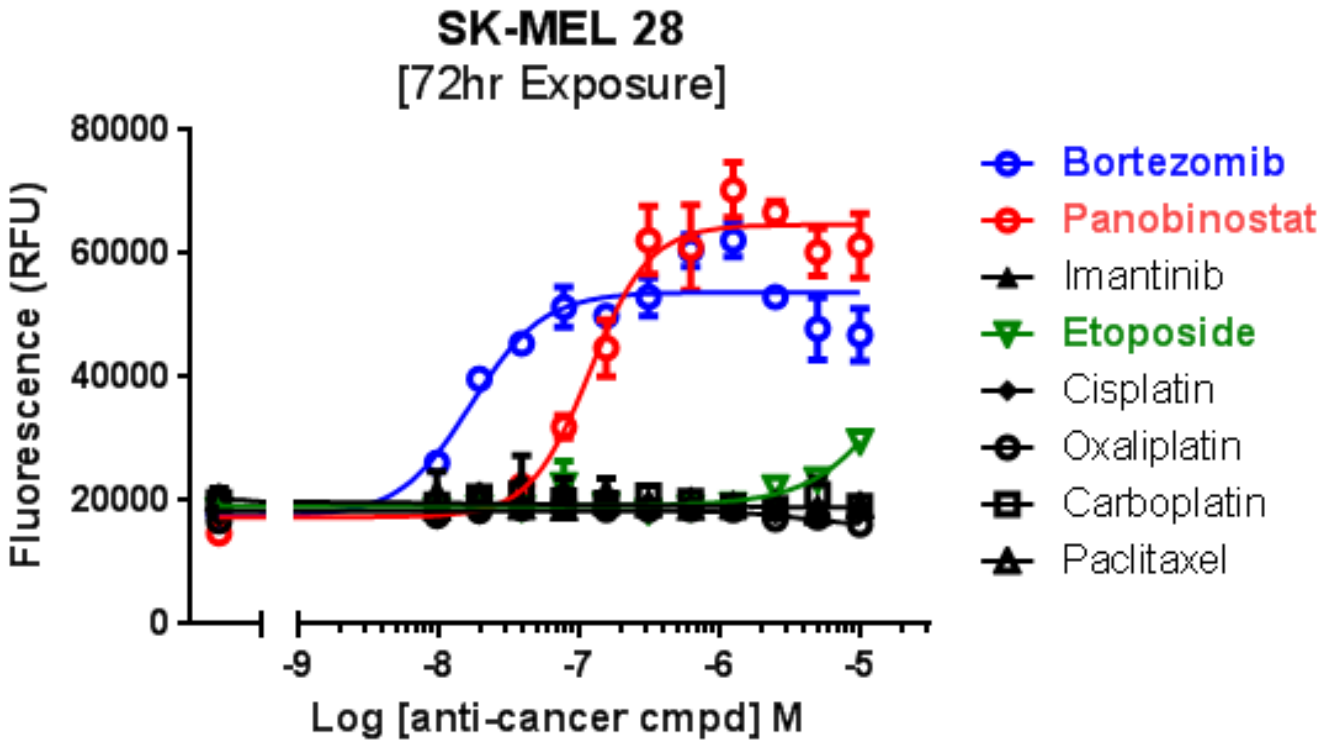
CellTox™ Green is non-toxic and doesn't influence dose-dependent cytotoxicity measures

Three Reagent Delivery Formats

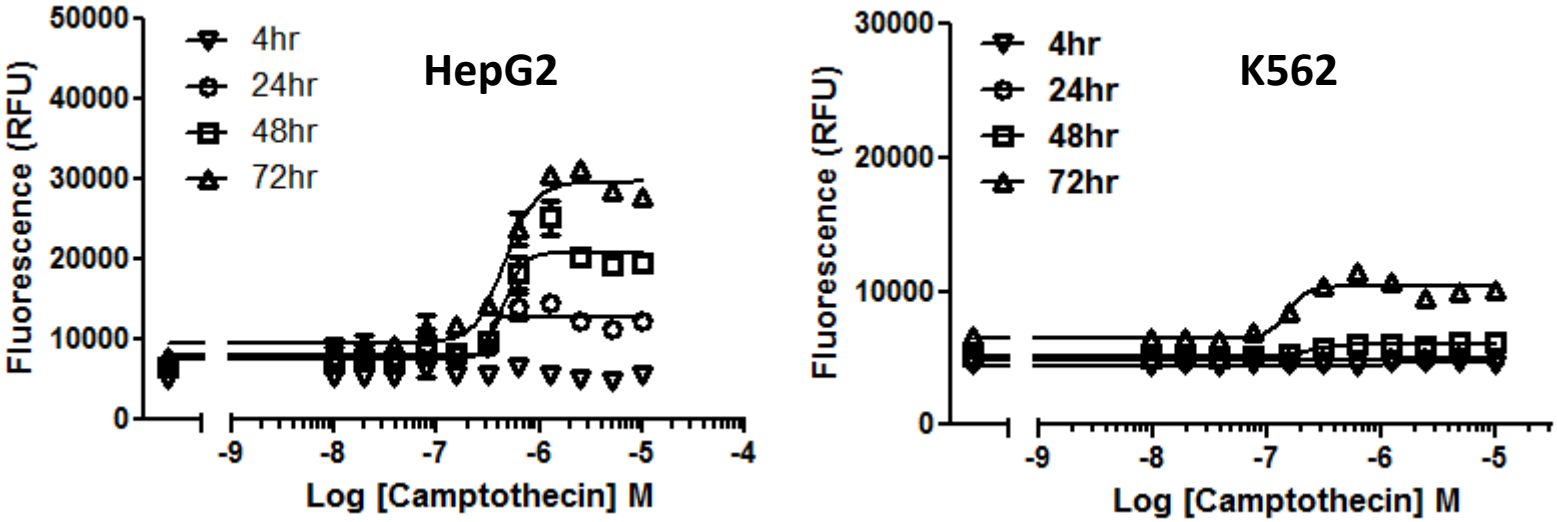


Rank Order Cytotoxicity for Chemo-sensitivity Testing

No confounding results due to cell death detection chemistry used.



Real-Time Cytotoxicity Format Reduces Effort



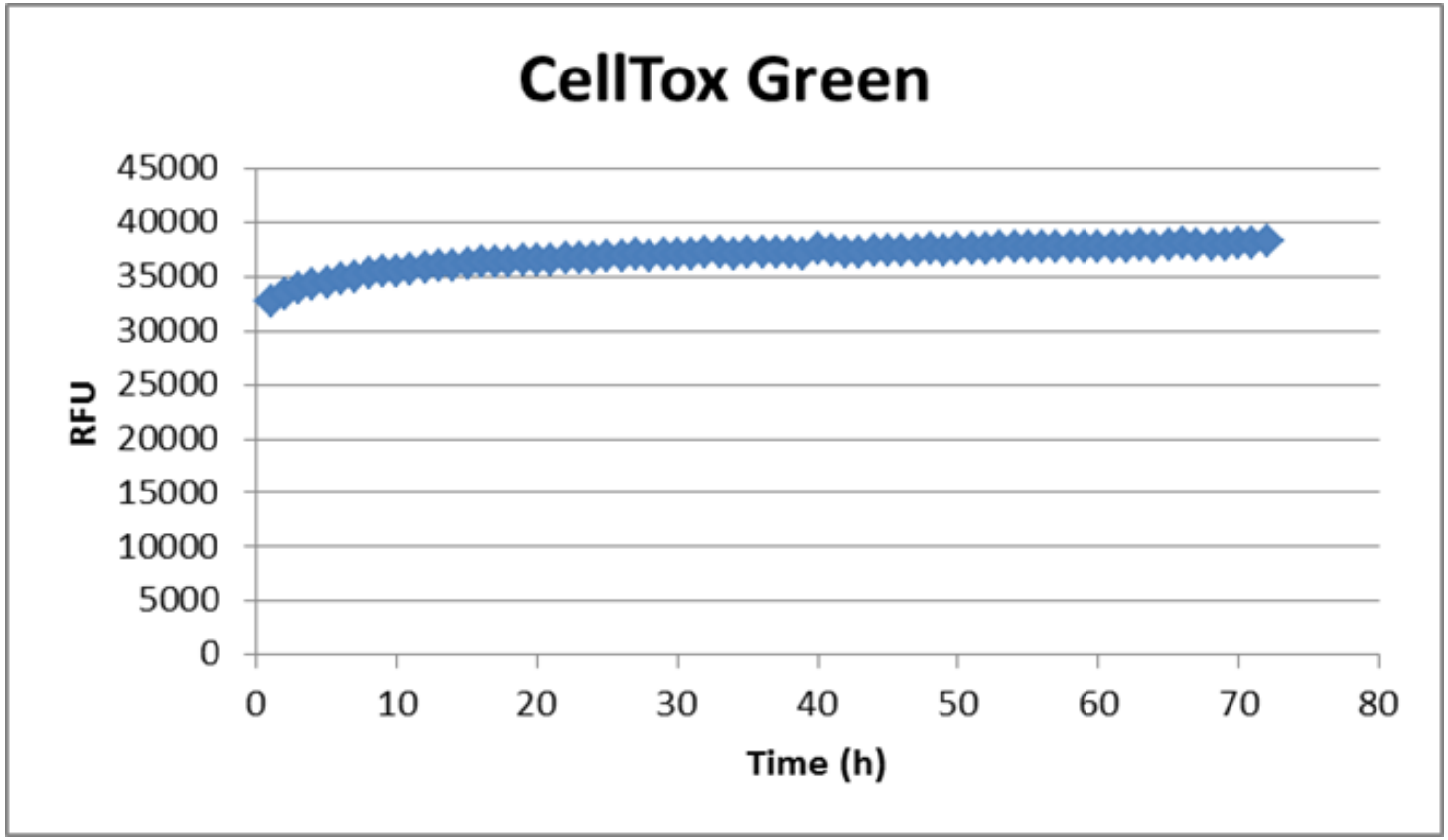
CellTox™ Green Dye added at plating or dosing:

1 plate measured/re-measured at 4-72 hours

Typical End-Point Assays:

4 plates to measure at 4, 24, 48 and 72 hours

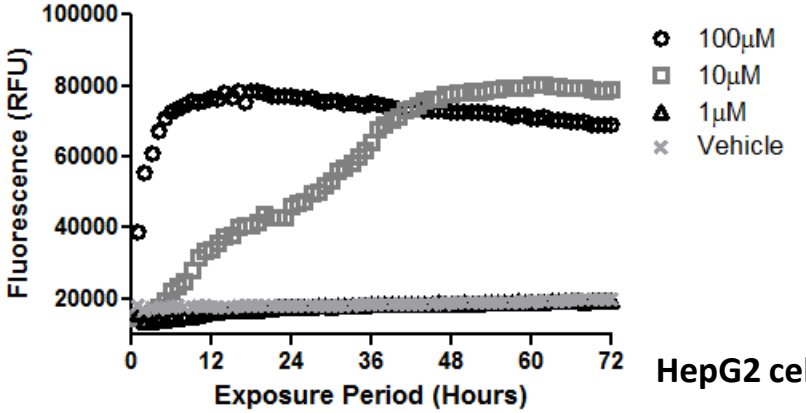
Photo-Stable Reagent Format



Fluorescence meaured every hour for 72h

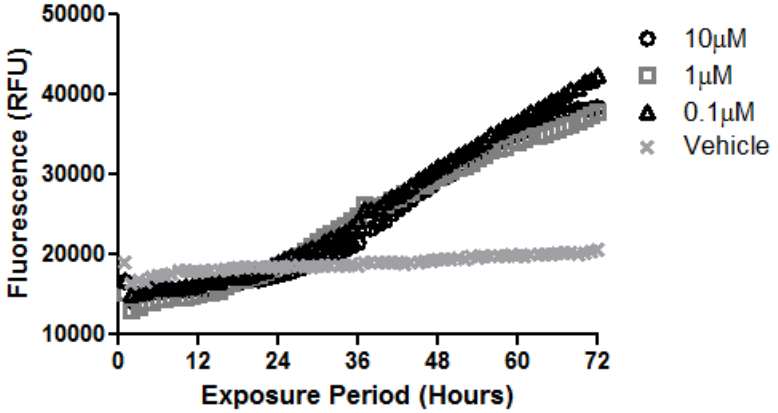
Real Time Kinetic Emergence

Terfenadine



HepG2 cells

Nocodazole



CellTox™ Green Dye added at dosing. Fluorescence measured every hour using Tecan Infinite™ 200 Pro with Gas Control Module™.

Examining Cytotoxic MOA by Multiplexing

Viable → "Stress" biomarkers → anti-proliferative effects → MOA death

Elapsed time

Dose Cells in the presence of CellTox™ Green

Monitor for increases in fluorescence

Luminescent Viability

Anti-proliferative?

(Terminal endpoint)

Fluorescent Viability

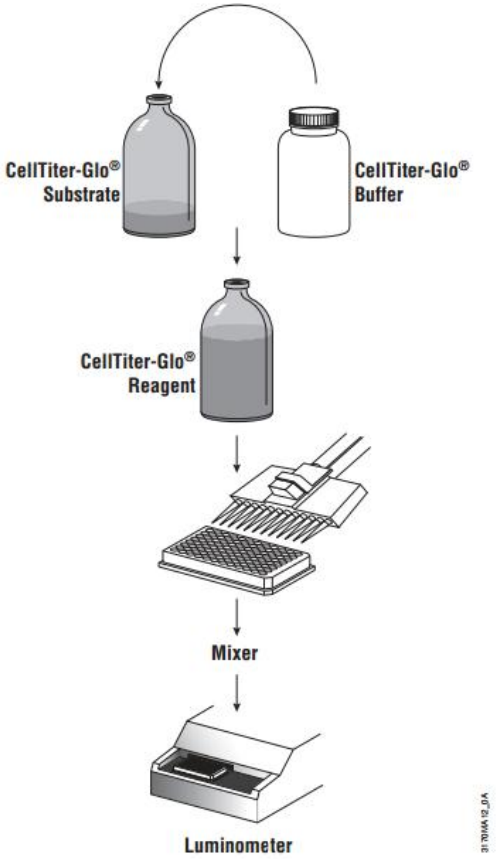
Anti-proliferative?

Luminescent Apoptosis Assay

Caspase Activation?

Luminescent and fluorescent cell-based assays can be multiplexed in a single well, in a defined sequential order!

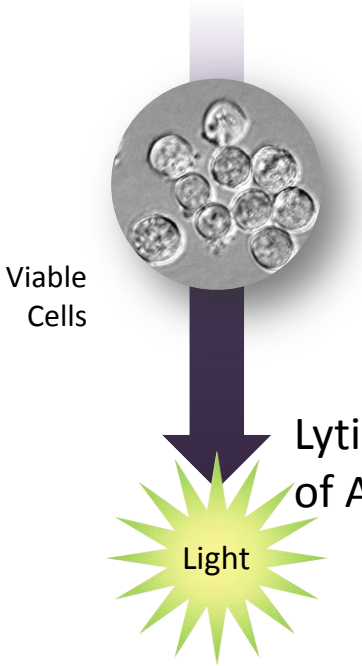
CellTiter-Glo®: An ATP Viability Assay



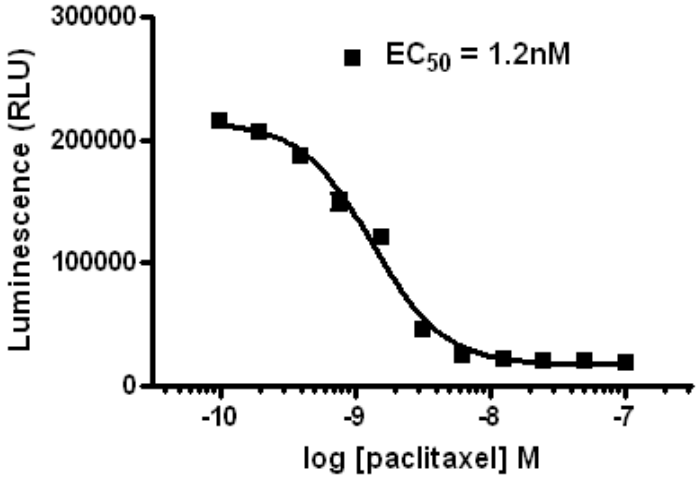
Measure **Luminescence**

CellTiter-Glo® Reagent

(Ultra-Glo™ Luciferase
Luciferin)



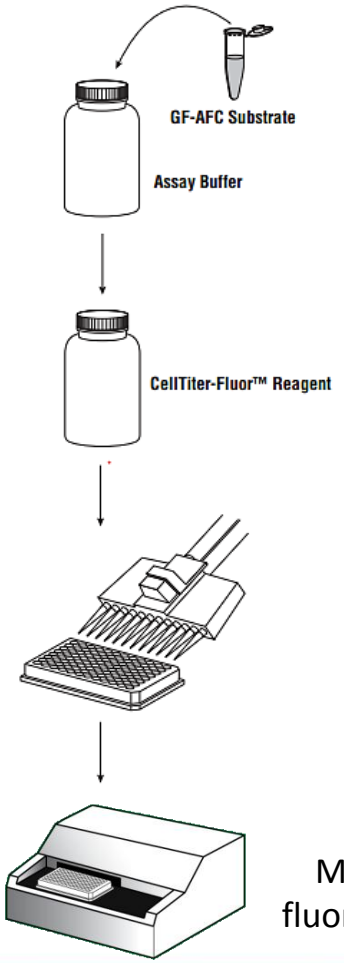
Light production is proportional to live cell number



Advantages:
- Sensitivity
- Scalability
- Speed to first result

Disadvantages:
-Terminal assay
- Requires luminometer

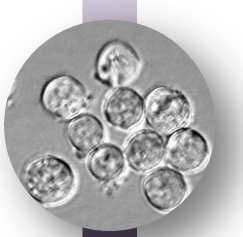
CellTiter-Fluor™: A Protease Viability Assay



Measure fluorescence

CellTiter-Fluor® Reagent

Cell-permeable profluorogenic peptide substrate

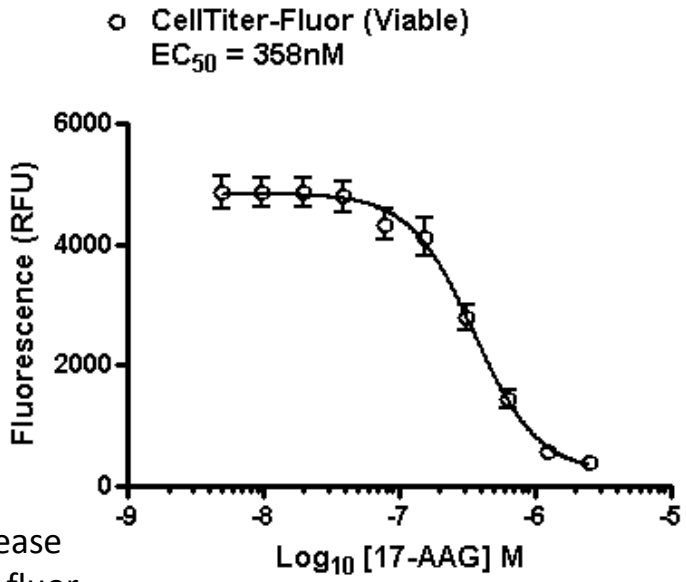


Viable Cells

Live cell protease liberates AFC fluor

AFC

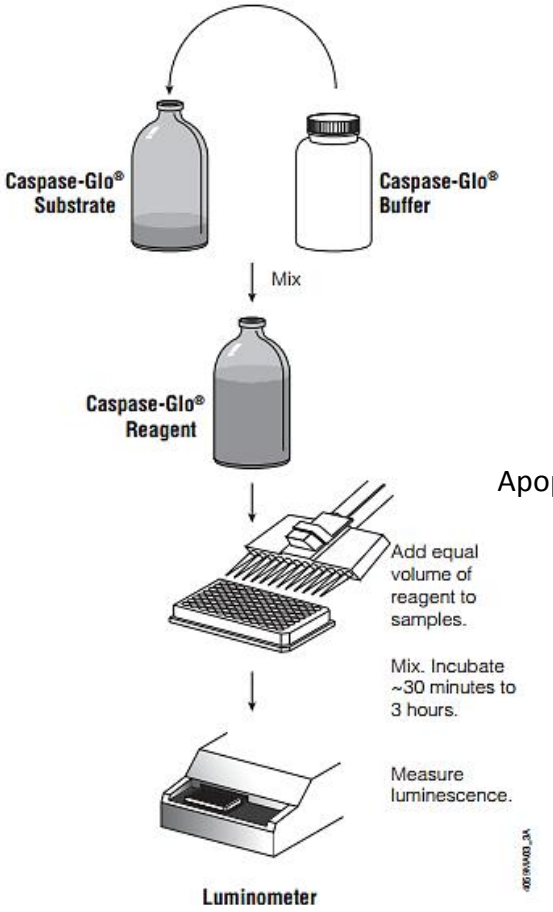
AFC production is proportional to live cell number



Advantages:

- Sensitive
- Scalable
- Compatible for multiplexing

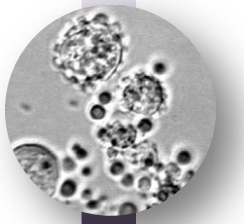
Caspase-Glo® 3/7: Caspase Activity Assay



Measure Luminescence

Caspase-Glo® 3/7 Reagent

Z-DEVD-Luciferin
Ultra-Glo™ Luciferase
ATP

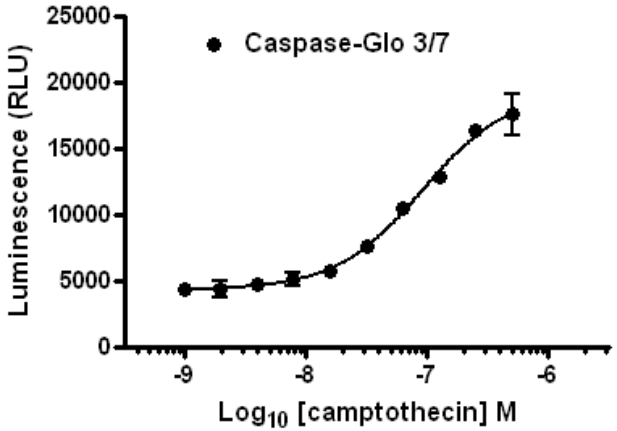


Apoptotic Cells

Activated caspase cleaves substrate liberating aminoluciferin

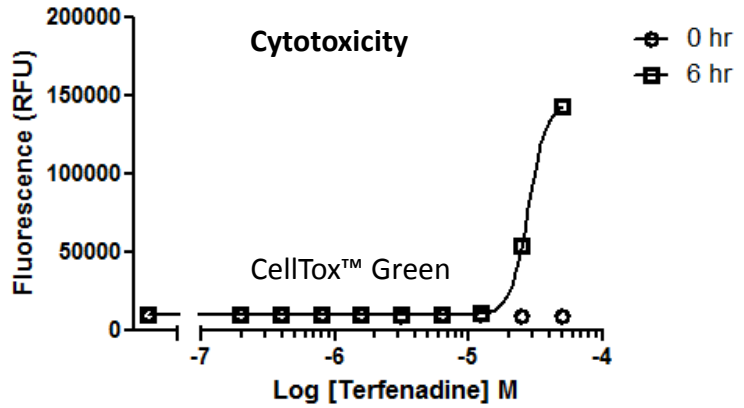
Light

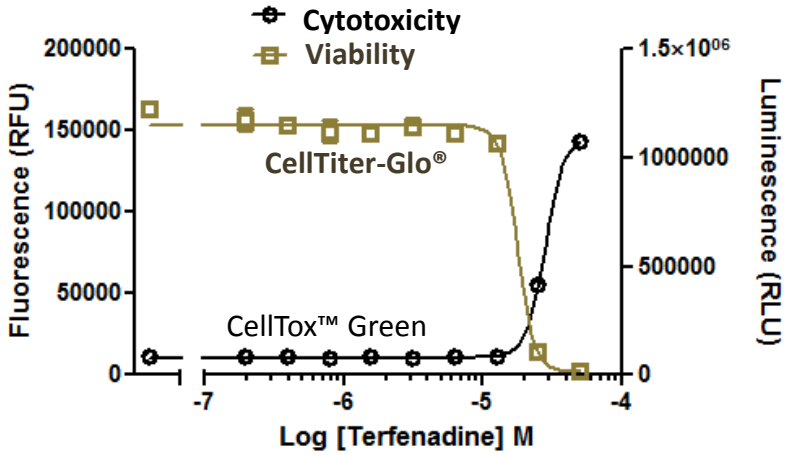
Light production is proportional to caspase-3/7 activity

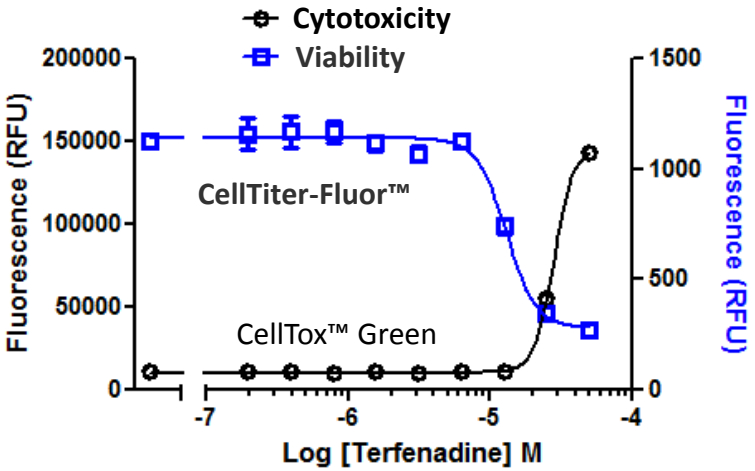


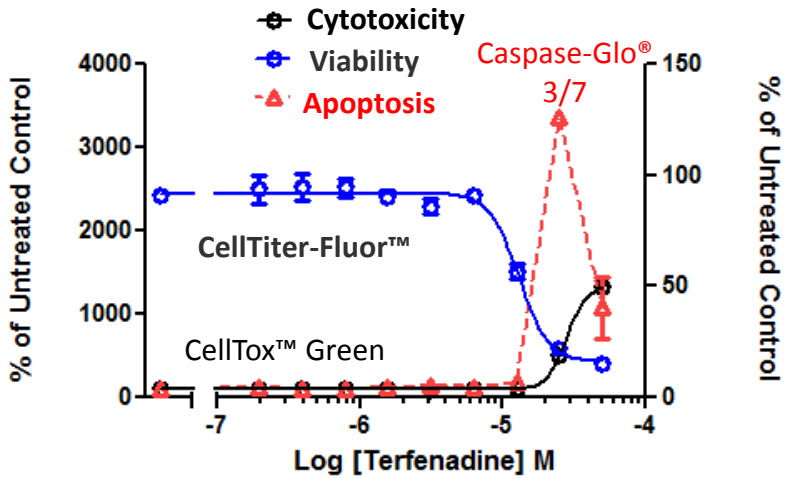
Caspase activity is definitive for apoptosis

Terfenadine (Non-Sedating Antihistamine)



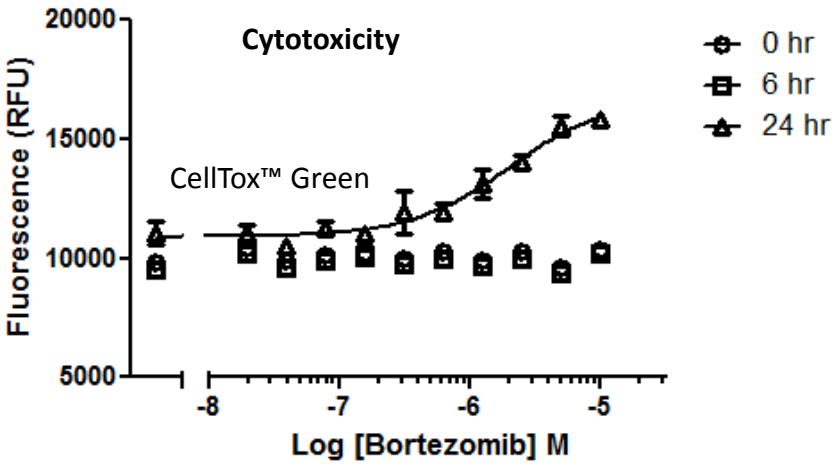


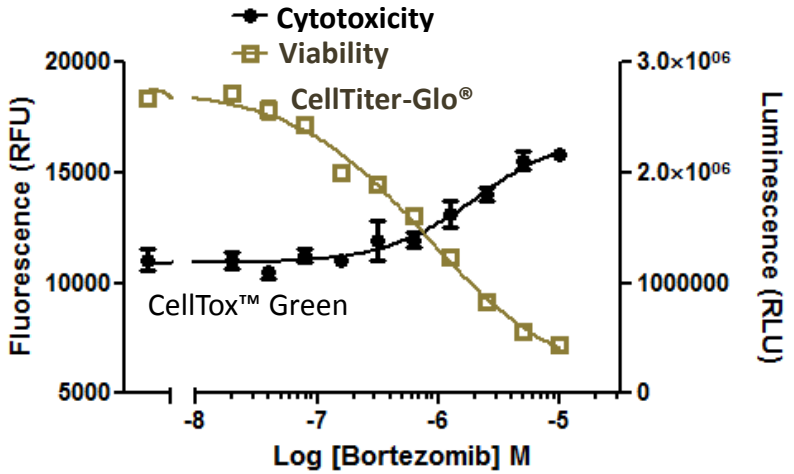


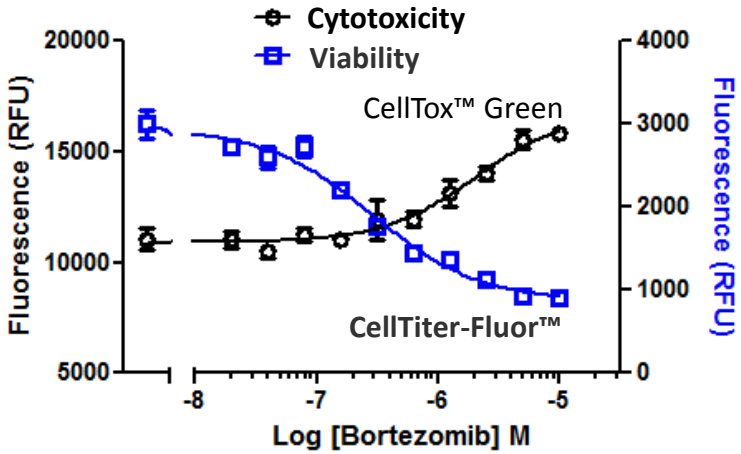


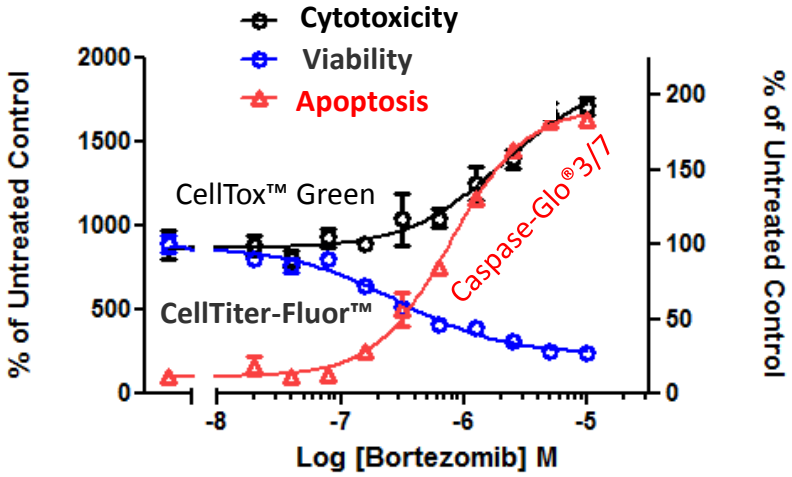
MOA = Fast Apoptosis

Bortezomib (Proteasome Inhibitor)



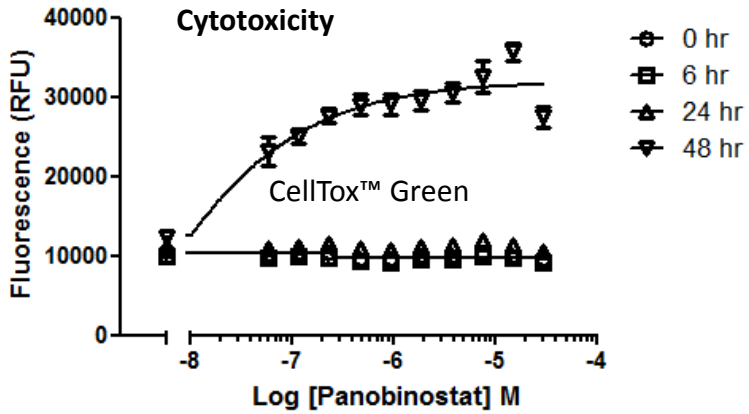


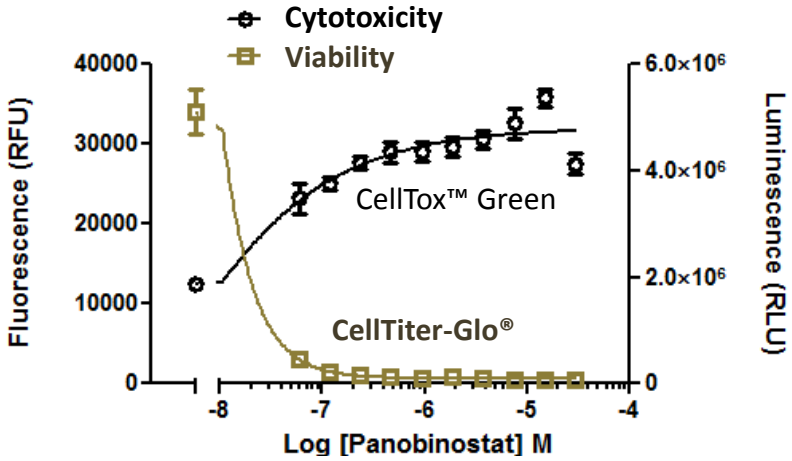


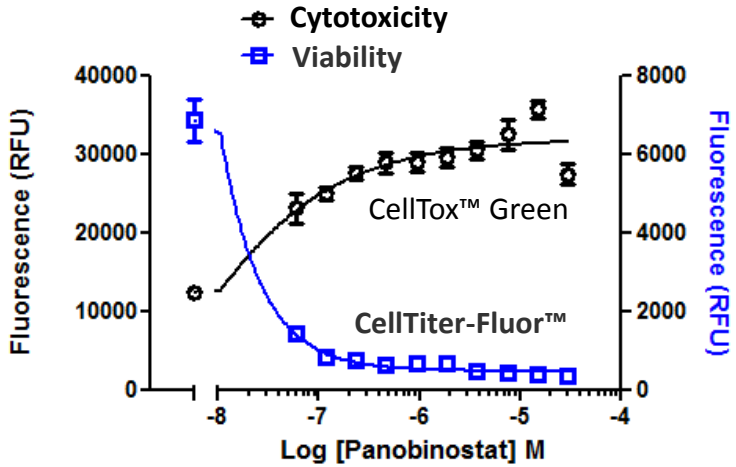


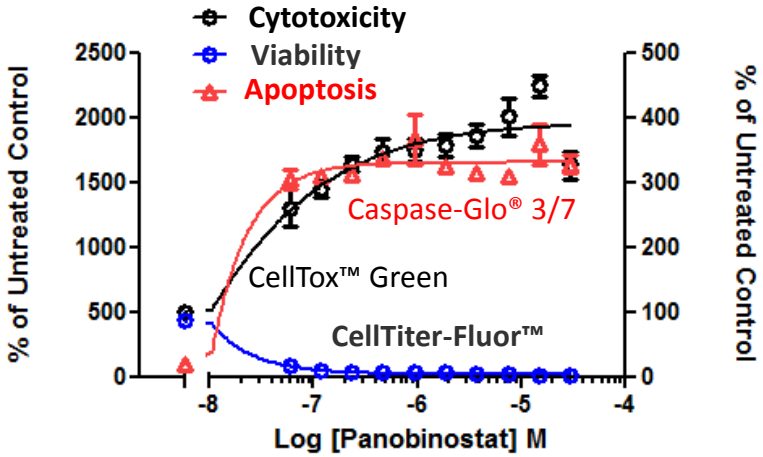
MOA = Intermediate Term Apoptosis

Panobinostat (HDAC Inhibitor)



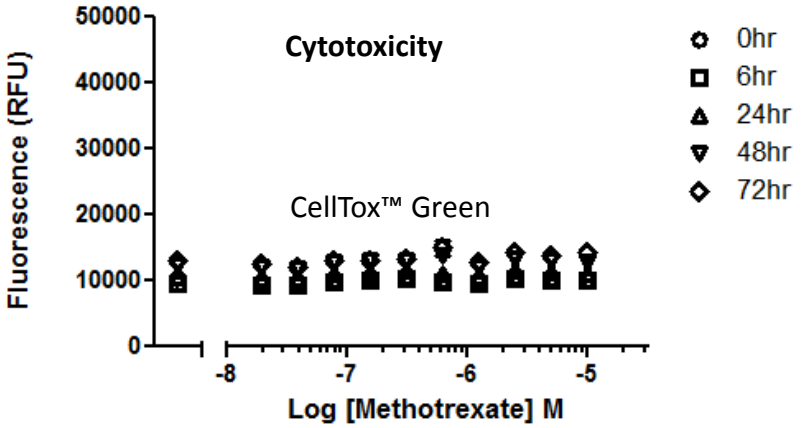


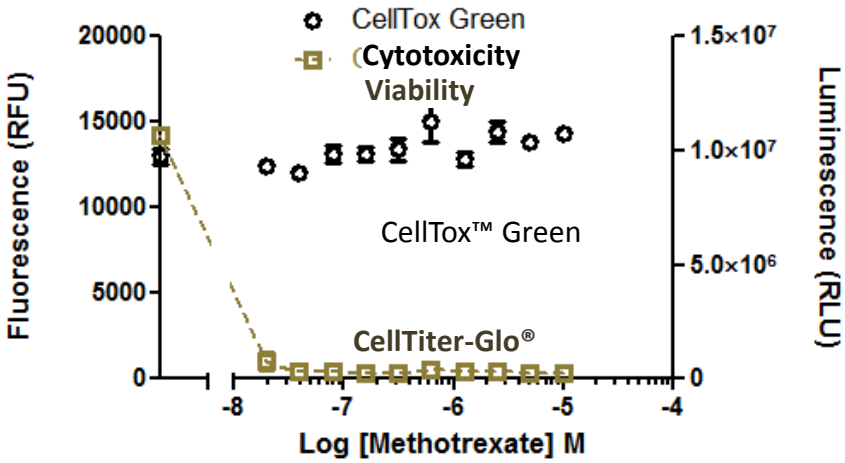


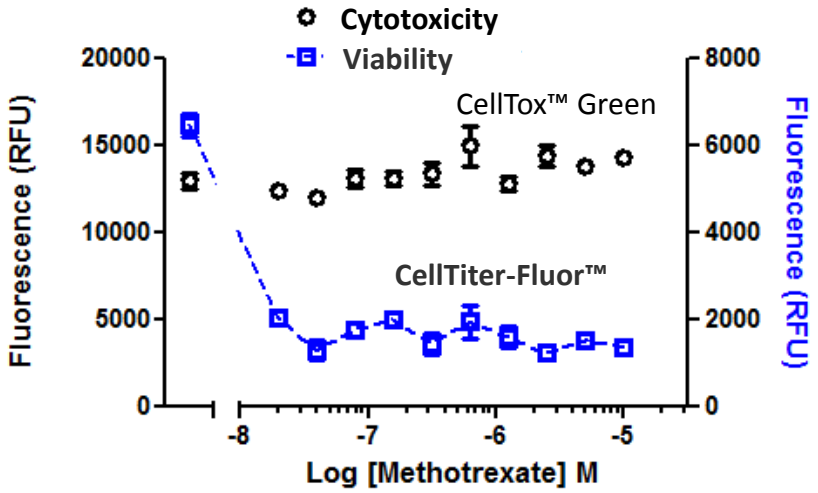


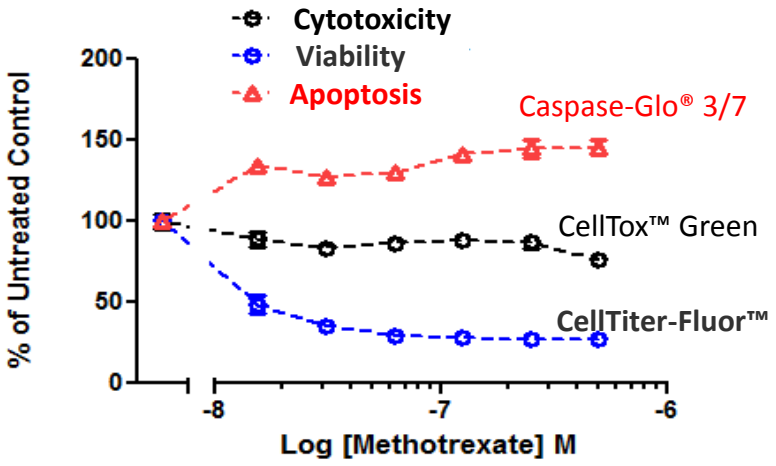
MOA = Later Term Apoptosis

Methotrexate (anti-cancer metabolite)



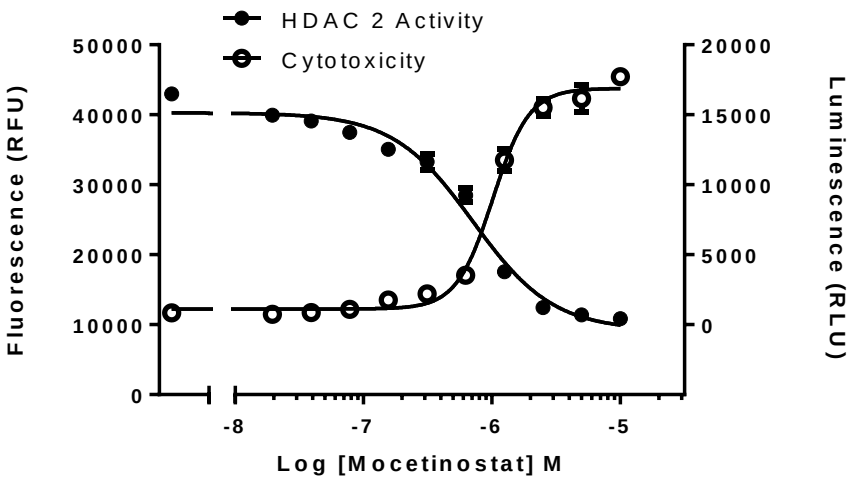


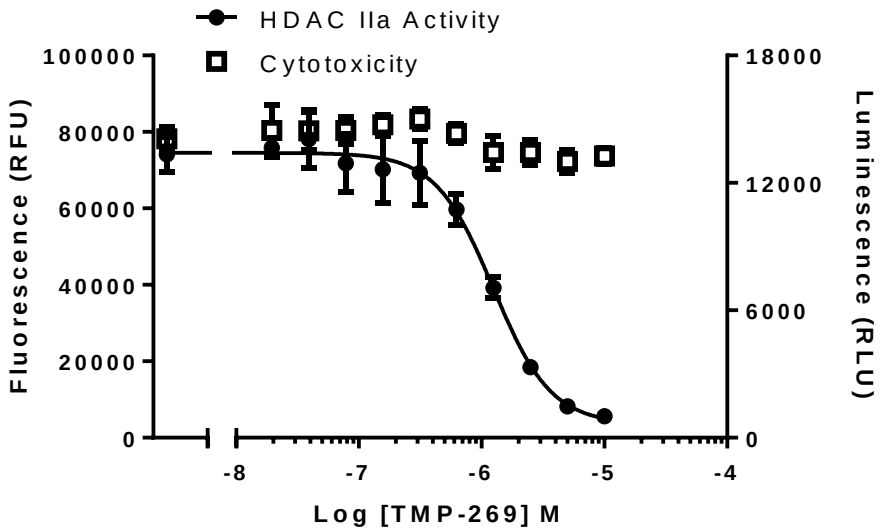




MOA = Cell Cycle Arrest, Early Apoptosis?

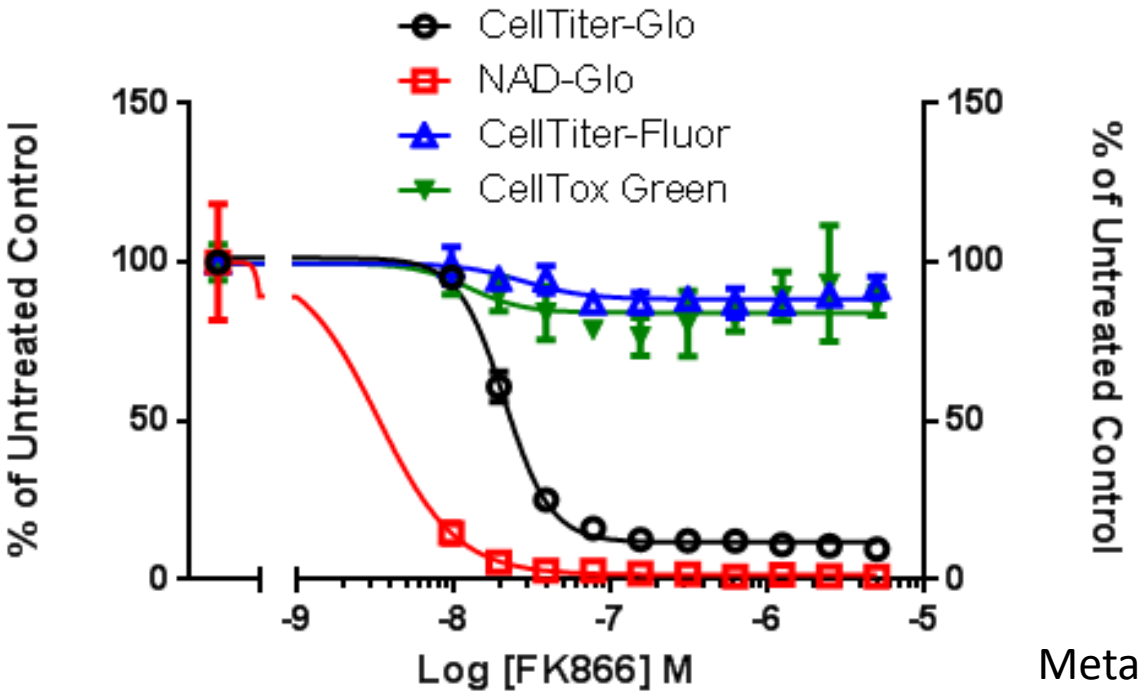
Consequences of Selective HDAC Class Inhibition





K562 were dosed with HDACi for 72hr with CellTox™ Green. Cytotoxicity fluorescence was gathered then HDAC 2 or IIa activity determined by HDAC-Glo 2 or IIa Assays in a multiplexed, same-well format.

General vs. Selective Pathway Effects



Metabolism biomarkers (NAD+ and ATP) can be reduced without loss of membrane integrity (CellTox Green) or reduction in non-metabolic biomarkers (CellTiter-Fluor)

Summary and Conclusions

Determination of cytotoxicity is greatly dependent upon:

- Dosage (“The dose makes the poison.”- Paracelsus in 1500’s)
- Exposure period with cells
- Mechanism of Action (primary vs. secondary necrosis, cytostasis)
- Intrinsic Cell Susceptibility (on- vs. off-target toxicity)

Not all cytotoxicity assays are created equal.

- Activity assays using endpoints are useful but imperfect
- CellTox™ Green allows for real-time measurement of cytotoxicity, and sequential multiplexing with viability and MOA assays for more complete characterization of the response

Acknowledgements

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Thank You!