A Real-Time, Bioluminescent Annexin V Assay for the Assessment of Apoptosis

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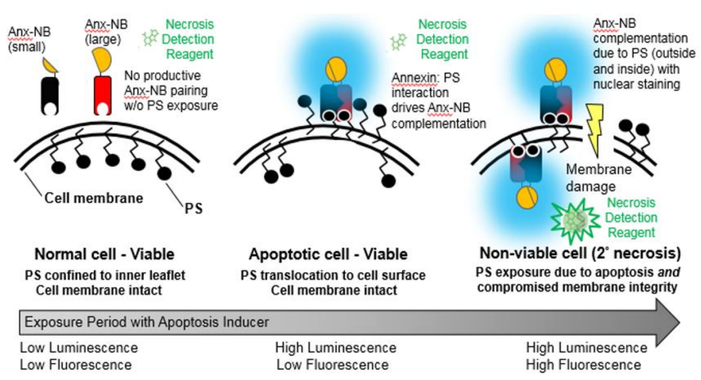
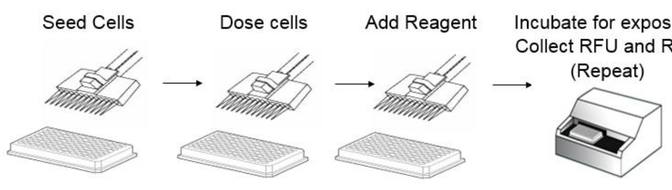
1. Introduction

Dose and exposure are the most important parameters used to define acute toxicological risk. However, *in vitro* duration of xenobiotic exposure is often relegated to convenient but arbitrary chronological endpoints. Therefore a methodological need exists to capture the kinetic detail of cytotoxic responses. A homogeneous, bioluminescent annexin V reagent was developed which can be applied to cells in plate-based formats at the time of dosing to monitor the real time progression of necrosis or apoptosis. The assay reagent utilizes two annexin fusion proteins which have been engineered to harbor separate and distinct complementary subunits of a binary luciferase. These proteins respond to the kinetic emergence of phosphatidylserine (PS) exposure by proximity-based enzyme complementation. The reagent uses a time-released luciferase substrate to produce a stable bioluminescent signal proportional to PS exposure. The reagent also contains a cell impermeant, profluorescent DNA dye that measures real time loss of membrane integrity. Together, the resulting luminescent and fluorescent profiles help define cell death mechanism of action (MOA).
This pilot study was conducted to examine the utility of the real time annexin reagent in a HepG2 hepatotoxicity model. Specifically, paclitaxel was employed as a control apoptosis induction agent to establish cell model response magnitudes, associated signal persistence, and to explore concordance between annexin and caspase activity biomarker measures. Compounds associated with direct or idiosyncratic hepatotoxicity (terfenadine, menadione, acetaminophen, diclofenac and aflatoxin B1) were tested to explore the kinetic relationship between potency and exposure, and MOA.
Although extrapolation of *in vitro* toxicity data for risk assessment continues to be a challenge, we conclude that this approach enables kinetic cytotoxicity profiles that allow for rank ordering potential risk.

2. Bioluminescent Annexin Assay Principle



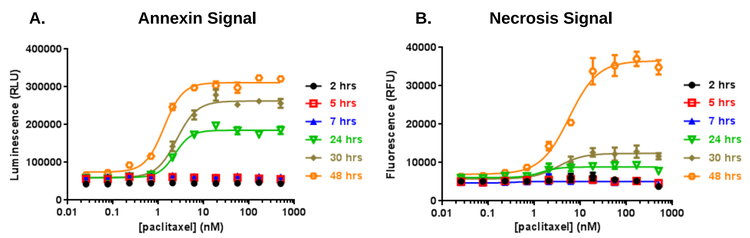
3. Real-Time Workflow and Model Hepatotoxicants



Test Agent	Indicated Use	Mechanism of Toxicity
Paclitaxel	Chemotherapeutic	Microtuble stabilizer
Acetominophen	Mild analgesic	Metabolite liver toxicity
Diclofenac	NSAID, analgesic	Mitochondrial impairment
Terfenadine	Anti-histamine	Arrhythmia/liver metabolism
Menadione	Pro-vitamin[1]	Reactive oxygen species
Aflaxtoxin B1	Pro-toxin found in food stuffs	Reactive epoxides

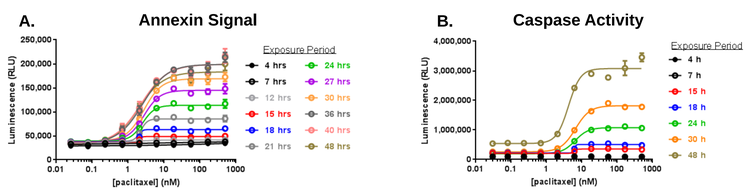
[1]Banned in economically developed countries

4. Kinetic Progression of Apoptosis and 2° Necrosis



HepG2 cells were dosed with paclitaxel for 48h in the presence of the real-time Annexin V assay reagent containing a pro-fluorescent necrosis detection probe. Luminescent and fluorescent measures were gathered periodically over the time course.
Panel A. Dose-dependent increases in PS exposure resulting from apoptosis emerged between 7 and 24h, with increases in the potency and magnitude of the response continuing throughout 48h. **Panel B.** Loss of membrane integrity was first measurable at 24h. The temporal lag between PS exposure and loss of membrane integrity are consistent with the apoptotic phenotype.

5. Concordance with an Orthogonal Apoptosis Method



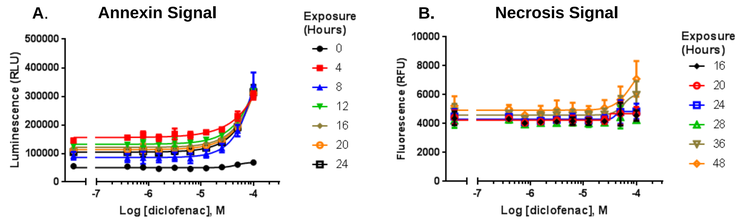
C.

Exposure	Caspase EC_{50}	Annexin EC_{50}
15h	~ 6nM	~ 2nM
18h	~ 6nM	~ 2nM
24h	6.5nM	2.5nM
30h	6.6nM	2.7nM
48h	4.2nM	1.9nM

HepG2 were dosed with paclitaxel for 48h.

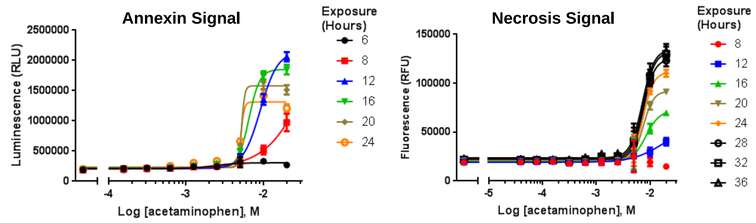
Panel A. Bioluminescent annexin data were collected in real-time using one plate with one addition. **Panel B**. Caspase-3/7 activity data were collected in parallel plates at various time points using the Caspase-Glo™ 3/7 Assay in endpoint format using seven separate plates. **Panel C**. Caspase activity and bioluminescent annexin EC_{50} values are concordant.

6. Rapid Inducers of Apoptosis



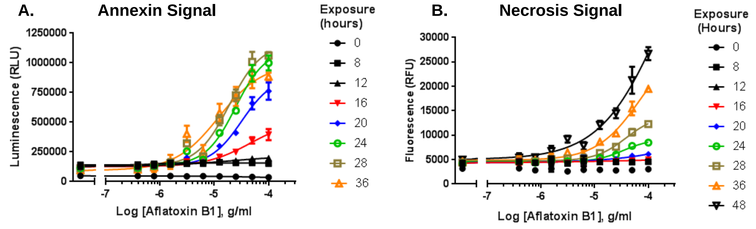
HepG2 were dosed with serial dilutions of diclofenac or terfenadine for 48h as described previously.
Panel A. Diclofenac induced a rapid (<4h) induction of apoptosis that did not increase in potency or magnitude as a function of additional exposure.
Panel B. Induction of apoptosis consequently produced a modest loss of membrane integrity and cell death beginning at 16-20h.
Results with terfenadine were similar to diclofenac (data not shown).

7. Intermediate Inducers of Apoptosis



HepG2 were exposed to serial dilutions of acetaminophen or menadione for 48h as described previously.
Panel A. Acetaminophen producedPS exposure at an intermediate time period of about 8h. Potency and magnitude of response continued to increase as a result of additional compound contact.
Panel B. Loss of membrane integrity was first evident at 12h and increased as induced cells proceeded to the secondary necrosis phenotype.
Menadione data was similar to acetaminophen (data not shown).

8. Late Inducer of Apoptosis



HepG2 were exposed to serial dilutions of aflatoxin B1 for 48h as described previously.
Panel A. Aflatoxin B1 produced PS exposure beginning at about 16h which progressed in magnitude and potency until peaking at 28h. This late induction phenotype likely reflects the kinetic rate of biotransformation of the parent molecule by HepG2 into the more toxic and reactive epoxide form.
Panel B. Loss of membrane integrity as a result of apoptosis was first evident beginning at 24h and progressed throughout the time course of exposure.

9. Conclusions

The bioluminescent Annexin V assay requires one homogenous addition step
- It requires no washes or other processing steps
- It can be applied to cells at the time of dosing

The assay accurately reports apoptosis in real time
- It produces substantial signal:background ratios for both PS exposure and necrosis
- The real-time PS exposure data correlate with endpoint caspase activity measures

The assay can resolve the kinetics of apoptosis induction
- It can define minimal and maximal exposure periods for an apoptotic response
- It can characterize the relationship between potency and magnitude of response

It can be employed with various cell models exploring safety and exposure
- The assay may help stratify chemical risk based on in vitro exposure
- The assay may identify potency thresholds for risk assessment

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