Improved DAMP Assays for the In Vitro Assessment of Immunogenic Cell Death

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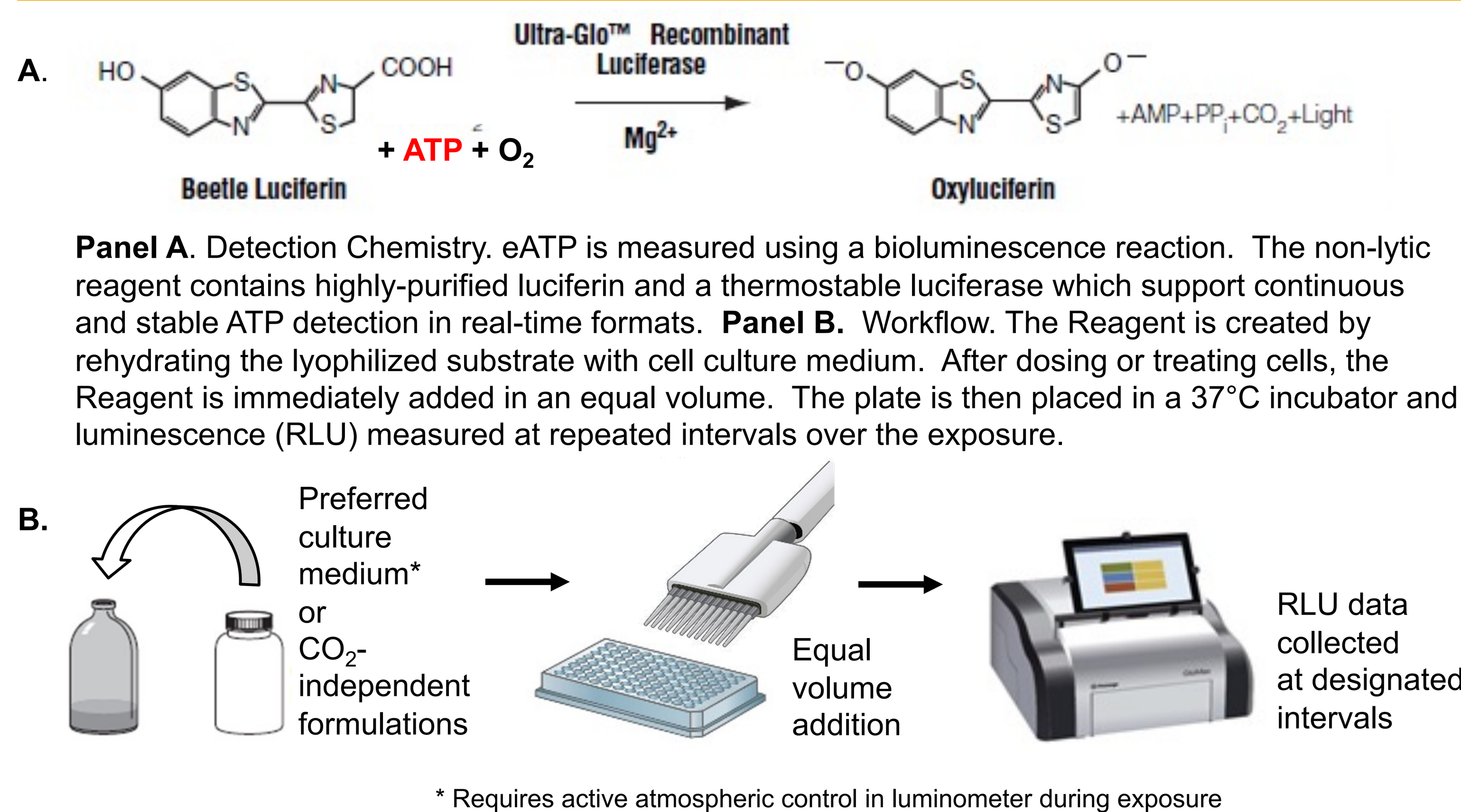
Abstract # 1933

1. Introduction

The extracellular levels of damage-associated molecular patterns (DAMPs) released during immunogenic cell death (ICD) are positively correlated with the magnitude and efficacy of the resulting in vivo immune response. Therefore, extracellular ATP (eATP) and high mobility group box 1 (HMGB1) have been identified as key biomarkers for their predictive capacity during in vitro ICD screening activities. Current methods for identifying eATP and HMGB1 inducers are laborious and costly and are hampered by poor throughput. To overcome these challenges, we have developed easy-to-use, homogeneous, bioluminescent assays that measure dose-dependent release of these immunostimulatory agents directly in cell culture. The eATP assay utilizes an optimized ATP detection chemistry that can be employed directly to assess live-cell kinetic responses for up to 24 h, or in longer exposures with a staggered reagent addition approach. The HMGB1 assay measures the protein's concentration at exposure endpoint in the same sample well using complementary, luciferase fragment-labelled, monoclonal antibodies. We tested the utility of the assays using U20S, Jurkat and U937 cells dosed with serial dilutions of known ICD inducers (doxorubicin, idarubicin, mitoxantrone, and bortezomib). The resulting data suggest the potency of eATP and HMGB1 release is dependent upon cell model and agent but can be reproducibly and robustly measured in 96 and 384 well environments. Further, the eATP assay produced remarkable early dose-dependent response resolution whereas the HMGB1 data provided a confirmatory post-mortem ICD parameter. This new ICD biomarker detection workflow helps to efficiently define and rank-order the capacity of new chemical entities to induce apoptosis and immunogenic cell death.

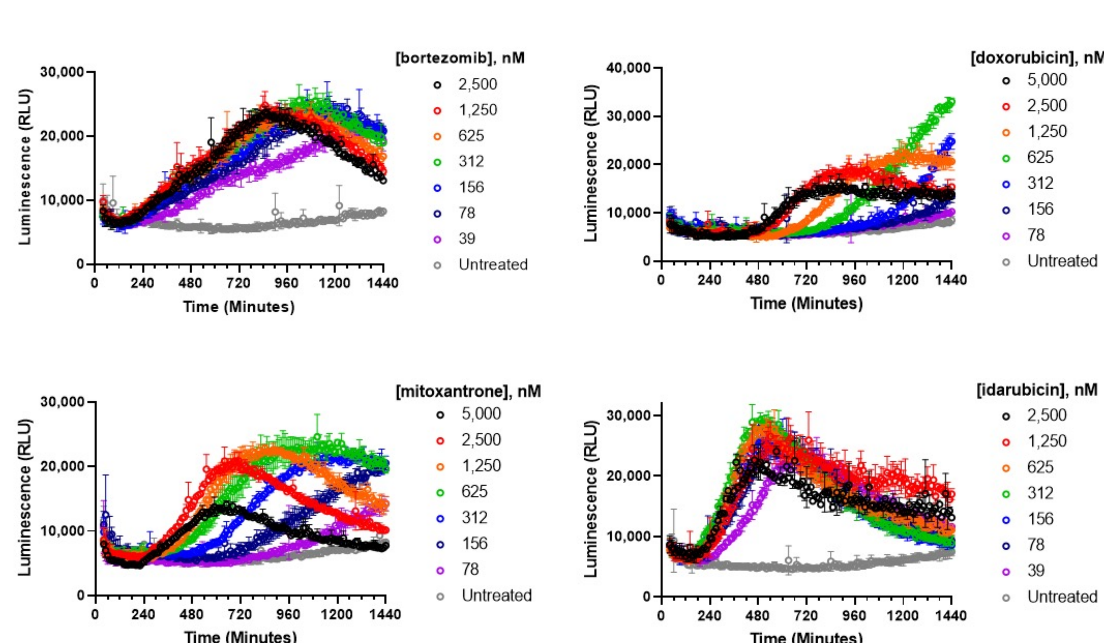
2. Real-Time Detection of eATP



Panel A. Detection Chemistry. eATP is measured using a bioluminescence reaction. The non-lytic reagent contains highly-purified luciferin and a thermostable luciferase which support continuous and stable ATP detection in real-time formats. **Panel B.** Workflow. The Reagent is created by rehydrating the lyophilized substrate with cell culture medium. After dosing or treating cells, the Reagent is immediately added in an equal volume. The plate is then placed in a 37°C incubator and luminescence (RLU) measured at repeated intervals over the exposure.

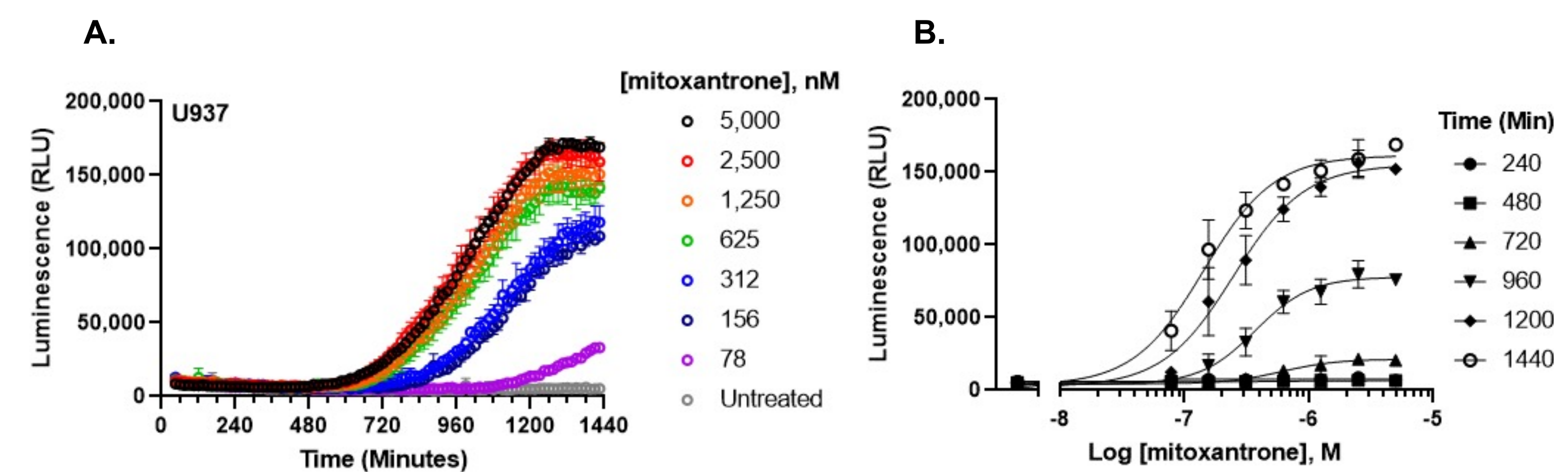
* Requires active atmospheric control in luminometer during exposure

3. Tool Agents Produce Differential eATP Release Profiles



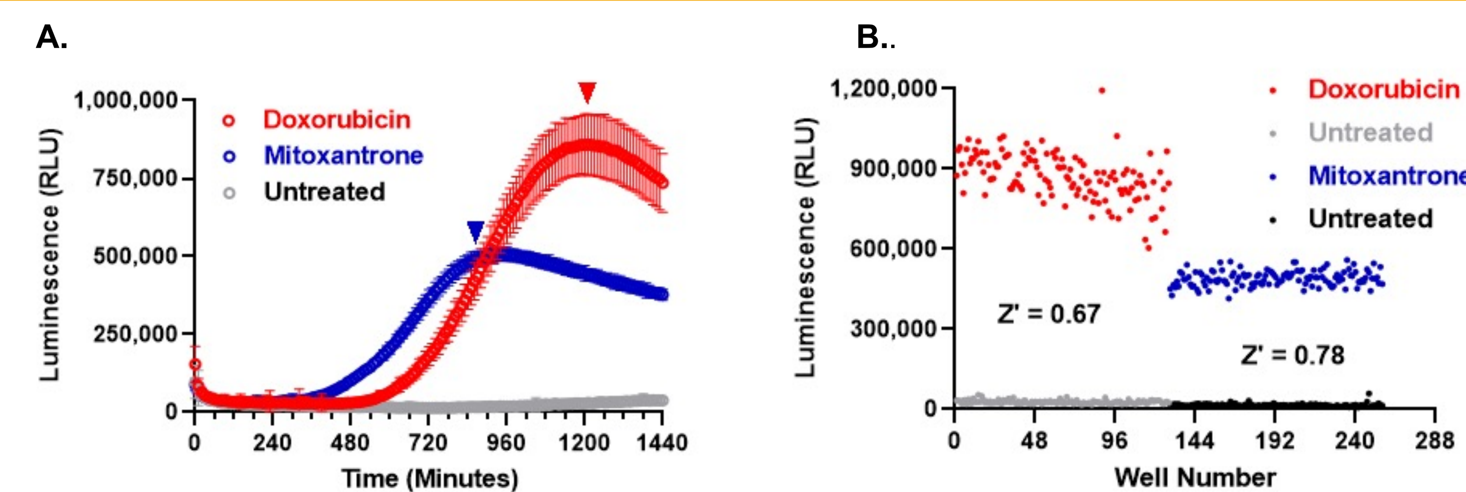
Well-characterized ICD-inducing compounds were two-fold serially diluted in RPMI 1640 + 10% FBS in a white 96 well plate. Jurkat were added at a density of 10,000 cells/well. Next, eATP detection reagent was added and luminescence measured in 10 min intervals for 24 h using a BMG CLARIOstar® with atmospheric control (ACU). The agents all provoked eATP release, albeit at different kinetic intervals and with varying dose proportionality.

4. Dose- and Time-Dependent eATP Release



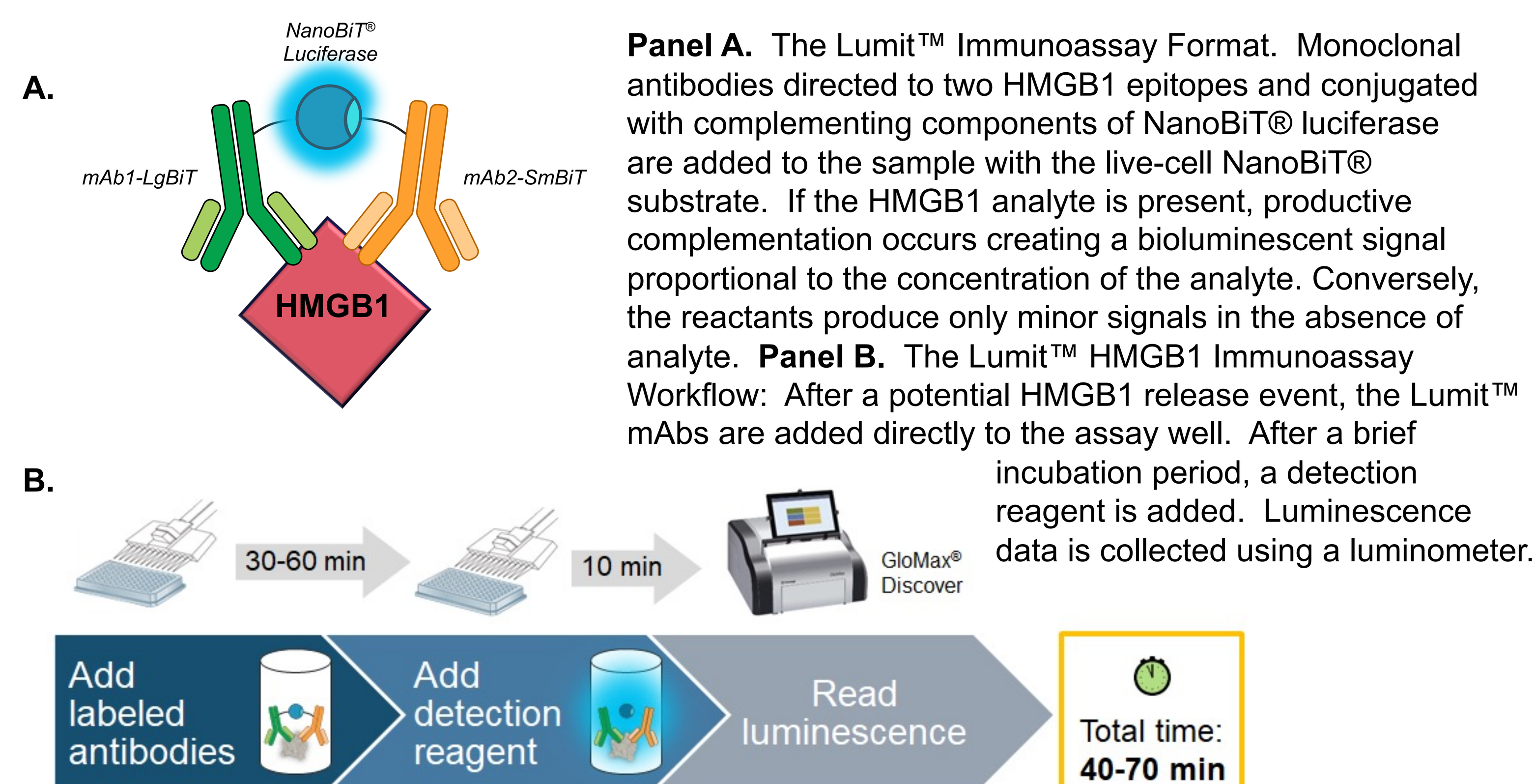
Mitoxantrone was two-fold serially diluted in CO_2-Independent medium supplemented with 10% FBS in a white 96 well plate. U937 cells were harvested, resuspended in the CO_2-Independent medium formulation, and added at a density of 10,000 cells/well. The eATP detection reagent was prepared with the same medium and added to each assay well. Luminescence measures were gathered every 15 min for 24 h using a Promega GloMax® Discover plate reader adjusted to 37°C. **Panel A.** Mitoxantrone produced dose- and time-dependent eATP release. **Panel B.** The data collected at 4, 8, 12, 16, 20 and 24 h were fitted using GraphPad Prism software to illustrate the increase in potency vs. time.

5. High Throughput Scalability of eATP Reagent



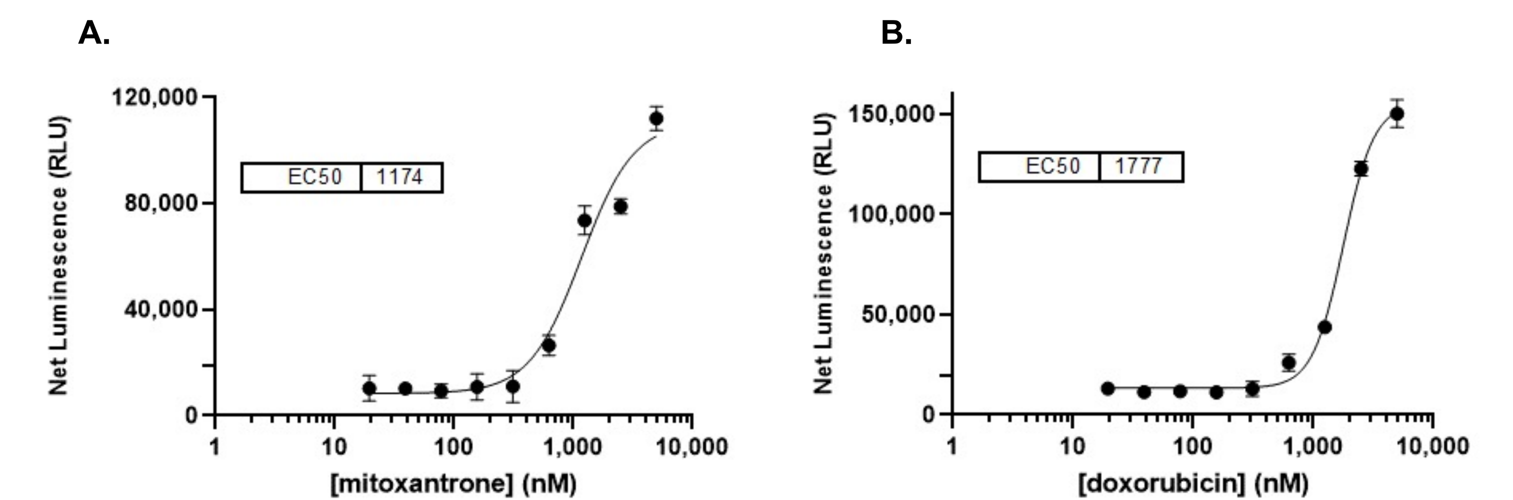
U937 cells were dosed with either doxorubicin or mitoxantrone (5 µM final concentration) in RPMI 1640 with 10% FBS and seeded into a Costar 384 well plate at a density of 2,500 cells/well. DMSO-matched medium + cells served as untreated control. The cells were contacted with an equal volume of eATP detection reagent and luminescence measured every 10 min for 24 h using a BMG CLARIOstar® with ACU at 37°C. **Panel A.** A kinetic trace of luminescent values was plotted for induced and uninduced populations. Peak eATP release was achieved at 14 h for mitoxantrone and 20 h for doxorubicin. **Panel B.** A scatter plot was created using data collected at peak eATP induction. The Z-prime values of 0.67 and 0.78 demonstrated single assay screening suitability (Z' $\geq$ 0.5) and overall HTS fitness.

6. Lumit™ HMGB1 Immunoassay Format and Workflow



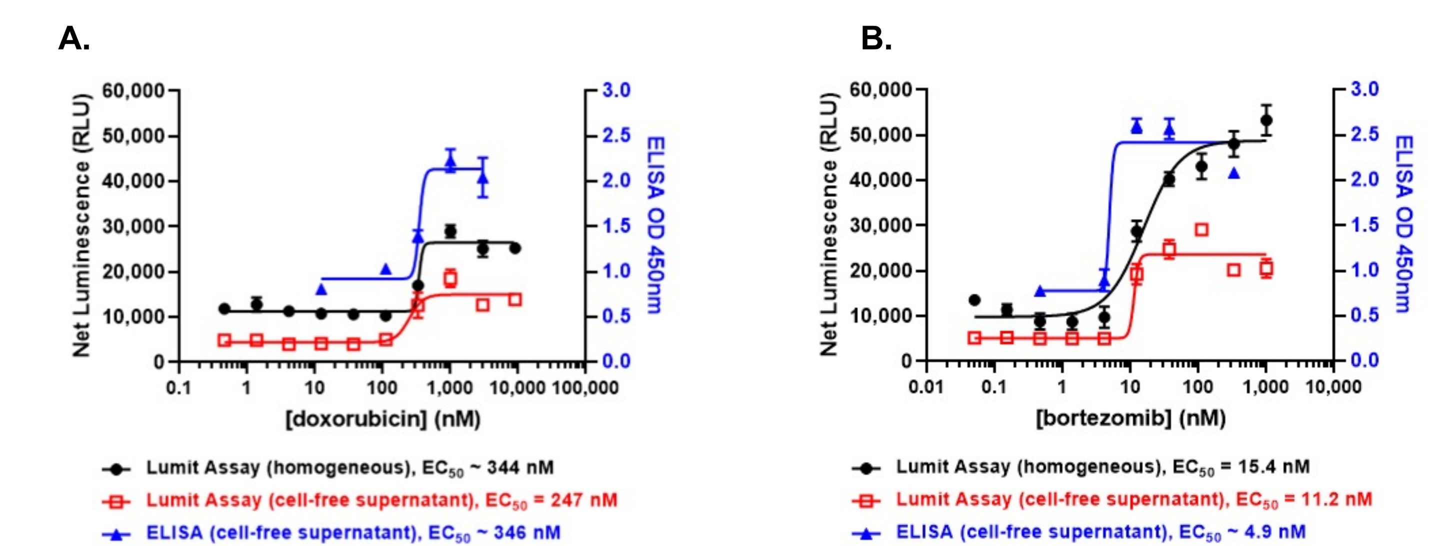
Panel A. The Lumit™ Immunoassay Format. Monoclonal antibodies directed to two HMGB1 epitopes and conjugated with complementing components of NanoBiT® luciferase are added to the sample with the live-cell NanoBiT® substrate. If the HMGB1 analyte is present, productive complementation occurs creating a bioluminescent signal proportional to the concentration of the analyte. Conversely, the reactants produce only minor signals in the absence of analyte. **Panel B.** The Lumit™ HMGB1 Immunoassay Workflow: After a potential HMGB1 release event, the Lumit™ mAbs are added directly to the assay well. After a brief incubation period, a detection reagent is added. Luminescence data is collected using a luminometer.

7. Potency Determinations for HMGB1 Release



U20S were seeded into a white 96 well plate at a density of 10,000 cells/well in DMEM + 10% FBS and allowed to attach. Two-fold serial dilutions of mitoxantrone or doxorubicin were prepared and added in an equal volume. After the 24 h compound exposure, the Lumit™ HMGB1 Immunoassay mAbs were added and allowed to incubate for 90 min. The live cell NanoBiT® luciferase substrate was added, and luminescence collected using a Promega GloMax® Discover plate reader. **Panel A**. Mitoxantrone produced a half-maximal effect at 1174 nM. **Panel B.** Doxorubicin achieved a potency of 1777 nM during the exposure.

8. Concordance with ELISA Method



U937 cells were seeded into 12 well plates at a density of 400,000 cells/well in RPMI 1640 + 10% FBS and dosed with either doxorubicin or bortezomib for 22 h. 50µl volumes of cells + supernatant or cell-free supernatant were assayed in a separate 96 well plate using the Lumit™ HMGB1 Immunoassay. A subset of cell-free supernatants (and dilutions of supernatant) were added to a HMGB1 ELISA plate (IBL International) and processed as directed by the manufacturer. **Panel A.** The homogeneous and cell-free formats produced potency data that were concordant with the ELISA format after doxorubicin exposure. **Panel B.** Bortezomib also produced similar HMGB1 release profiles when compared to ELISA.

9. Conclusions

- Extracellular ATP and HMGB1 are important biomarkers which characterize an immunogenic cell death response.
- The RealTime-Glo™ Extracellular ATP Assay measures eATP:
  - in real-time throughout at least a 24 h exposure with conventional luminometers
  - in a fully homogeneous, "add-mix-measure" format
  - release potential and potency as a function of time
  - in both 96 and 384 well formats
- The Lumit™ HMGB1 Immunoassay measures HMGB1 release:
  - in a homogeneous format without the need for supernatant sample processing
  - in a 40-90 min time frame without washes or extended incubations required with ELISA formats
  - that is concordant with ELISA methods without sample dilution to accommodate assay range
- Together, the assays represent an efficient means of evaluating the ICD inducing potential of new chemical entities or other treatment modalities.

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