Getting the conditions right: platelet cytotoxicity assays to understand the effects of BH3 mimetics and PROTAC DT2216 on platelet viability *in vitro*

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Background

BH3-mimetics in development as anti-cancer drugs targeting BCL-XL have been limited by on-target and dose-limiting thrombocytopenia.

The PROTAC (Proteolysis Targeting Chimera) DT2216 was designed to avoid the on-target platelet toxicity of the Bcl-2 inhibitor ABT263 (Navitoclax).

DT2216 shares the ABT263 warhead (Diagram 1), was initially reported to be platelet sparing *in vitro*,[1,2]. However, moderate on target platelet toxicity of DT2216 has been reported in animal models *in vivo*[3].

We investigated the *in vitro* platelet toxicity effects of DT2216 and explored experimental methodology to investigate the differences reported by *in vitro* and *in vivo* platelet toxicology testing.

Modes of Action

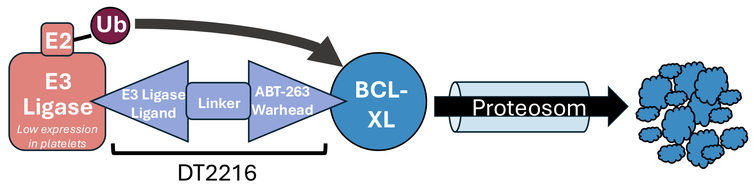


Diagram 1. Schematic representing the PROTAC DT2216 interaction with the anti-apoptotic protein target BCL-XL.
DT2216 is comprised of a modified ABT-263 warhead, that binds anti-apoptotic proteins, and an E3 ligase ligand. Recruitment of E3 ligase to the target protein leads to the transfer of ubiquitin (Ub) and degradation of the target protein.

Conclusion

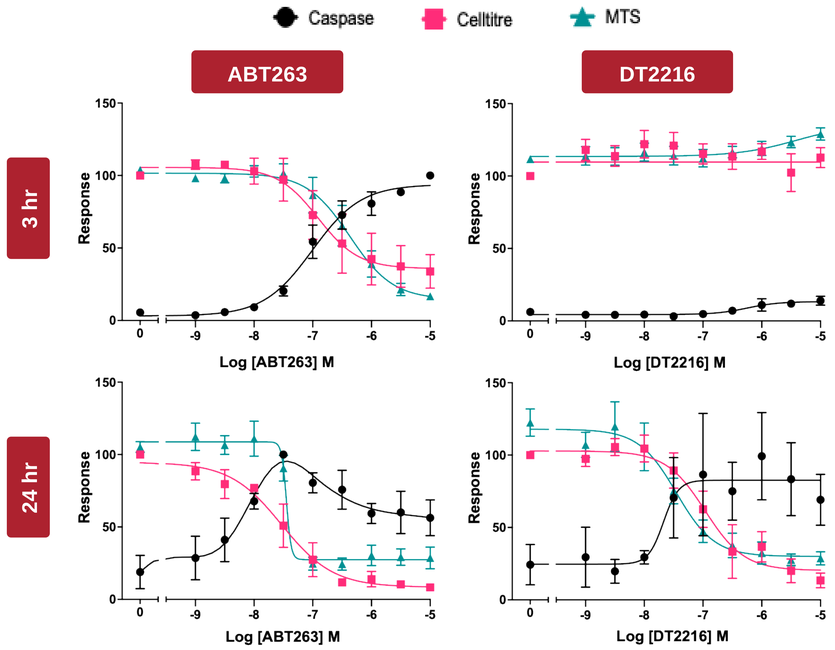
Key to reliably measuring and understanding the effects of new drug entities on platelet toxicity is the selection of the most appropriate conditions for platelet viability assays that best mimic *in vivo* conditions.

ABT-263 reduced platelet viability within 3 hr treatment. DT2216 also decreased platelet viability but required 24 hr treatment at 37℃, which contrasts with in vitro data in the literature but better mirrors the early results of *in vivo* studies in animal models.

Using BH3-mimetics with known *in vivo* platelet toxicity effects, we have demonstrated the suitability of sensitive, multiplexed higher throughput *in vitro* platelet cytotoxicity assays that could be used for better prediction of *in vivo* platelet toxicity to de-risk new therapeutics in development.

Results 1

The effects of the compounds ABT263 and DT2216 were assessed in human washed platelets at 37℃, initially using the MTS assay (assess metabolic activity). A subsequent study was performed with CellTiter-Blue (metabolic activity) and caspase 3/7 activity assays (assesses apoptotic signalling), as they are compatible with multiplexing.



- ABT263 both reduced platelet metabolic activity and increased caspase 3/7 activity after 3 hr treatment and its potency increased at 24 hr.
- DT2216 was shown to reduce platelet metabolic activity and increase caspase 3/7 activity following 24 hr incubation.
- There was good correlation between two metabolic activity assays – MTS and CellTiter-Blue.

Figure 1. The effects of ABT263 and PROTAC DT2216 on human washed platelets viability assessed by MTS, CellTiter-Blue and Caspase 3/7 activity
Compounds were incubated with washed platelets for 3 and 24 hr before analysis by MTS (n=5-6), CellTiter-Blue (n=3-4), and caspase 3/7 (n=3-4). Caspase response = % of maximal effect seen with ABT263. All data are mean ± SEM.

Results 2

The latter observation with DT2216 did not replicate previously published in vitro data[1], where effects were assessed at room temperature. Therefore, next we investigated the impact of incubation temperature on the effects of ABT263 and DT2216 on platelet viability using the CellTiter-Blue assay.

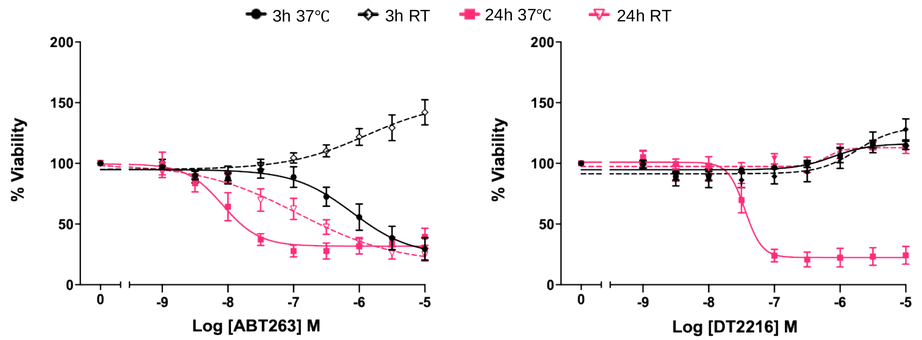


Figure 2. The effects of ABT-263 and PROTAC DT2216 on human washed platelets viability assessed by CellTiter Blue
Compounds were incubated with washed platelets for 3 and 24 hr at 37℃ and room temperature (RT) before analysis. All data are mean ± SEM, n=4.

- ABT263 was less potent after 3 and 24 hr treatment at room temperature compared with 37℃.
- DT2216, as seen previously, did not affect platelet viability at 3 hr; after 24 hr treatment it was only shown to reduce platelet viability at 37℃ with no effect at room temperature, which was in line with previously published data.

References: (1) A selective BCL-X_L PROTAC degrader achieves safe and potent antitumor activity (S. Khan et al., 2019), (2 DT2216-a Bcl-xL-specific degrader is highly active against Bcl-xL-dependent T cell lymphomas (Y. He et al., 2020). (3) Discovery of BCL-XL heterobifunctional degrader with potentially improved therapeutic window and minimal platelet toxicity for hematological malignancies (Y. Xie et al., 2023).

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