An *ex vivo* Platform to Evaluate the Effects of Compounds on Different Stages of Platelet Development

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Introduction

Thrombocytopenia is an on-going problem in drug development. Small animal *in vivo* models, which are commonly used to assess thrombocytopenia risk at the development stage, often do not translate well into non-human primate (NHP) models and human clinical trials. To address the need for a more predictive, quantitative and clinically relevant means of assessing thrombocytopenia risk at the development stage, we set out to develop primary cell based assays that could allow for the evaluation of thrombocytopenia risk in both NHPs and humans in a manner that is both biologically relevant and that allows for insight into the biological mechanism of the toxicity. In this poster, we describe three *ex vivo* primary cell based assays that can be used to assess the risk of thrombocytopenia in both NHPs and humans. The assays, which use primary bone marrow cells cultured under conditions that mimic the *in vivo* microenvironment, allow for quantitative readouts. When combined, the assays can allow for the assessment of toxic effects on cells ranging from CD34+ bone marrow progenitors through to mature platelets. Since the assays are performed *in vitro*, they allow for better control of experimental conditions and are more amenable to downstream proteomic, genomic and epigenetic analysis.

Objective

The goal of this work is to create assays that can accurately predict the risk of thrombocytopenia associated with investigational biotherapeutics. To be considered predictive, the assay should, as a starting point, recapitulate *in vitro* the known *in vivo* biology of thrombopoiesis in that megakaryocytes and platelets should develop from progenitor cells in a manner that is biologically relevant, reproducible and quantitative.The assays must be suitable for routine use in a biotherapeutics development program, must allow for meaningful comparisons between test and control articles, and ideally should correlate with *in vivo* results in NHPs and humans.

In order to evaluate the performance of the assay, we assessed four therapeutic compounds representing four distinct compound classes: (a) 5-Fluorouracil, an antineoplastic, (b) Abexinostat, an HDAC inhibitor, (c) Lenalidomide, a thalidomide-derived immunomodulator and (d) Anagrelide, a phosphodiesterase inhibitor.

Materials and Methods

Four drugs from different classes were selected for testing in the megakaryocyte-based platform based on reported potential thombocytopenia effects. Bone marrow mononuclear cells from normal human donors (n=3, ReachBio, Seattle, WA) and NHP donors (n=2, AllCells, Alameda, CA) were mixed with the compounds over an extended concentration range in MegaCult™, a collagen based medium (StemCell Technologies, Vancouver BC) and plated in 35 mm dishes (three replicates per concentration). The cultures were incubated in a humidified incubator at 37ºC, 5% CO_2. CFU-Mk were enumerated on day 14, following fixation and staining for CD41, and IC_{50} values were determined for each drug.

CD34+ cell populations derived from normal human bone marrow (NorCal Biologics, Placerville, CA) and NHP (fresh marrow received from the NIH Primate Center, University of Washington and processed at ReachBio) were cultured in X-Vivo15 containing stem cell factor and thrombopoietin in a 96 well format over a 14 day period in the presence and absence of two concentrations of each of the four drugs. At various times, a cocktail of antibodies containing DAPI, CD34-PE and CD41-FITC (Beckman-Coulter, Hebron, KY) were used to stain the cultured cells. Flow cytometry analysis was then performed using a BD LSR-2 flow cytometer. Additionally, a replicate well from the same experiment was mixed and a sample removed for platelet analyses. The cells were incubated with a 1% ammonium oxalate solution and counted manually using a hemacytometer.

Results

CFU-Mk assays measure the effect of test compounds on primitive progenitors. Data between NHP (n=2) and Human (n=3) CFU-Mk IC_{50} values were comparable (Table 1 and Figure 1).

Compound	CFU-Mk IC_{50} NHP (n=2)	CFU-Mk IC_{50} Human (n=3)
5-FU (µg/mL)	0.28	0.23
Abexinostat (nM)	300	300
Anagrelide µM	31	34
Lenalidomide µM	432	> 597

Table 1. IC_{50} values for 4 drugs on human and NHP megakaryocyte progenitors (CFU-Mk).

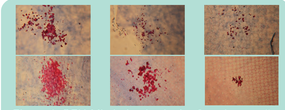


Figure 1. CFU-Mk derived colonies from human (top panels) and NHP (bottom panels) depicting primitive (left panels), intermediate (middle panels) and mature (right panels) progenitors.

Culturing CD34+ cells (derived from either NHP or human bone marrow) in the presence of Tpo and SCF drives them along a differentiation pathway, thus allowing assessment of compounds on various distinct populations as defined by CD41 expression (Figure 2). The effect of the test compounds on three distinct populations CD41dim and low side scatter (early megakaryocyte cells), CD41mid and low side scatter (intermediate megakaryocytes) and CD41bright and high side scatter (mature megakaryocytes) can be determined (Figure 3). For the human platform, day 10 was optimal at assessing changes in megakaryocyte cell populations whereas for the NHP platform, day 7 was optimal (Table 2).

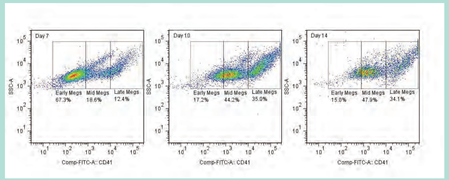


Figure 2. Kinetic profile of megakaryocyte development from Day 7 through Day 14 in liquid culture.

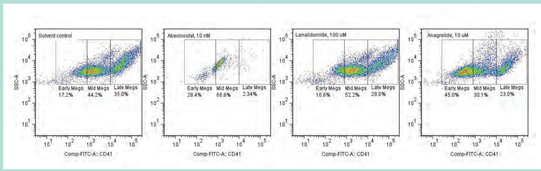


Figure 3. Day 10 flow cytometry analysis of early-, mid- and late-stage megakaryocyte development in the presence of abexinostat, lenalidomide and anagrelide. Abexinostat arrests both the proliferation and maturation of megakaryocytes, lenalidomide has limited effect and anagrelide arrests maturation of megakaryocytes without restricting proliferation.

Culturing CD34+ cells in the presence of Tpo and SCF supports platelet development over a 14 day period. Platelets are initially detected as early as day 7 and continue to increase over the next 7 days. The generation of platelets from CD34+ cells is donor dependent (Figure 3) and does not correlate with the starting purity of CD34+ cells.

5-FU caused a significant decrease of CFU-Mk, and at 1.0 and 0.3 µg/mL, only the more mature progenitors were evident in the cultures. At 1.0 µg/mL, 5-FU also affect platelet production and this seemed more pronounced in the human than in the NHP model (Table 3).

Abexinostat at 10 and 100 nM inhibited the proliferative and differential potential of CD34+ cells towards megakaryocytes in a liquid culture system in both human and NHP models (Table 2). Abexinostat decreased the early megakaryocyte maturation at concentrations which had no effect on the CFU-Mk. The decrease in early megakaryocytes translated into decreased platelet counts (Table 3).

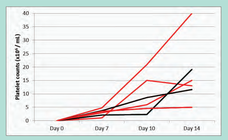
Lenalidomide had limited effects on the proliferative and differential potential of CD34+ cells towards megakaryocytes (Table 2), but decreased platelet counts at day 14 as compared to solvent control cultures (Table 3).

Anagrelide appeared to arrest cells in the early megakaryocyte stage (Table 2) and, at 35 µM, caused a reduction in platelet counts in the human platform (Table 3).

NHP Donor	Drug	Dose	Units	Megakaryocyte Stage of Development, % of Total Megakaryocyte-Lineage Cells: Early	Intermediate	Mature
120	SC			13	16	66
	5-FU	1	ug/mL	12	55	28
	5-FU	0.1	ug/mL	29	33	33
	Abexinostat	100	nM	14	68	16
	Abexinostat	10	nM	5	79	16
	Lenalidomide	386	µM	2	30	61
	Lenalidomide	100	µM	2	20	70
	Anagrelide	35	µM	8	66	18
	Anagrelide	10	µM	14	22	55
122	SC			37	13	43
	5-FU	1	ug/mL	9	47	39
	5-FU	0.1	ug/mL	13	18	60
	Abexinostat	100	nM	12	64	12
	Abexinostat	10	nM	8	76	12
	Lenalidomide	386	µM	14	43	33
	Lenalidomide	100	µM	4	21	67
	Anagrelide	35	µM	3	61	25
	Anagrelide	10	µM	47	20	29

Human Donor	Drug	Dose	Units	Megakaryocyte Stage of Development, % of Total Megakaryocyte-Lineage Cells: Early	Intermediate	Mature
806	SC			17	44	35
	5-FU	1	ug/mL	46	42	11
	5-FU	0.1	ug/mL	12	51	35
	Abexinostat	100	nM	6	93	1
	Abexinostat	10	nM	28	69	2
	Lenalidomide	386	µM	18	53	27
	Lenalidomide	100	µM	17	52	28
	Anagrelide	35	µM	61	23	15
	Anagrelide	10	µM	45	30	23
820	SC			13	47	37
	5-FU	1	ug/mL	26	56	17
	5-FU	0.1	ug/mL	8	47	42
	Abexinostat	100	nM	4	90	6
	Abexinostat	10	nM	4	90	5
	Lenalidomide	386	µM	14	51	29
	Lenalidomide	100	µM	11	47	34
	Anagrelide	35	µM	53	31	14
	Anagrelide	10	µM	37	32	28

Table 2. The effect of drugs on in vitro megakaryocyte development after 7 days in culture (NHPs) and 10 days in culture (human)



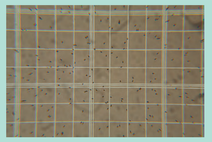


Figure 3. Kinetic profile of megakaryocyte development from Day 7 through Day 14 in liquid culture.

NBM 806	Solvent Control	5-FU (1.0 µg/mL)	5-FU (0.1 µg/mL)	Abexinostat (100 nM)	Abexinostat (10 nM)	Lenalidomide (386 µM)	Lenalidomide (100 µM)	Anagrelide (35 µM)	Anagrelide (10 µM)
Day 7	1.1	0.74	1.14	1.26	6.46	1.03	1.89	2.18	4.86
Day 10	15	4.83	3.7	4.7	8.8	4	12.5	3.1	10
Day 14	13	2.4	4.9	2.3	13.9	3.3	2.7	1.6	12.4
NHP 122	Solvent Control	5-FU (1.0 µg/mL)	5-FU (0.1 µg/mL)	Abexinostat (100 nM)	Abexinostat (10 nM)	Lenalidomide (386 µM)	Lenalidomide (100 µM)	Anagrelide (35 µM)	Anagrelide (10 µM)
Day 7	3.7	3.5	1.6	2.5	3.36	1.9	4.6	2.28	4.8
Day 10	8.6	1.8	10.7	4.2	8.3	3.2	9.8	7.8	5.7
Day 14	11.6	9.2	9.8	3.5	10.8	5.2	7	14.2	15

Table 3. Kinetic assessment of platelet counts (million per mL) for solvent control and drug-treated cultures.

Conclusions

Assays that can evaluate drugs at the various stages of platelet development facilitate an understanding of the mechanism of thrombocytopenia. CFU-Mk assays evaluate the effect of compound on primitive progenitors and these have been useful in predicting thrombocytopenia (Pessina *et al,* 2009).

Some compounds appear to have a limited effect on the CFU-Mk derived progenitors, but still cause thrombocytopenia. For these compounds, evaluating the development of megakaryocytes may prove useful. In these studies, the optimal day for evaluating NHP megakaryocyte development is Day 7 and for the human platform Day 10 is optimal. Abexinostat had limited effects on the CFU-Mk assay, but arrested megakaryocyte development in its early stage.

CD34+ derived cells from both NHP and human donors can be cultured and platelet assessments made after 7 days in culture. The optimal time for assessing platelets is Day 14. There appears to be donor variability in the numbers of platelets generated and to date, this has not correlated with the age of the donor or the starting CD34+ cell purity.

References

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