THE CELL BIOLOGY EXPERTS™

JENNIFER O'MALLEY
EMER CLARKE, Ph.D.

Hematopoietic Colony Forming Cell (CFC) Assay

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The preclinical screening method that helps clients eliminate bad players earlier in the drug development process.

CFC WHITE PAPER // 01

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HEMATOPOIETIC CFC ASSAY

For Drug Safety and Toxicity

Hematopoietic progenitor cells in bone marrow give rise to mature blood cells of the myeloid, erythroid and megakaryocytic lineages. These precursor cells are particularly vulnerable to off-target perturbation by a broad range of therapeutics, including, but not limited to, chemotherapeutics, antibiotics, and anti-inflammatory drugs. In fact, administered alone or in combination with standard of care drugs, preclinical drug candidates targeting virtually any disease class have the potential for serious hematotoxic clinical consequences, including neutropenia, severe anemia, and thrombocytopenia.

High throughput, cell line-based screening methods don't capture the fastidious growth requirements of primary bone marrow progenitor cells, and have led to late-stage drug failure due to unacceptable toxicity in the clinic. When performed by highly trained, experienced personnel, the well validated colony forming cell (CFC) assay is predictive of these consequences. Further, data from this type of assay is often requested by regulatory bodies, including the FDA, before clinical trials can proceed.

CFC assays designed to predict neutropenia or severe anemia are performed by incubating test drugs with bone marrow cells in a semi-solid, methylcellulose-based medium containing specific cytokine combinations to induce differentiation along the myeloid or erythroid pathway. A collagen-based assay is also used at ReachBio for megakaryocytic lineage studies to predict thrombocytopenia. Changes to the expected numbers of lineage-specific colonies that develop from the progenitor cells, relative to controls, as well as changes to colony morphology, are assessed by trained microscopists.

Drug classes that can be assessed include small molecules, monoclonal antibodies, bispecific antibodies, antibody-drug conjugates (ADCs), oligonucleotide therapeutics, CAR-T cells, novel biologic constructs, and others.

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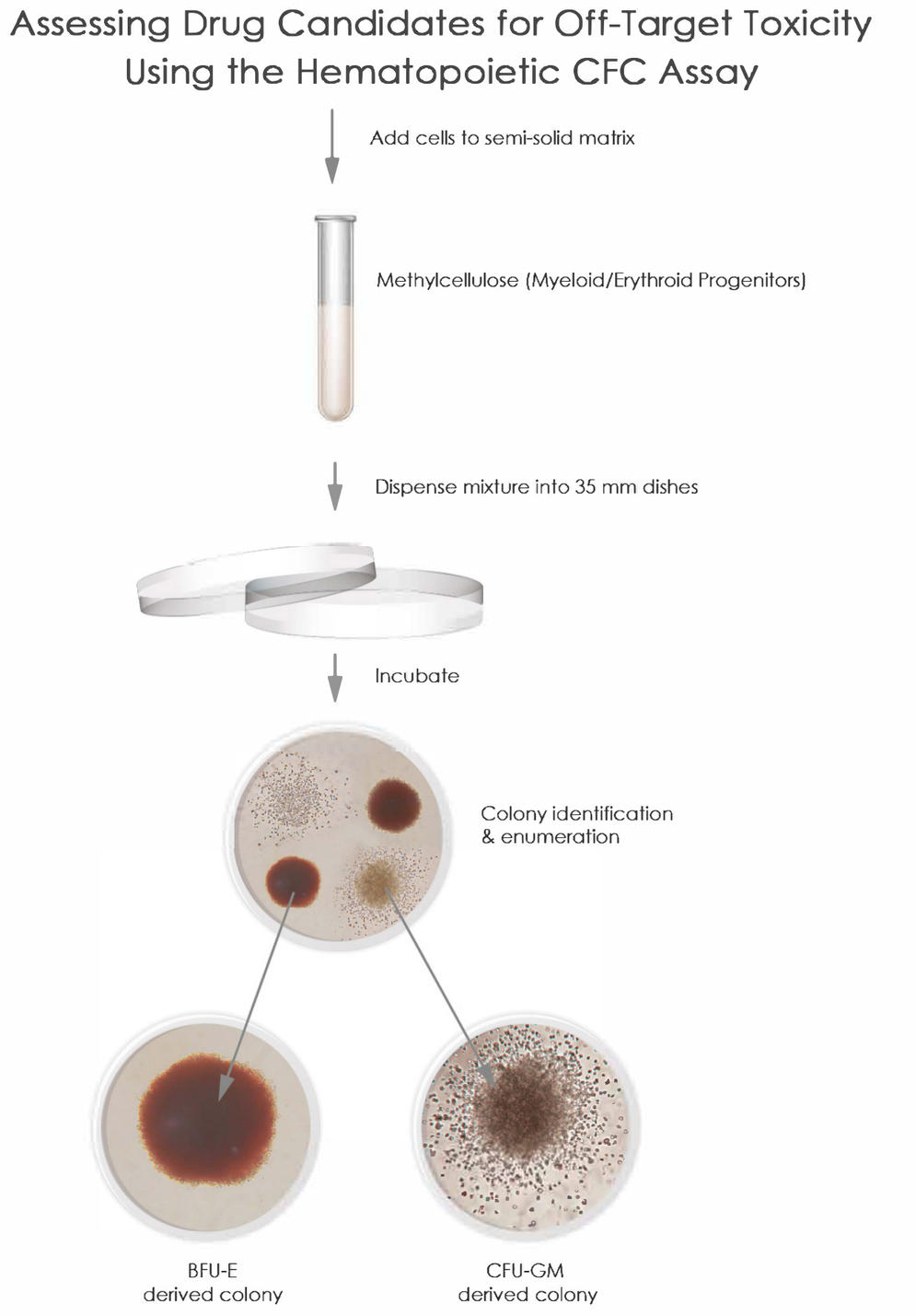
In this paper, we present data from internal ReachBio studies specific to neutropenia. The first section demonstrates the clinical relevance of CFC assays by correlating known clinical myelotoxicity outcomes of approved tyrosine kinase inhibitor (TKI) drugs with CFC data using the same drugs. This study also examines the use of bone marrow from multiple species, with comparisons to human samples. The second study looks at CFC assays using drug combinations. Because drug combinations can have additive or other synergistic effects on hematotoxicty, and because drugs are rarely administered to treatment-naïve patients, combination studies can provide useful preclinical information.



DIFFERENTIAL TOXICITIES OF KINASE INHIBITORS

on Hematopoietic Progenitor Cells from Different Species

INTRODUCTION

More than two decades after the approval of Imatinib, which targets the ABL tyrosine kinase in CML, development of rationally designed kinase inhibitors (KIs) has expanded to target various other cancer types, as well as inflammatory diseases. Although more successful than many conventional therapies, myelotoxicity is often a major side effect of KIs. We previously showed that there was a direct correlation (R^2=0.81)[1] between the in vitro human CFU-GM IC_{50} values for various KIs and clinical neutropenia. Interestingly, studies have revealed significant differences between human, dog, rat and mouse CFU-GM sensitivities to certain pharmaceuticals. Since multiple animal models are often used in toxicity testing, we have now compared the IC_{50} values between human, non-human primate, dog, rat, and mouse for Imatinib, Erlotinib, Dasatinib, Sorafenib, and Sunitinib. Lapatinib was also tested using human cells.

MATERIALS AND METHODS

Based on our previous data set generated using human bone marrow (Figures 1 and 2), five KIs were selected for testing: Imatinib, Erlotinib, Dasatinib, Sorafenib, and Sunitinib. These KIs have different target and disease specifications (Table 1) and also have reported differences in their clinical toxicity profiles. Clonogenic progenitor assays of the human, non-human primate, dog, rat, and mouse myeloid (CFU-GM) lineages were set up in ColonyGEL® methylcellulose-based media formulations optimized for each species with appropriate species-specific cytokines (ReachBio, WA). Lapatinib was tested using human cells but not with other species as human progenitor cell toxicity was not observed. The KIs were added to the various media formulations to give final concentrations ranging from 100 to 0.0001 µM. Solvent control cultures were also initiated.

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The cultures were set up in triplicate 35 mm dishes with cells derived from normal human bone marrow (3 x 10^4/ culture), non-human primate bone marrow from both rhesus and macaque (3 x 10^4/ culture), dog bone marrow (2 x 10^5/culture), rat femoral bone marrow (5 x 10^4/culture), and mouse femoral bone marrow (2 x 10^4/culture). The experiment was set up using cells from three individual human donors. Following incubation at 37°C, 5% CO_2, for 7-16 days (depending on the species), the colonies were assessed and scored by trained personnel and IC_{50} values were determined for each drug for each species.

Table 1 : Target and Disease Specifications of Kinase Inhibitors Tested

TKI	TARGET	TREATMENT
Imatinib	ABL/ PDGF/ KIT	CML
Dasatinib	ABL/ PDGF/ KIT/ src	CML
Sorafenib	VEGFR2/ KIT/ PDGF/ RAF/ FLt3	Renal cell carcinoma/ myeloma
Sunitinib	VEGFR1-3/ KIT/ PDGF/ CSFR1/ FLt3	Renal cell carcinoma
Erlotinib	RGFR/ mutant JAK2 kinase	Lung cancer and possibly PV and MF

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Figure 1: Correlation between CFU-GM IC_{50} Values and Clinical Neutropenia for Kinase Inhibitors

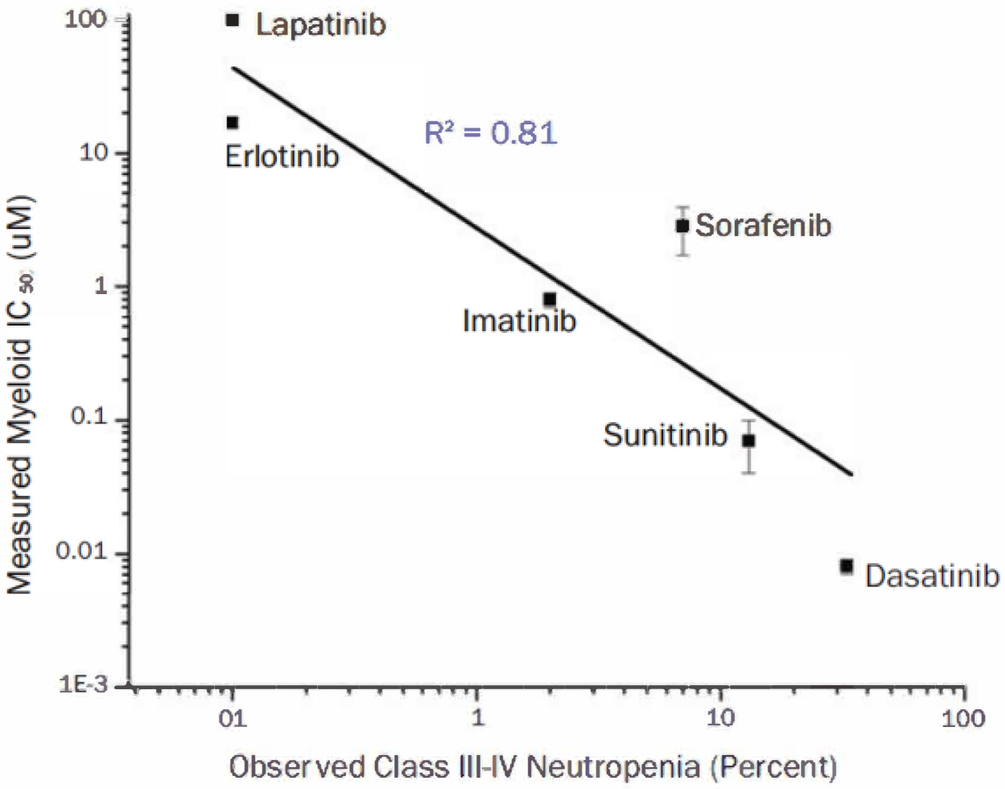
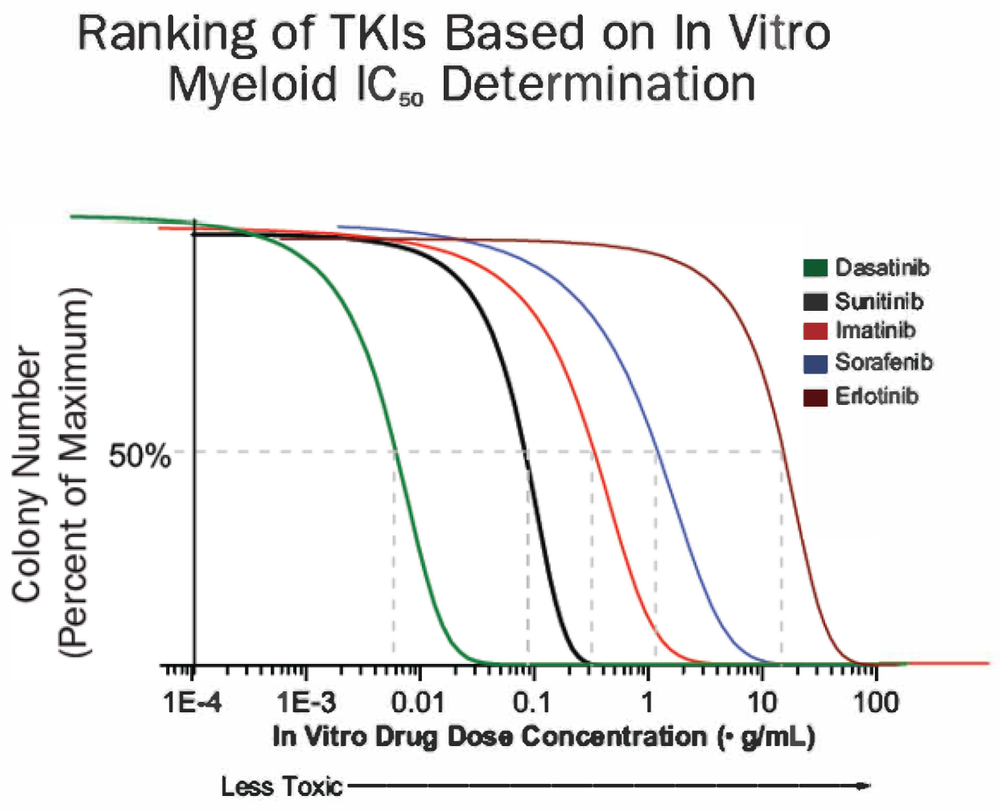


Figure 2: Kinase Inhibitor Myelotoxicity May be Ranked Based on CFU-GM Assay



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DIFFERENTIAL TOXICITIES OF KINASE INHIBITORS

on Hematopoietic Progenitor Cells from Different Species

RESULTS

IC_{50} values determined for the 5 KIs using non-human primate and dog marrow compared well with human values (Table 2, Figure 2). There was no difference in the non-human primate IC_{50} values, whether the marrow was derived from a rhesus or a macaque monkey. The rank order of in vitro toxicity of the 5 KIs was also the same in human, non-human primate, and dog CFU-GM assays, mirroring their human clinical myelosuppression rankings. In contrast, the IC_{50} values for the same drug in rat and mouse for CFU-GM assays differed significantly from human values, with over a log difference in IC_{50} values for Imatinib and Dasatinib (Table 2). When tested with mouse and rat bone marrow, the KIs also did not rank in the same order as they did in human, non-human primate, and dog assays (Table 2, Figure 3), or as they do with respect to their reported human clinical myelosuppressive levels. Representative photos of the CFU-GM derived colonies of each species are presented in Figure 4.

Table 2: CFU-GM IC_{50} Values for Kinase Inhibitors in Different Species

	Human CFU-GM	NHP CFU-GM	Dog CFU-GM	Rat CFU-GM	Mouse CFU-GM
	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)
Erlotinib	16	9.3	10.4	41	24
Sorafenib	3.5	5.7	2.9	43	18
Imatinib	2.6	3.1	2.6	25	34
Sunitinib	0.09	0.08	0.02	0.4	0.36
Dasatinib	0.008	0.02	0.012	0.34	0.49

Figure 3: Comparison of CFU-GM IC_{50} Values for TKIs from Different Species

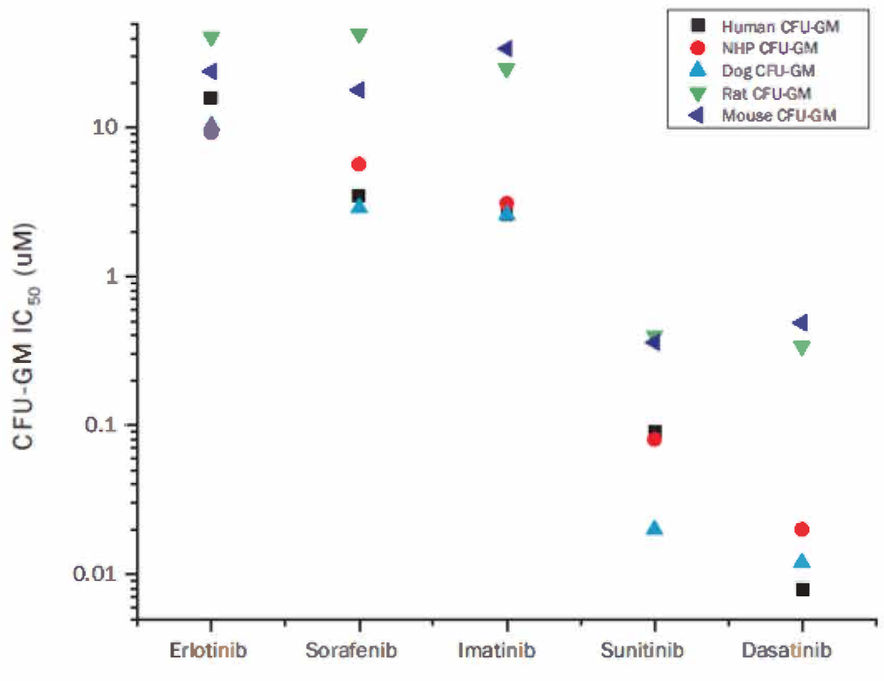
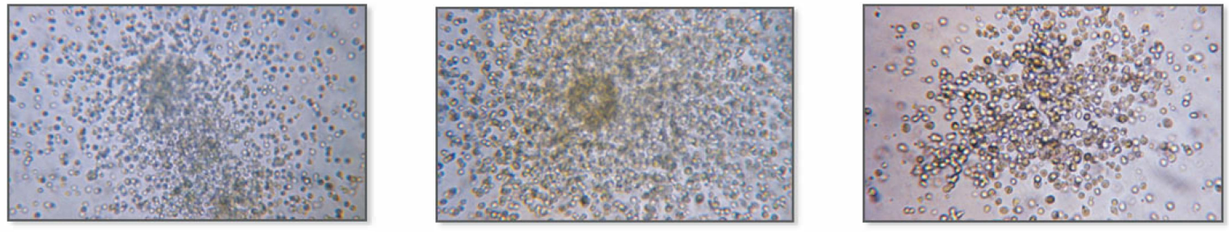
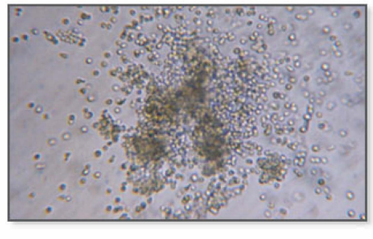


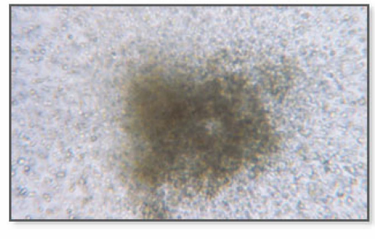
Figure 4: Representative Photos of CFU-GM Derived Colonies from Various Species



Human CFU-GM



NHP CFU-GM



Dog CFU-GM

Mouse CFU-GM

Rat CFU-GM

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CONCLUSIONS

The IC_{50} values of the five KIs tested in the non-human primate and dog marrow CFU-GM assays compared well with human CFU-GM IC_{50} values. The rank order of the compounds in terms of toxicity (as measured by the CFU-GM IC_{50} value) was the same for human, non-human primate, and dog, and corresponded with their rankings in terms of human clinical myeloid suppression level.

The IC_{50} values for the KIs in mouse and rat CFU-GM assays differed significantly from those obtained in human CFU-GM assays, with values greater than a log difference for Imatinib and Dasatinib. The rank order of toxicity of the KIs in mouse and rat CFU-GM assays was also different to their order in the human CFU-GM assay and in the clinic.

It is possible that data generated from rat and mouse assays for KIs may not as accurately predict the toxicity of these compounds on the human hematopoietic system as data from human, non-human primate, or dog assays. Further, some in vivo toxicity models using rat or mouse models may not predict, or may underestimate, clinical findings. The use of rat or mouse CFC assays may provide additional information and clarity around this issue.

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DID YOU KNOW?

THERE ARE A VARIETY OF APPLICATIONS FOR THE

HEMATOPOIETIC CFC ASSAY

- Assess off-target hematopoietic toxicity of drug candidates, including :
  - Small molecules
  - Antibody-drug conjugates (ADCs)
  - Bispecific antibodies
  - CAR-T/engineered T cells
  - Oligonucleotide therapeutics
  - Monoclonal antibodies
  - Novel biological entities
  - and others
- Predict neutropenia, severe anemia, and thrombocytopenia at the preclinical stage
- Pair the CFC assay with our neutrophil assay to assess both off-target toxicity and linker stability of ADCs
- Evaluate blood cancer (MM, AML etc) progenitor cell killing efficacy of test therapeutics
- Cytokine mimetic studies
- Study the ability of a test drug to reverse drug-induced hematotoxicity
- Human, non-human primate, dog, rat, and mouse assays
- Combination drug studies
- The CFU-GM assay has been validated by The European Centre for the Validation of Alternative Methods (ECVAM)[2]

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CFC ASSAYS ARE PREDICTIVE OF INCREASED CLINICAL NEUTROPENIA

with Combination Therapies

INTRODUCTION

Combination therapies are commonly employed to treat diseases where monotherapies do not yield the desired clinical outcomes. While combining two or more drugs can have positive synergistic benefits, certain combinations can result in significantly increased levels of myelosuppression/neutropenia. Chemotherapeutic combinations run a well-known risk of these adverse events, and this is an important preclinical consideration, particularly since new cancer therapeutics are rarely administered alone or to treatment-naïve patients. This risk is not limited to cancer therapeutics, however, and has also been observed with drug combinations targeting a variety of other disease classes, including with concurrent treatment of different diseases, as in the use of Serotonin Reuptake Inhibitors (SSRIs) and Linezolid[3].

In the study described earlier in this paper, we used CFC assays to rank the in vitro toxicity caused by a panel of therapeutic tyrosine kinase inhibitors and were able to correlate the IC_{50} values obtained therein with the level of clinical neutropenia caused by the same agents, as reported in the literature. The aim of the next study, outlined here, is to assess whether the CFC assay can also be used to predict the increased toxicity of drug combinations relative to the toxicity caused by the single agents alone.

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with Combination Therapies

MATERIALS and METHODS

Six drugs were selected for testing based upon their different target and disease specifications as well as reported differences in their clinical toxicity profiles. The compounds were tested both alone and in the following paired combinations:

-Imatinib and Hydroxyurea

-Linezolid and Fluoxetine

-Doxorubicin and Bortezomid (Velcade)

Bone marrow cells from normal donors (n=3) were mixed with the single compounds listed above (extended concentration range) in ColonyGel™ 1102 complete methylcellulose-based medium (ReachBio) and plated in 35mm dishes (three replicates per concentration). The cultures were incubated in a humidified incubator at 37°C and 5% CO_2. CFU-GM were enumerated on day 14 and IC_{50} values were determined for each drug. The drugs were then tested in the combination pairs indicated above, using the same assay system, and again using marrow from multiple donors. Additional toxicity was evaluated using a matrix design (Table 1). The percentage of CFU-GM inhibition was determined by comparing colony numbers in the treatment conditions to those in the solvent control cultures.

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Table 1 : Matrix Template

		COMPOUND 1					
		30 µM	10 µM	3 µM	1 µM	0.3 µM	0 µM
COMPOUND 2	200 µM						
	50 µM						
	10 µM						
	5 µM						
	1 µM						
	0 µM						

Matrices can be set up with any compounds and at appropriate concentrations for each one.

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RESULTS

Individual IC_{50} values ranged from 0.012µM Bortezomid (Velcade) to 109µM (Linezolid), Table 2. Where two compounds were combined together at their IC_{50} equivalent values, there was an additional inhibition of CFU-GM: 84% inhibition with Imatinib and Hydroxyurea in combination (see Table 3 for percent inhibition at multiple concentrations of both drugs), 75% inhibition with Fluoxetine and Linezolid in combination (see Table 4 for percent inhibition at multiple concentrations of both drugs) and 69% inhibition with Velcade and Doxorubicin in combination (see Table 5 for percent inhibition at multiple concentrations of both drugs).

Table 2: IC_{50} Values of Individuals Compounds

DRUG	IC_{50} VALUE (µM)
Bortezomib	0.012
Doxorubicin	0.028
Imatinib	2.6
Hydroxyurea	31
Linezolid	109
Fluoxetine	27

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Table 3: Effect of Imatinib & Hydroxyurea Alone and in Combination on CFU-GM Inhibition

		IMATINIB					
		30 µM	**10 µM**	**3 µM**	**1 µM**	**0.3 µM**	**0 µM**
HYDROXYUREA	200 µM	100%	100%	100%	100%	100%	100%
	50 µM	98%	98%	92%	86%	84%	76%
	10 µM	96%	92%	**76%**	44%	42%	42%
	5 µM	96%	88%	70%	36%	18%	8%
	1 µM	96%	84%	74%	10%	12%	4%
	0 µM	94%	86%	64%	20%	0%	

The IC_{50} for Imatinib is 2.6 µM and for hydroxyurea is 31 µM. We have highlighted the closest concentrations to these, showing CFU-GM inhibition and the inhibition of the combined concentrations.

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with Combination Therapies

Table 4: Effect of Fluoxetine & Linezolid Alone and in Combination on CFU-GM Inhibition

		FLUOXETINE					
		144 µM	**36 µM**	**12 µM**	**4 µM**	**1 µM**	**0 µM**
LINEZOLID	148 µM	100%	85%	83%	83%	76%	78%
	120 µM	100%	80%	71%	66%	59%	59%
	100 µM	100%	**83%**	71%	58%	42%	41%
	50 µM	100%	83%	58%	48%	25%	20%
	25 µM	100%	69%	47%	22%	14%	3%
	0 µM	100%	64%	31%	17%	7%	

The IC_{50} for Fluoxetine is 27 µM and for Linezolid is 109 µM. We have highlighted the closest concentrations to these, showing CFU-GM inhibition and the inhibition of the combined concentrations.

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Table 5: Effect of Doxorubicin & Bortezomib (Velcade) Alone and in Combination on CFU-GM Inhibition

		DOXORUBICIN					
		0.1 µM	**0.03 µM**	**0.01 µM**	**0.003 µM**	**0.001 µM**	**0 µM**
VELCADE	0.1 µM	100%	100%	100%	100%	100%	100%
	0.03 µM	100%	100%	100%	100%	100%	100%
	0.01 µM	99%	**69%**	44%	31%	18%	42%
	0.003 µM	98%	47%	18%	4%	-1%	27%
	0.001 µM	98%	31%	1%	2%	-5%	-4%
	0 µM	96%	65%	24%	2%	1%	

The IC_{50} for Doxorubicin is 0.028 µM and for Bortezomib (Velcade) is 0.012 µM. We have highlighted the closest concentrations to these, showing CFU-GM inhibition and the inhibition of the combined concentrations.

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with Combination Therapies

CONCLUSIONS

The GFU-GM assay can be used to test single agents or multiple agents, and may be employed to evaluate drug combinations where there is a concern for increased toxicity. Since clinical trials rarely involve treatment naïve patients, this assay may facilitate an understanding of how the toxicity of new candidate compounds might be affected by the background presence of current standard of care drugs. CFC assays using this matrix design also allow for the discovery of compounds which may exert a protective effect on a known toxic compound. These assays can also be used to predict toxicity that may not be expected with specific combinations.

Further Studies conducted by Genentech and ReachBio:

"Effects of Tyrosine Kinase 2 Inhibitors on Megakaryocyte Development." H. Uppal[1], D. Danilenko[1], E. Harstad[1], J. Tarrant[1], E. Clarke[2], P. Dhawan[1], A. Kauss[1], B. McCray[1], D. Misner[1], and J. Singh[1]. 1 Safety Assessment, Genentech Inc., San Ramon, CA; 2 ReachBio LLC, Seattle, WA.

"Effects of Nicotinamide Phosphoribosyltransferase (NAMPT) Inhibitors on Platelet Development." J. Singh[1], T. Zabka[1], H. Uppal[1], D. Diaz[1], J. Tarrant[1], E. Clarke[2], T. Lin[1], N. La[1], B. McCray[1], T. Nguyen[1], P. Dhawan[1], E. Doudement[1], A. Kauss[1], D. Dambach[1], and D. Misner[1]. 1 Safety Assessment, Genentech, South San Francisco, CA; 2 ReachBio LLC, Seattle, WA.

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AUTHOR PROFILES + REFERENCES

JENNIFER O'MALLEY

Jennifer is the Business Development Manager for Contract Services at ReachBio. With over 20 years in the biotech field, her expertise is essential in connecting with preclinical pharmaceutical scientists and introducing them to our CRO assay services. She finds the field of drug research inspiring and humbling. Jen earned a BSc, Biology from Simon Fraser University in 1998.

DR. EMER CLARKE

Emer is a co-founder and the Chief Scientific Officer at ReachBio. She is instrumental in managing the scientific operations of the company, including research and development and client services. Dr. Clarke earned her Ph.D. in hematology from Trinity College Dublin. Emer is engaged in client affairs through one-on-one consultation, study design, and data analysis.

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2. Pessina A, Albella B, Bayo M, Bueren J, Brantom P, Casati S, Croera C, Gagliardi G, Foti P, Parchment R, et al. "Application of the GM-CFU Assay to Predict Acute Drug-Induced Neutropenia: An International Blind Trial to Validate a Prediction Model for the Maximum Tolerated Dose (MTD) of Myelosuppressive Xenobiotics." Toxicol Sci *75*: 355–367, 2003

3. Hachem RY, Hicks K, Huen A, et al. "Myelosuppression and serotonin syndrome associated with concurrent use of linezolid and selective serotonin reuptake inhibitors in bone marrow transplant recipients." Clin Infect Dis. 2003; 37(1):e8–e11.