Quantitative assessment of the sensitivity of dormant AML cells to the BAD mimetics ABT-199 and ABT-737.

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Key words: AML, ABT-199, ABT-737, dormancy

Abstract

Cells from patients with acute myeloid leukaemia (AML) that remain dormant and protected by stromal cells may escape effects of chemotherapy. We modelled dormancy *in vitro* and investigated the ability of Bcl-2 inhibitors ABT-199 and ABT-737 to overcome chemoprotection of dormant cells. CD34-enriched primary AML cells with aberrant leukaemia-associated phenotypes (LAPs) were cultured on stromal cells. The chemosensitivity of dormant ($PKH26^{high}$), CD34+, LAP+ cells was ascertained by 5-colour flow cytometric counting after 12 days. The $PKH26^{high}$, CD34+, LAP+ subset retained clonogenic capacity. The dormant fraction was completely resistant to ara-C (P=0.007). However, ABT-199 and ABT-737 were able to reduce the dormant fraction by 84% and 80% respectively of their effects on proliferating counterparts. In conclusion, we have elaborated a system for quantifying chemosensitivity in LAP+ dormant leukaemia cells thought to contribute to disease relapse, and shown sensitivity of dormant LAP+ cells to ABT-199 and ABT-737 in this system.

Introduction

Relapse is common in patients with acute myeloid leukaemia after remission-induction with nucleoside analogue/anthracycline-based chemotherapy. The cells which give rise to relapse are likely to have evaded the effects of treatment by lying dormant [1, 2]. The concept of a dormant cancer cell can initially seem counter-intuitive, but in AML the presence of these cells has been substantiated by both functional and phenotypic evidence [1, 2, 3, 4, 5, 6, 7]. Dormant AML cells are concentrated adjacent to stromal cells [1, 8]. Protection of AML cells from chemotherapeutic drugs by proximity to stromal cells has been shown for several compounds, including the standard chemotherapeutics daunorubicin and cytarabine [1, 9, 10] as well as FLT3 inhibitors [5, 11]. Alvares and colleagues combined dormancy and niche in a single model and were able to demonstrate resistance to a FLT3 inhibitor in a dormant AML subpopulation cultured *in vitro* on stromal cells [5].

A potential novel approach to AML therapy is to target the B-cell lymphoma 2 (BCL2) protein family and thus directly induce apoptosis [12]. Anti-apoptotic members of the BCL2 family, i.e. BCL2, $BCLX_L$, BCLW, BFL1 (A1), BCLB and MCL1, are considered as guardians of mitochondrial integrity by opposing pro-apoptotic BCL2 family proteins. In theory, oncogenic stresses may upregulate pro-apoptotic pathways, and therefore the successful cancer cell needs to have just enough functioning anti-apoptotic molecules to hold apoptosis in check. Cancer cells may therefore be dependent on ("addicted to") survival gene expression and/or be "primed" by pro-apoptotic BH3 only proteins to readily undergo apoptosis once the anti-apoptotic family members are inhibited [13, 14, 15]. ABT-737 and ABT-199 (a reverse engineering version of ABT-737's oral form ABT-263 that has high affinity for BCL-2 and lower affinity for $BCLX_L$), have exhibited promising anti-cancer activities *in vitro* and *in vivo* [16, 17] including activity against AML cells [18, 19, 20, 21, 22]. Furthermore, these agents are found to significantly potentiate certain other

chemotherapeutics [22, 23, 24, 25, 26, 27, 28, 29], suggesting a promising future for novel drug combinations.

In the current study, we compare the sensitivities of proliferating and dormancy-enriched primary AML cells to ABT-199 and ABT-737. We show that aberrant phenotypes can persist through several days of culture on MS-5 stromal cells and we were able to use this finding in order to quantify dormant AML cells labelled with the proliferation marker PKH26 with and without the addition of ABT-199 and ABT-737.

Materials and methods

Materials

Antibodies were from BD Biosciences (Cowley, UK) and other reagents were from Sigma-Aldrich (Poole, UK), unless otherwise stated. TG02, originally designated SB1317, was kindly provided by Tragara Pharmaceuticals (Carlsbad, CA, USA). ABT-737 was from Sequoia Research products (Pangbourne, UK). ABT-199 was from Active BioChem (Hong-Kong). All three agents were reconstituted in Dimethyl sulfoxide (DMSO) and stored at 10 mM.

AML Patient Samples

Fresh or cryopreserved bone marrow or peripheral blood samples of patients presenting with *de novo* or relapsed AML were collected after written consent at diagnosis at the Nottingham University Hospitals. Use of these samples was approved by the Nottingham Research Ethics Committee and the samples were anonymised. Mononuclear cells were isolated using a standard density gradient/centrifugation method with Histopaque and red blood cells depleted from the sample using ammonium chloride. Cryopreserved samples kept in liquid nitrogen were thawed and rested in RPMI-1640 medium (Sigma-Aldrich, Poole, UK) enriched with

20% FCS (First Link, UK), for 60 to 90 minutes before experimental handling. Only samples with > 85% post-rest viability, assessed by trypan blue exclusion, were used. Leukaemia associated phenotypes (LAPs) were assessed by published methods [30] prior to culture.

12-day Leukaemic cultures on stroma

MS-5 stromal cells [31] were maintained at a density of 5 x 10^4/mL in α-minimum essential medium (α-MEM; Gibco, Paisley, UK), supplemented with 10% heat-inactivated FCS, 1% penicillin/streptomycin and 2mmol/L L-glutamine. For 12-day co-culture studies, MS-5 cells were sub-cultured at 2 x 10^4/mL on a gelatin-coated 12-well flat-bottom plate for 24 to 48 hours to form a monolayer before addition of AML cells. $CD34^+$ cells from fresh or cryopreserved AML samples were enriched by magnetic bead separation using Mini-MACS CD34 isolation kit (MiltenyiBiotec, Bisley, UK) and then labelled with the permanent cell membrane dye PKH26 according to manufacturers' instructions. Labelled cells (at 2 x 10^6 cells/mL) were added to the MS-5 feeder layer and co-cultured in Gartner Medium [32].Cells were kept in culture for 12 days and demi-populated on day 7. Drugs were added to cells on day 8 of the cell culture. On day 12, cells were harvested and processed for clonogenic assay and flow cytometric assessment as detailed below.

Enumeration of viable, dormant LAP positive AML cells

Immunophenotyping assays were performed on a FACS Canto II cytometer equipped with FACS-DIVA II software (Becton Dickinson, San Jose, USA). On days 0,7 and 12, cells were labelled with anti-CD45-APC.Cy^{TM}7 and anti-CD34-APC clone BIRMA-K3 (Alere Ltd, Stockport, UK). Additional antibodies used to identify LAPs were anti-CD7- FITC (Beckman Coulter, Marseille, France) or anti-CD19-FITC. 7-AAD was used at 5μg/mL to exclude non-

viable cells. Dako CytoCount™ beads (Alere Ltd, Stockport, UK), were added as internal standard for cell counting.

Methylcellulose Colony Formation (CFU) Assay

Following 12-day culture, cells were labelled as above and sorted on a MoFlo™ XDP Cell Sorter (Beckman Coulter, Fullerton, CA, USA). Sorted cells were washed, re-suspended in StemSpan Expansion medium with 1% Stemspan CC100 cytokine cocktail for 3-4 days and transferred to Methocult H4034 colony medium (all from Stem Cell Technologies, Grenoble, France). Cells were plated onto 96-well flat-bottom plates at 2 x 10^4 cells/100 µL in triplicates and were incubated at 37 ˚C in 5% CO_2 for 14 days. Colonies of >50 cells were counted.

Statistics

Data were analysed using SPSS 21 software package (Chicago, IL, USA).

Results

Dormant primary leukaemia cells retaining clonogenic capacity can be identified after 12 days of co-culture and are effectively targeted by ABT-737 and ABT-199

We established and validated a system for measuring the chemosensitivity of dormant primary leukaemia cells (Figure 1). After labelling CD34+ primary cells with PKH26 and culturing on MS-5 stromal cells for 12 days, we ascertained that the leukaemic nature of the cells following culture could be assured through the selection of samples with a major leukaemia associated phenotype (LAP). The aberrant CD34/CD7 and CD34/CD19 status of cells identified in presentation samples was maintained in the dormant subset throughout the 12 days of culture (Table I). We further ascertained the clonogenicity of sorted CD45

$^{dim}CD34^{+}LAP^{+}PKH26^{high}$ (dormant) subpopulation following the 12 day culture. We found that this subpopulation retained almost all of the clonogenic capacity compared to the $CD45^{dim}CD34^{+/-}LAP^{+}PKH26^{low}$ cells that had already divided *in vitro* (Table II). In two samples, a comparison of stromal support by MS-5 and by mesenchymal stem cells was made, with healthy human adult BM-MSCs cultured as described [33]. Clonogenicity of cells cultured by both methods was measured, but major differences were not observed (Table II).

Drugs were added after eight days to primary AML cells in the MS-5 co-culture system. The drug concentrations used were chosen on the basis that these were the average 72 hour IC_{40} values determined in preliminary assays in suspension culture (data not shown). Chemosensitivity was ascertained by 5-colour flow cytometric counting using a suspension of CytoCount™ fluorospheres as a reference population. Six samples were studied with this protocol as shown in Table III. We have previously shown that the multi-kinase inhibitor TG02, which targets transcriptional cyclin-dependent kinases, has efficacy against dormant AML cells [7] and we used this agent as a positive control. 200 nM Ara-C reduced the cell number by 49% in the cycling fraction, but there was no cell reduction at all in the dormant fraction (P=0.007, Figure 2). In contrast, exposure to ABT-199, ABT-737 and TG02 markedly reduced the number of both dormant and cycling cells. Although there is some favouring of cycled cells with these agents (Table III), we found that ABT-199 and ABT-737 were able to reduce the dormant cell fraction by 84% and 80% respectively of their effects in proliferating counterparts.

Discussion

The presence of dormant cells within a leukaemic clone presents the chemoresistance researcher with a distinct challenge, as most anti-neoplastic drugs have been designed to target proliferating cells as a surrogate for tumour cells, and therefore highly chemoresistant dormant cancer cells do not fit into the mainstream chemotherapeutic paradigm.

We built on a model previously described by Alvares and colleagues for identifying dormant cells grown on MS-5 stromal cells [5]. Additionally we used LAP markers to identify leukaemic cells against a background of potentially normal haematopoietic cells. We chose 12 days to approximate the length of time an AML cell would need to remain dormant to resist a course of chemotherapy. Guan and colleagues had demonstrated that the majority of *ex vivo* dormant AML cells rapidly re-enter the cell cycle *in vitro* [4], and thus phenotypic labelling of freshly isolated or thawed cells with dormancy markers might greatly overestimate the proportion of cells that would remain dormant, whereas the 12 day culture allowed us to be more confident that we were studying a clinically relevant subset of these cells. Our evaluation of this model indicated that not only could the expression of some LAP markers persist in the 12-day culture, but also that some dormant LAP^+ leukaemia cells maintained their clonogenic potential, thus alleviating our concern that the *in vitro* conditions may have rendered the cells senescent. A second argument against the *in vitro* induction of senescence in our model is that senescent cells are characterised by increased size and granularity and therefore have increased low-angle and orthogonal light scatter (forward scatter and side scatter) on flow cytometric analysis [34], whereas the CD34+/PKH^{high} cells selected for analysis in our experiments retained low scatter properties – this is illustrated in the Figure 1 gating. The variability in proliferative abilities between cells within a single sample has been appreciated for several decades [35, 36], but the reasons why clonogenic cells can remain dormant in culture with stromal cells and then form colonies in semi-solid medium suggests a contribution from the stromal cells to the state of dormancy. The retention of clonogenic potential in $PKH^{high,}$ but not PKH^{low} cells was very marked, and the use of LAP markers in the sorting process ensured that non-leukaemic cells were not affecting the output.

The efficacy of ABT-199 and ABT-737 in dormant cells cultured on MS-5 was at least 80% of their efficacy against cycled cells and the contrast to Ara-C, which completely failed to eradicate dormant primary cells when cultured on stromal cells, was pronounced. Primary samples differ enormously in their rates of cycling and therefore the proportion of cells likely to be vulnerable to ara-C [37]. Morevoer, the osteoblastic niche has been shown to protect a dormant subset of AML cells from this drug [1]. The *in vitro* model system used thus enabled us to incorporate a protective stromal microenvironment, to discriminate between leukaemic and residual normal cells based on LAPs and

to contrast the effects of novel agents with the established drug araC which is selective for proliferating cells. The success of this model in assessing drug sensitivity in both cycling and dormant cell compartments, whilst unlikely to eliminate the need for *in vivo* models, has the potential to refine the number and scale of such studies.

2/6 samples used in this study were from patients with complex cytogenetics. A further patient with normal cytogenetics was studied with refractory disease. A fourth patient had transformed CMML and was unsuitable for intensive treatment. There were no good cytogenetic risk samples included. The high proportion of CD7+ aberrant phenotypes probably contributed to this bias. There have been at least 20 studies of aberrant CD7 expression in AML with very mixed results as to its prognostic value. However, the majority consensus appears to be that it is associated with normal or adverse cytogenetics and with multidrug resistance [38, 39]. This is therefore an interesting cohort to study with a view to identifying therapies from which hard-to-treat patients might benefit.

Evidence that BCL2 is therapeutically relevant in AML was established in our laboratory and by others nearly two decades ago [40, 41], but has been refined recently with the emergence of first ABT-737 and then ABT-199 as research tools and as drugs. An additional important discovery is that cells with low levels of reactive oxygen species (ROS-low), functionally defined as leukaemic stem cells, aberrantly over-express BCL-2 and are selectively targeted by ABT-737 [20]. ROS-low AML cells are enriched for dormant (Ki-67 negative) cells and therefore overlap the subpopulation we have investigated in the current work. An observation that AML cells with high BCL2 expression tend to have an impaired ability to enter the cell cycle has also been documented [42]. ABT-737 causes prolonged thrombocytopenia associated with $BCLX_L$ targeting, but ABT-199, which is more specific for Bcl-2, is of huge clinical interest: one report has been published [43] and 12 clinical studies are currently (September 2017) listed for ABT-199 (also known as venetoclax) in AML by clinicaltrials.gov. Our data suggest that this agent may have efficacy against cells protected by niche and dormancy. Analysis of post-treatment biopsies would be of great help to establish the cell cycle status of residual cells and thus whether venetoclax can eradicate dormant leukaemic cells in the clinic.

In summary, we have established and validated a system for the direct comparison of the chemosensitivity of intra-sample cycling and dormant leukemic cells. Using this methodology we have shown a differential response to araC in cycling versus dormant leukaemia cells, however ABT-199 and ABT-737 demonstrated effective targeting of both cellular compartments emphasising the potential importance of these agents in the clinical eradication of all leukaemic cells.

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Contributions

NY designed the study, developed methodology, performed and analysed experiments, and wrote the manuscript.

CS performed and analysed experiments and edited the manuscript.

NR designed the study, provided samples and demographics, and edited the manuscript.

MP designed the study, performed and supervised experiments, and wrote the manuscript.

FIGURE LEGENDS

Figure 1. 12 day culture of dormant primary AML cells

(A) Schema for dormant leukaemia cell chemosensitivity assay.

(B) and (C). Flow cytometric gating for dormant cells chemosensitivity assay. (B) On Day 0 the fluorescence of PKH-labelled CD34+ cells is recorded. (C) On day 12, (i) scatter, (ii) 7-AAD and (iii) CD45-gated plots combine to distinguish viable myeloid cells. (iv) LAP positive cells are identified (CD7 in this example). (v) Although most cells have decreased/lost PKH-26 and/or CD34 compared with Day 0, a sub-population of non-dividing ($PKH26^{high}$) cells is detected in CD34+, LAP+ myeloid blasts.

Figure 2. Sensitivity to ABT-199 and ABT-737 in 12 day culture of dormant primary AML cells

The bar chart shows percentage surviving cells compared with untreated controls in the dormant and cycled fractions of AML blasts after 12 days culture on MS-5 stromal cells (n=5, mean + standard deviation). 100 nM ABT-199, 100 nM ABT-737, 25 nM TG02 or 200 nM Ara-C were added on Day 8.

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Table I. Retention of aberrant surface marker (LAP) expression after 12-day culture of primary AML cells

AML sample	Days in culture	LAP marker	LAP cells as % of total AML cells	LAP cells in the $CD45^{dim}CD34^{+}PKH26^{high}$ compartment (%)
603	**Day 0**	CD7	85.5	79.1
	Day 7		68.1	77.4
	Day 12		13.8	77.2
596	**Day 0**	CD7	84.6	89.1
	Day 7		73.6	83.4
	Day 12		12.5	83.9
582	**Day 0**	CD19	43.1	54.7
	Day 7		38.5	44.8
	Day 12		37.3	41.3
536	**Day 0**	CD7	61.3	85.1
	Day 7		55.4	84.3
	Day 12		53.9	80.4
444	**Day 0**	CD7	86.4	98.4
	Day 7		73.1	87.2
	Day 12		41.4	86.9
601	**Day 0**	CD7	32.9	92.1
	Day 12		26.3	72.9

Table II. Colony frequency in sorted fractions from *de novo* AML samples after 12-day culture

AML sample	Subset	Total sorted cells	Total colony count	Standardised colony growth rate (per 10,000 cells)
603	dormant	1471	11	75
	cycled	344	0	0
596	dormant	717	17	237
	cycled	8510	2	2
582	dormant	6530	31	47
	cycled	9830	3	3
536	dormant	10390	55	53
	cycled	230750	3	1
444	dormant	6306	54	86
	cycled	10757	0	0
*603 MSC**	dormant	1070	4	37
	cycling	128	0	0
*596 MSC**	dormant	352	15	426
	cycling	3359	1	3

*Although MS-5 cells were used in all cases, in two cases (#603 and #596) we also used primary mesenchymal stem cells (MSC) so that the clonogenic yield from each support system could be compared.

Table III. Samples studied in 12-day culture

Demographics						
Sample	603	582	596	444	536	601
patient age	64	42	67	71	40	61
LAP	CD34 CD7	CD34 CD19	CD34 CD7	CD34 CD7	CD34 CD7	CD34 CD7
NPM1 status	wildtype	wildtype	mutant	wildtype	wildtype	wildtype
FLT3 ITD status	wildtype	mutant	mutant	wildtype	wildtype	mutant
FLT3 point mutation status	wildtype	wildtype	wildtype	wildtype	wildtype	wildtype
Cytogenetics	complex	MLL, +11	46XX del(2)(q11q21) der(7)t(1;7)(q2q22), add(11)(q2)[9]	normal	complex	normal
%CD34+	76	76	10	91	16	10.5
%CD34+ CD38-	0.2	0.031	2.407	not done	0.04	2.1
category	de novo	de novo	de novo	refractory	relapse after transplant	transformed CMML
Percent cell survival						
araC						
cycled	52	51	62	97	48	n/a*
dormant	104	117	105	111	97	100
ABT-737						
cycled	43	8	31	39	37	n/a*
dormant	49	39	46	32	60	61
ABT-199						
cycled	39	30	29	7	32	n/a*
dormant	47	36	40	14	60	42
TG02						
cycled	48	17	40	59	42	n/a*
dormant	65	41	62	47	81	73

* This sample lacked a cycled subset after 12 days. (It was therefore excluded from the comparative graph)

Figure 1

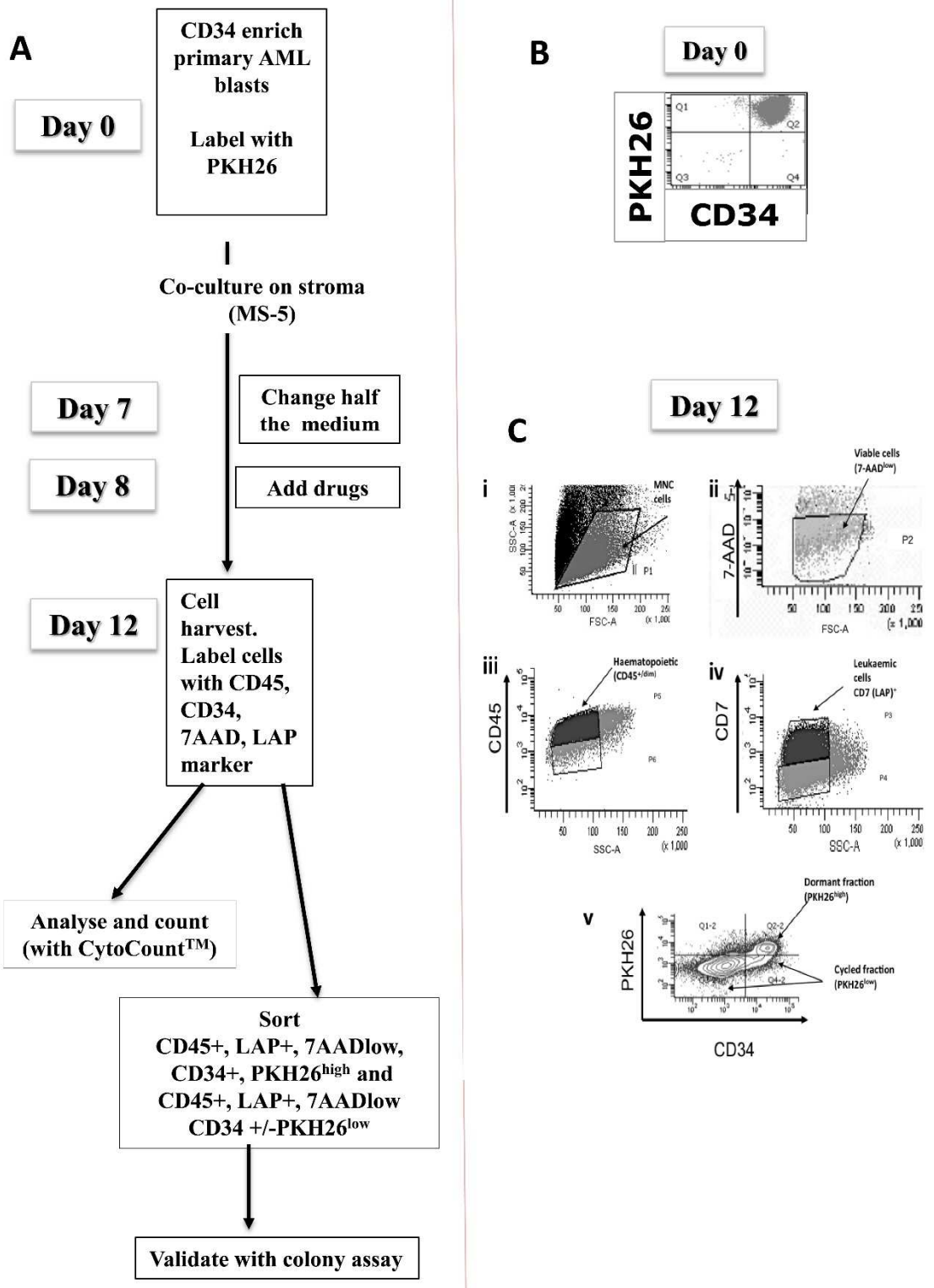
A
CD34 enrich primary AML blasts
Label with PKH26
Day 0
Co-culture on stroma (MS-5)
Day 7
Change half the medium
Day 8
Add drugs
Day 12
Cell harvest. Label cells with CD45, CD34, 7AAD, LAP marker
Analyse and count (with CytoCount™)
Sort CD45+, LAP+, 7AADlow, CD34+, PKH26high and CD45+, LAP+, 7AADlow CD34 +/-PKH26low
Validate with colony assay
B
Day 0
PKH26
CD34
Q1
Q2
Q3
Q4
C
Day 12
i
MNC cells
P1
SSC-A
FSC-A
ii
Viable cells (7-AADlow)
P2
7-AAD
FSC-A
iii
Haematopoietic (CD45+/dim)
P5
P6
CD45
SSC-A
iv
Leukaemic cells CD7 (LAP)+
P3
P4
CD7
SSC-A
v
Dormant fraction (PKH26high)
Cycled fraction (PKH26low)
Q1-2
Q2-2
Q4-2
PKH26
CD34

Figure 2 **Table III. Samples studied in 12-day culture**

Demographics						
Sample	603	582	596	444	536	601
patient age	64	42	67	71	40	61
LAP	CD34 CD7	CD34 CD19	CD34 CD7	CD34 CD7	CD34 CD7	CD34 CD7
NPM1 status	wildtype	wildtype	mutant	wildtype	wildtype	wildtype
FLT3 ITD status	wildtype	mutant	mutant	wildtype	wildtype	mutant
FLT3 point mutation status	wildtype	wildtype	wildtype	wildtype	wildtype	wildtype
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Percent cell survival						
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dormant	49	39	46	32	60	61
ABT-199						
cycled	39	30	29	7	32	n/a*
dormant	47	36	40	14	60	42
TG02						
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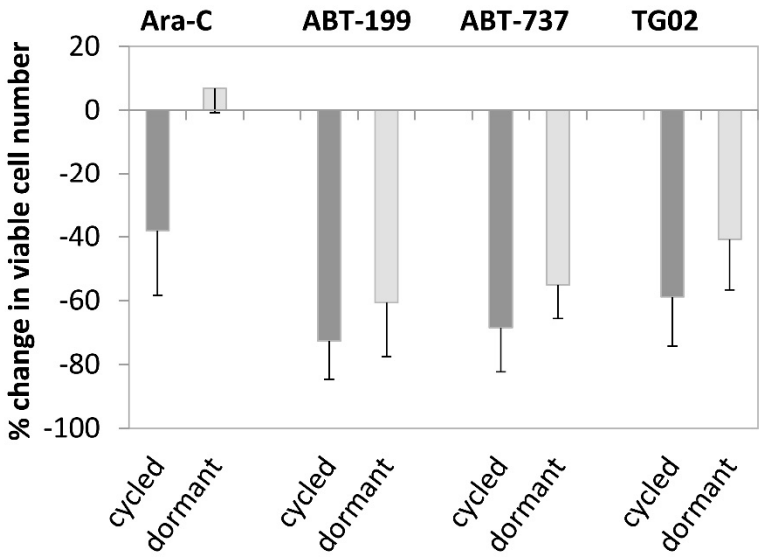
dormant	65	41	62	47	81	73

* This sample lacked a cycled subset after 12 days. (It was therefore excluded from the comparative graph)


Ara-C
ABT-199
ABT-737
TG02
% change in viable cell number
20
0
-20
-40
-60
-80
-100
cycled
dormant
cycled
dormant
cycled
dormant
cycled
dormant