bjh research paper

Combination of a mitogen-activated protein kinase inhibitor with the tyrosine kinase inhibitor pacritinib combats cell adhesion-based residual disease and prevents re-expansion of *FLT3*-ITD acute myeloid leukaemia

Joanna Zabkiewicz, Michelle Lazenby, Gareth Edwards, Ceri A. Bygrave, Nader Omidvar, Lihui Zhuang, Steve Knapper, Carol Guy, Robert K. Hills, Alan K. Burnett and Caroline L. Alvares

Academic Department of Haematology, University of Cardiff, Heath Park, Cardiff, UK

Received 24 February 2020; accepted for publication 23 March 2020

*Correspondence: Caroline Alvares, Academic Department of Haematology, Cardiff University, Cardiff CF14 4XN, UK.
E-mail: AlvaresC@cardiff.ac.uk

*(formally)

Abstract

Minimal residual disease (MRD) in acute myeloid leukaemia (AML) poses a major challenge due to drug insensitivity and high risk of relapse. Intensification of chemotherapy and stem cell transplantation are often pivoted on MRD status. Relapse rates are high even with the integration of first-generation FMS-like tyrosine kinase 3 (FLT3) inhibitors in pre- and post-transplant regimes and as maintenance in *FLT3*-mutated AML. Pre-clinical progress is hampered by the lack of suitable modelling of residual disease and post-therapy relapse. In the present study, we investigated the nature of pro-survival signalling in primary residual tyrosine kinase inhibitor (TKI)-treated AML cells adherent to stroma and further determined their drug sensitivity in order to inform rational future therapy combinations. Using a primary human leukaemia-human stroma model to mimic the cell–cell interactions occurring in patients, the ability of several TKIs in clinical use, to abrogate stroma-driven leukaemic signalling was determined, and a synergistic combination with a mitogen-activated protein kinase (MEK) inhibitor identified for potential therapeutic application in the MRD setting. The findings reveal a common mechanism of stroma-mediated resistance that may be independent of mutational status but can be targeted through rational drug design, to eradicate MRD and reduce treatment-related toxicity.

Keywords: acute myeloid leukaemia, tyrosine kinase inhibitors, drug resistance, microenvironment, minimal residual disease.

The FMS-like tyrosine kinase-3 internal tandem duplication (*FLT3*-ITD) mutation confers a poor prognosis in acute myeloid leukaemia (AML) due to a high relapse rate, and is a major therapeutic target.[1,2] Tyrosine kinase inhibitors (TKIs) are currently approved for *FLT3*-mutated AML.[3–5] Many clinical pathways now risk-stratify patients according to whether minimal residual disease (MRD) is detectable or not after initial chemotherapy and intensification of treatment including stem cell transplantation (SCT) is pivoted on MRD status. The bone marrow (BM) microenvironment is implicated in drug resistance through activation of pro-survival signalling, stromal cytokine secretion and direct contact of leukaemia cells at the stromal interface.[6,7] The key focus is now on identifying combination therapies with targeted agents that can potentiate toxicity against AML in the context of a cytoprotective microenvironment,[8] in order to achieve MRD-negative status at the earliest time-point and before SCT, to reduce relapse risk and optimise patient outcomes. Informing the rationale design of such therapies requires an understanding of the signalling pathways activated on adherence of AML cells to stroma and its functional consequences. Recent studies have reported efficacy of TKI and azacytidine combinations to eliminate stromal protection of blasts[9]; however, the signalling dependencies of niche attached AML cells have not been characterised. Coupling therapies able to specifically off-set stroma-dependent AML signalling may help to eliminate MRD and reduce treatment-related toxicity.

doi: 10.1111/bjh.16665

Signalling through the FLT3 receptor can activate many pathways including: phosphatidylinositol 3-kinase (PI3K)/phosphatase and tensin homologue (PTEN)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR), Ras/Raf/mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK), and Janus kinase/signal transducers and activators of transcription (JAK/STAT)[10,11]; however, the signalling profiles of stroma-adherent AML cells may differ from non-adherent blasts. *FLT3*-ITD has been implicated in regulating leukaemia cell adhesion through α4β1 integrin,[12] but the reliance of TKI-resistant cells on stromal contact for survival is unknown. High throughput screening of TKIs using stroma-conditioned media suggests that synergy between JAK2 and FLT3 inhibitors may override the secretory stromal protection of *FLT3*-mutant AML.[13] It is already established that pacritinib, a dual FLT3/JAK2 inhibitor, can inhibit *FLT3*-ITD AML growth in xenografts, and interestingly, reduces intra-pulmonary leukaemic deposits in these models, suggesting that it may mitigate the ability of AML cells to seed onto extramedullary sites.[14] Given that leukaemic cell lines may not have a dependency on stromal contact for their survival, we investigated the oncogenic signalling pathways active in both stroma-adherent and non-contact primary AML cells that may contribute to drug-resistant MRD in a TKI context. The ability of several TKIs, including pacritinib, to kill primary AML cells was examined, in a system that models primary AML–stromal interactions and residual disease, thus providing clinically relevant therapeutic insight.

Materials and methods

Cell lines and reagents

Pacritinib was supplied by CTI Biopharma Corp. (Seattle, USA). PD0325901 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Cytosine arabinoside (Ara-C) was supplied by the University Hospital of Wales pharmacy. Ponatinib was supplied by Ariad Pharmaceuticals (Cambridge, MA, USA) and crenolanib by AROG pharmaceuticals (Dallas, TX, USA). Ruxolitinib was purchased from Cell Guidance Systems Ltd (Babraham Bioscience Campus, Cambridge, UK). MS-5 and MV4;11 cells (DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany) were cultured in α-minimum essential medium (MEM) (Sigma-Aldrich) and Iscove's modified Dulbecco's media (IMDM), respectively, supplemented with 10% v/v fetal calf serum (FCS), 200 mmol/l glutamine (Sigma-Aldrich) and 20 μg/ml gentamycin. HS5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-high glucose (Sigma-Aldrich), 10% FCS, 1% Glutamax (Gibco by Life Technologies, Carlsbad, CA, USA) and 20 μg/ml gentamycin. Stromal cells were not used beyond passage 4.

Primary samples

Primary AML peripheral blood and BM samples were obtained from patients with *de novo* AML at diagnosis (Table 1) following written informed consent and in accordance with the National Cancer Research Institute (NCRI) AML trial bank regulations and the Declaration of Helsinki. Mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation. Primary cells (>75% viability) were cultured in IMDM with 10% FCS, 200 mmol/l glutamine (Sigma-Aldrich) and 20 μg/ml gentamycin for short-term assays. Primary mesenchymal stromal cells (MSCs) from healthy subjects or patients with AML at diagnosis were cultured in α-MEM, 15% fetal bovine serum (FBS), 1% L-glutamine (Gibco by Life Technologies) and 20 μg/ml gentamycin. Normal BM (NBM)- and AML-derived MSCs were used at passage 3 for assays. Multi-lineage potential of MSCs was verified as described in Ref. [15].

Cytotoxic drug response and synergy assays

Primary AML blasts were seeded at a density of 1×10^6/ml in 96-well plates and Cell Titer Glo assays set up according to manufacturer's instructions (Methods S1).

Annexin V apoptosis assay

Primary AML cells were seeded in triplicate for 24, 48, 72 h and 7 days in IMDM/10% FCS in the presence of pacritinib concentrations up to 1 μmol/l. Cells were stained with propidium iodide (PI) for cell cycle status or Annexin V/PI for apoptosis (Bender Medsystems Inc., Burlingame, CA, USA) and analysed by flow cytometry for late apoptotic (annexin V+/PI+) fractions.

Intracellular flow cytometry

Primary AML cells were co-cultured with stroma for 3 h, pacritinib or dimethyl sulphoxide (DMSO, vehicle control) was added and further incubation maintained for 18 h. Floating AML cells were removed and adherent AML cells isolated by trypsinization. Cells were fixed and permeabilised in Phosflow Perm II/III buffer according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA). CD45 distinguished AML and stromal populations. Co-staining with Vybrant® (Thermo Fisher Scientific, Inc., Waltham, MA, USA) cell viability dye excluded dead cells prior to analysis on the Accuri flow cytometer (BD Biosciences).

Leukaemic outgrowth assays

Primary AML cells were seeded on 80% confluent HS5. AML cells were cultured in long-term culture-initiating cell (LTC-IC) assay media [α-MEM, 12·5% heat-inactivated v/v FCS, 12·5% v/v heat-inactivated horse serum (Labtech International Ltd, Heathfield, East Sussex, UK), 20 μg/ml gentamycin,

Table 1. Patients' characteristics and EC_{50} (pacritinib)

Characteristic	Number	EC_{50}, nmol/l, median (range)	*P* (relation between EC_{50} and characteristic)
All data	63	112 (11–12 400)	
Age, years			
0–29	10	99 (25–779)	s
30–39	6	90 (19–361)	
40–49	16	93·5 (11–732)	
50–59	20	191·5 (16–2600)	
60+	11	64·5 (13–12 400)	
Median (range)	47 (0–70)		0·4†
Sex			
Male	31	127 (11–1100)	
Female	32	109·5 (11·6–12 400)	0·6*
WBC count, $\times 10^9$/l			
0–9·9	2	Values: 415, 700	
10–49·9	24	121 (22–779)	
50–99·9	18	123·5 (13–12 400)	
100+	19	56 (11–732)	
Median (range)	61·4 (1·8–386·5)		0·005†
Type of AML			
De novo	58	113·5 (11–12 400)	
Secondary	5	111 (20–380)	0·6*
Cytogenetic group†			
Favourable	9	133 (20–1100)	
Intermediate	43	111 (11·6–12 400)	
Adverse	3	732 (361–779)	
Unknown	8	96·5 (11–2600)	0·3†
FLT3 status			
ITD wt	40	147 (19–2600)	
ITD mutant	23	85 (11–12 400)	0·01*
ITD allelic ratio			
ITD WT	40	147 (19–2600)	
ITD mutant <50%	13	109 (11·6–12 400)	
ITD mutant ≥50%	5	46 (28–11 530)	0·03†
ITD mutant ratio unknown	5		
TKD WT	51	108 (11–12 400)	
TKD mutant	12	138 (24–1100)	0·3*
NPM1 status			
WT	41	115 (11·6–12 400)	
Mutant	22	110·5 (11–2600)	0·6*
ITD/NPM1 status			
ITD wt/NPM1 wt	30	172·5 (19–1100)	
ITD wt/NPM1 mut	10	121 (43–2600)	
ITD mut/NPM1 wt	11	56 (11·6–12 400)	
ITD mut/NPM mut	12	90·5 (11–222)	0·09*
NRas status			
NRas wt	51	147 (11–12 400)	
NRas mutant	12	100 (11–322)	0·4
Runx1 status			
Runx1 wt	55	100 (11–12 400)	
Runx1 mutant	8	361 (97–732)	0·01*
CEBPa status			
CEBPa wt	56	115 (11–12 400)	
CEBPa mutant	7	40 (19–176)	0·02*

Table 1. (*Continued*)

Characteristic	Number	EC_{50}, nmol/l, median (range)	*P* (relation between EC_{50} and characteristic)
WT1 status			
WT1 wt	54	111 (11–2600)	0·01*
WT1 mut	9	732 (60–12 400)	

CEBPa, CCAAT/enhancer-binding protein alpha; ITD, internal tandem duplication; NPM1, nucleophosmin 1; TKD, tyrosine kinase domain; WBC, white blood cells; WT1, Wilms tumour 1.

*Wilcoxon rank-sum/Kruskal test for difference between groups.

†Spearman correlation coefficient for continuous data/ordered groups.

200 mmol/l L-glutamine, 57·2 μmol/l β-mercaptoethanol (Life Technologies, Gaithersburg, MD, USA), 1 μmol/l hydrocortisone (Sigma-Aldrich), 20 ng/ml interleukin 3 (IL-3), 20 ng/ml granulocyte-colony stimulating factor (G-CSF), 20 ng/ml thrombopoietin (TPO; PeproTech Inc., London, UK)] with drug or DMSO. Control plates were set up without stromal monolayers. Cells were incubated for 7 days with drugs, and cell viability tracked by Trypan blue (Sigma-Aldrich). After suspension cell removal and drug washout, re-population from residual stroma-adherent blasts was monitored over 7 days. CD45-allophycocyanin (APC) was used to mark AML cells and CD90-phycoerthyrin (PE) to distinguish stromal cells.

Genotyping for FLT3-ITD, tyrosine kinase domain (TKD) and nucleophosmin 1 (NPM1)

See Methods S1.

Aldehyde dehydrogenase (ALDH) staining, LTC-IC and secondary limiting dilution analysis (LDA) assays

Primary AML cells were stained for ALDH with Aldefluor according to the manufacturer's instructions (Stem Cell Technologies UK, Cambridge, UK), co-labelled for leukaemia stem cell (LSC) populations with CD34 and CD38, and CD34+ CD38−ALDHint populations cell sorted (BD Aria flow cell sorter; BD Biosciences, San Jose, CA, USA) onto HS5 stroma in 96-well plates for LTC LDA assays. Cultures were replenished with fresh media and drugs or vehicle control on a bi-weekly basis and maintained for 5 weeks. H4435 methylcellulose was added after 5 weeks (Stem Cell Technologies) and colonies scored after 2 weeks. Secondary colony-forming unit (CFU) read-out was performed as described in Ref. [16]. LTC-IC frequency was calculated using the on-line ELDA software package (http://bioinf.wehi.edu.au/software/elda/).

Antibodies and Western blot analysis (WB)

See Methods S1.

In vivo experiments

All the experimental procedures were approved by Cardiff University Animal Welfare and Ethical Review Body and conform to the UK Home Office regulations. We established humanised models of leukaemia in sub-lethally irradiated adult (aged 7–10 weeks) NOD/SCID/IL2R$^{-/-}$ mice (NSG, one dose 200 cGy) by intravenous transplantation of 10^5 MV4;11 cells. At 14 days after the transplantation, mice were split into two equal groups and given intraperitoneal injections of vehicle or pacritinib:PD0325901 (0·6:3·0 mg/kg respectively) daily for 8 days. Mice were continually monitored for symptoms of leukaemia and tumour load was assessed at 6 weeks after inoculation. BM harvests were analysed by fluorescence-activated cell sorting (FACS), with engraftment of human donor cells defined as hCD45 positive.

Statistical analysis

Significant differences between AML cell populations were determined using a two-tailed paired *t*-test or a Mann–Whitney *U*-test in GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). The relationships between patient characteristics and pacritinib half maximal effective concentration (EC_{50}) were determined using Wilcoxon rank-sum/Kruskal–Wallis test for difference between groups and Spearman correlation coefficient for continuous data ordered groups (Table 1).

Results

Human stroma modulates the TKI sensitivity of primary AML cells

Both pacritinib and ponatinib showed significantly greater sensitivity than crenolanib on primary AML blasts (Fig 1A). Second-generation FLT3 inhibitors showed good efficacy in both wild-type (WT) and *FLT3* mutants (Table 1) and (Fig 1C,D). Pacritinib sensitivity was significantly greater in the 23 patients with a *FLT3*-ITD (Fig. 1B), although a sensitive WT group could be identified, suggesting a *FLT3* mutation-independent effect. As our interest was in the stroma-mediated effects of TKIs, we focussed on its activity in our primary models. For FLT3 independent effects of pacritinib, drug efficacy in sensitive FLT3 WT patient samples was compared to ruxolitinib, a JAK1/JAK2 inhibitor. Ruxolitinib

EC_{50}s were significantly >1000 nmol/l for all samples except for one AML, noted to have higher pJAK2 levels (Figure S1A,B). There were no *JAK2* mutations detected in any of the *FLT3*-WT or *FLT3*-mutant samples, suggesting pacritinib sensitivity in this subset to be independent of *JAK2* or *FLT3*-ITD mutation status.

The presence of stroma reduced the response to TKIs by increasing EC_{50} (Fig 1C,E,F) and reducing apoptosis of AML cells. Pacritinib did not produce any significant toxicity to stroma (Figure S3A,B). The induction of pro-survival signalling was greater in human:human co-cultures (Figure S3C) and therefore HS5 was chosen for longer term co-culture assays.

Pro-survival signals in primary AML cells are up-regulated by contact with human stroma and are differentially attenuated by TKIs

MSCs can support human haematopoietic stem cells (HSCs)[17] and maintain leukaemia-initiating cells (LICs) in AML.[18] Two populations of cells can be recognised in co-culture: (i) non-adherent AML cells exposed to stroma secreted cytokines and (ii) AML cells adherent to stroma requiring trypsinization for detachment. The latter population is of interest because of direct leukaemia–stroma contact, which may activate pro-survival signalling, promote drug resistance[19] and in LSC populations contribute to relapse.[6] We observed significant activation of phosphorylated STAT5 (pSTAT5) and pERK1/2 in both suspension and adherent AML cells (Fig 2A, $P < 0{\cdot}05$), confirmed by WB analysis (Fig 2B). Contact of AML blasts with HS5 produced the greatest increase in pERK, whilst pJAK2 signalling was not induced. Pacritinib treatment suppressed pERK and pSTAT5 in all fractions more potently than crenolanib and ponatinib, but higher drug concentrations were required in co-culture (Fig 2B,D,E, $P < 0{\cdot}05$; Figure S2A).

Wnt/-β-catenin signalling is frequently activated in *FLT3*-ITD AML and has been implicated in microenvironmental mediated leukaemogenesis.[20] Active β-catenin significantly increased on adherence of *FLT3*-ITD AML cells to stroma (seven of 10 samples), and was sustained at 24 h (Fig 2A, $P < 0{\cdot}05$). This was adherence dependant, greater with HS5 and not increased in suspension AML cells, indicating a requirement for direct stromal cell contact. On primary AML-derived MSCs, a similar significant increase was observed that was not present with cultures of NBM-derived MSCs ($P < 0{\cdot}01$). Pacritinib reduced active β-catenin levels in a dose-dependent manner ($P < 0{\cdot}01$, Fig 2C). Ruxolitinib did not show any significant attenuation of pERK, pSTAT, active β-catenin or pJAK2, confirming lack of efficacy in AML and indicating stromal alterations of pro-survival pathways by pacritinib to be independent of JAK2 (Figure S2D).

The MEK inhibitor PD0325901 can synergise with pacritinib to counteract stroma induced pERK feedback

Given the high induction and sustained expression of pERK in co-culture assays, we assessed the synergy of pacritinib with the MEK inhibitor, PD0325901, to counteract stromal up-regulation of this pathway. Dose–response curves for single and combination treated AML cells revealed synergy in ITD and WT primary AML samples across a range of drug effects at an optimum ratio of 5:1 (pacritinib: PD0325901) (Fig 3A,B), with a median (range) combination index (CI) value of 0·25 (0·007–1·9) ($n = 26$). Pacritinib: Ara-C had a limited synergistic effect with a modest CI value of 0·63 at a ratio of 1:100 (Fig 3A), which could not be improved with further dose ratios (data not shown). Pacritinib/PD0325901 treatment produced significantly greater pSTAT5 and pERK attenuation compared to single agents in co-culture conditions (Fig 3C,D), suggesting specificity of ERK targeting and a potential role of MEK inhibitors, to counter TKI-induced MEK feedback. B-cell lymphoma 2 (Bcl-2) was significantly reduced with the combination (Fig 3E) and greater apoptosis seen (Figure S4).

Dual therapy with pacritinib and the MEK inhibitor PD0325901 reduces leukaemic outgrowth of stroma-adherent cells and suppresses LIC in vitro and in vivo

Stromal outgrowth assays were set up to monitor 're-population' of the flask from residual adherent AML cells (Fig 4A–C). Pacritinib was more effective than ruxolitinib in reducing adherent cells and their outgrowth, an effect augmented by the MEK inhibitor PD0325901 (Fig 4D). WT FLT3 AML also showed some sensitivity, suggesting this combination can effectively target both WT and *FLT3*-mutant AML cells on stroma (Fig 4E,F).

Secondary CFU are considered a surrogate for *in vivo* drug efficacy and to assess drug effects on residual disease not modelled through xenograft studies. Residual drug-treated colonies are re-plated for secondary CFU. Pacritinib: PD0325901 combination therapy reduced CFU in AML compared to NBM CD34+s, indicating a potential therapeutic window for use. In contrast, ruxolitinib treatment showed significant toxicity to NBM (Fig 5A).

Pacritinib treatment of $CD34+CD38-ALDH^{int}$ AML *FLT3*-ITD cells, known to have leukaemic reconstitution potential and to predict relapse in patients,[21] markedly inhibited the ability of $CD34+CD38-ALDH^{int}$ AML cells to form cobblestone areas at 5 weeks on HS5 stroma and significantly reduced LTC-IC frequency (Fig 5B, $n = 6$; $P < 0{\cdot}01$ at 1 μmol/l pacritinib; Figure S6). Similar results were seen in MS5 co-cultures (data not shown). There were no cobblestones with combination treatment.

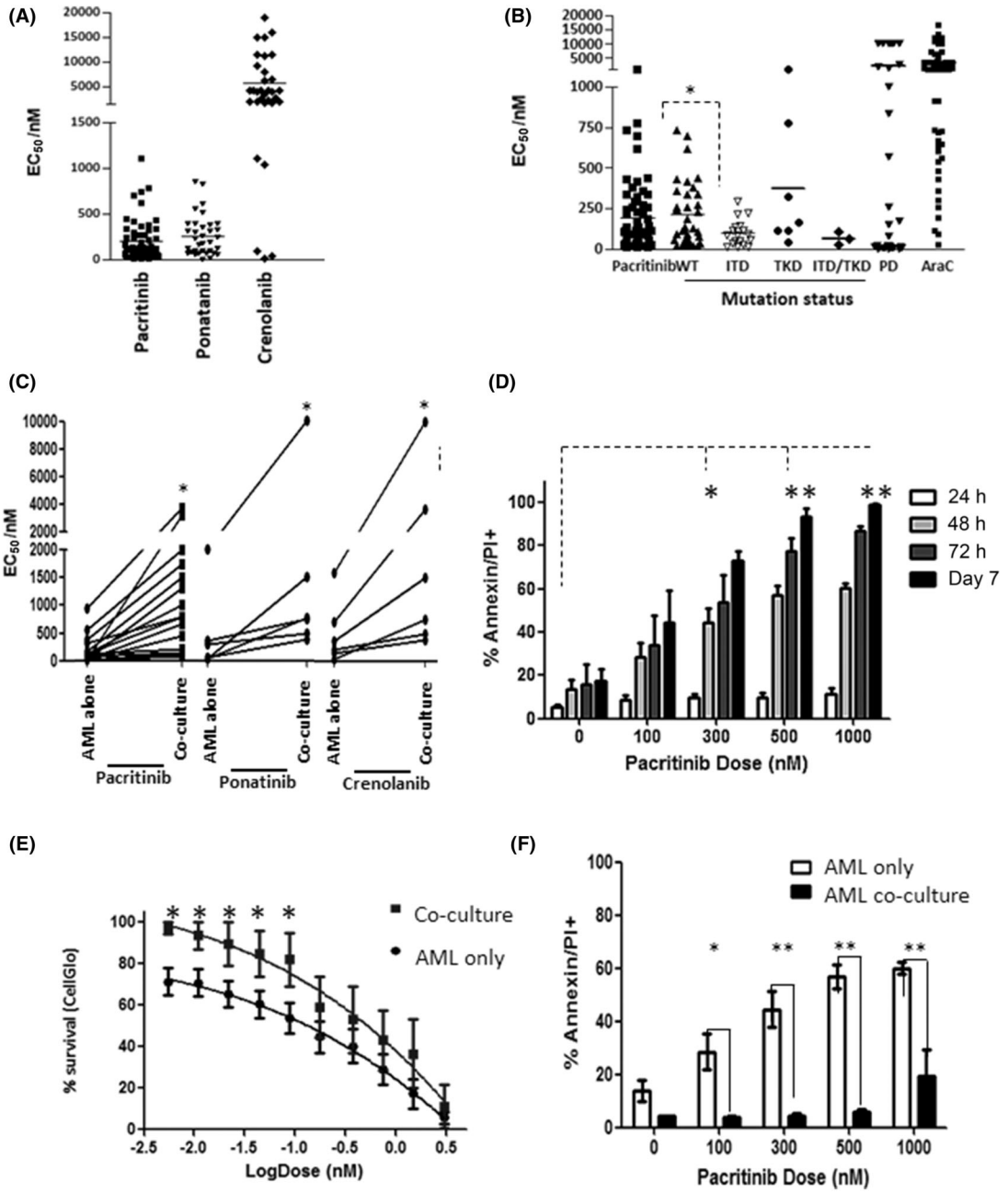

Figure 1. *In vitro* sensitivity of primary AML cells to potent FLT3 inhibition is suppressed in co-culture. (A) Primary AML sample efficacy assessment with pacritinib, ponatinib and crenolanib. EC_{50} values determined through cell titre glo readout of dose–response curves over 10 doses (n = 63 AMLs). Mean EC_{50} pacritinib 191·6 nmol/l ± 217, ponatanib mean EC_{50} 253.8 nmol/l ± 218, crenolanib mean EC_{50} 5588 nmol/l ± 5193 (*P < 0·001). (B) Efficacy of pacritinib, PD0325 and standard therapeutic agent Ara-C. Pacritinib efficacy sub-divided according to *FLT3*-mutation status. Mean EC_{50} ITD = 92·3 nmol/l *versus* WT 229·4 nmol/l, (*P < 0·01, Mann–Whitney U-test [MWU]). (C) Comparison of EC_{50} values at 48 h on co-culture (*P < 0·01, pacritinib n = 15 AMLs, ponatinib n = 6 AMLs, crenolanib n = 6 AMLs). (D) Primary samples stained for apoptotic induction with Annexin V and propidium iodide (PI) after pacritinib treatment (n = 7, *P < 0·04, **P < 0·01 MWU). Pacritinib treatment of *FLT3*-ITD cells induced a dose-dependent increase in apoptosis in Annexin V/PI assays (P < 0·05 at 300 nmol/l, P < 0·01 at ≥500 nmol/l at 72 h) with concomitant induction of cleaved caspase-3. (E) Dose-response analysis of pacritinib sensitivity in short-term 48 h stromal co-cultures with HS5 stromal layers (n = 15 ITD-mutant AML samples, P < 0·03 MWU). (F) Annexin V and PI apoptotic fractions at specified pacritinib doses +/− HS5 stromal culture (n = 8 primary AMLs, *P < 0·05, **P < 0·01 MWU).

Discussion

Modification of microenvironment-mediated drug resistance is an important therapeutic strategy to sensitise niche protected AML cells to current agents and to combat MRD. Relapse from *FLT3*-ITD disease is frequent and aggressive, and as stromal cells can protect *FLT3*-ITD progenitors from FLT3 inhibitors[22,23] strategies modifying stroma-based AML

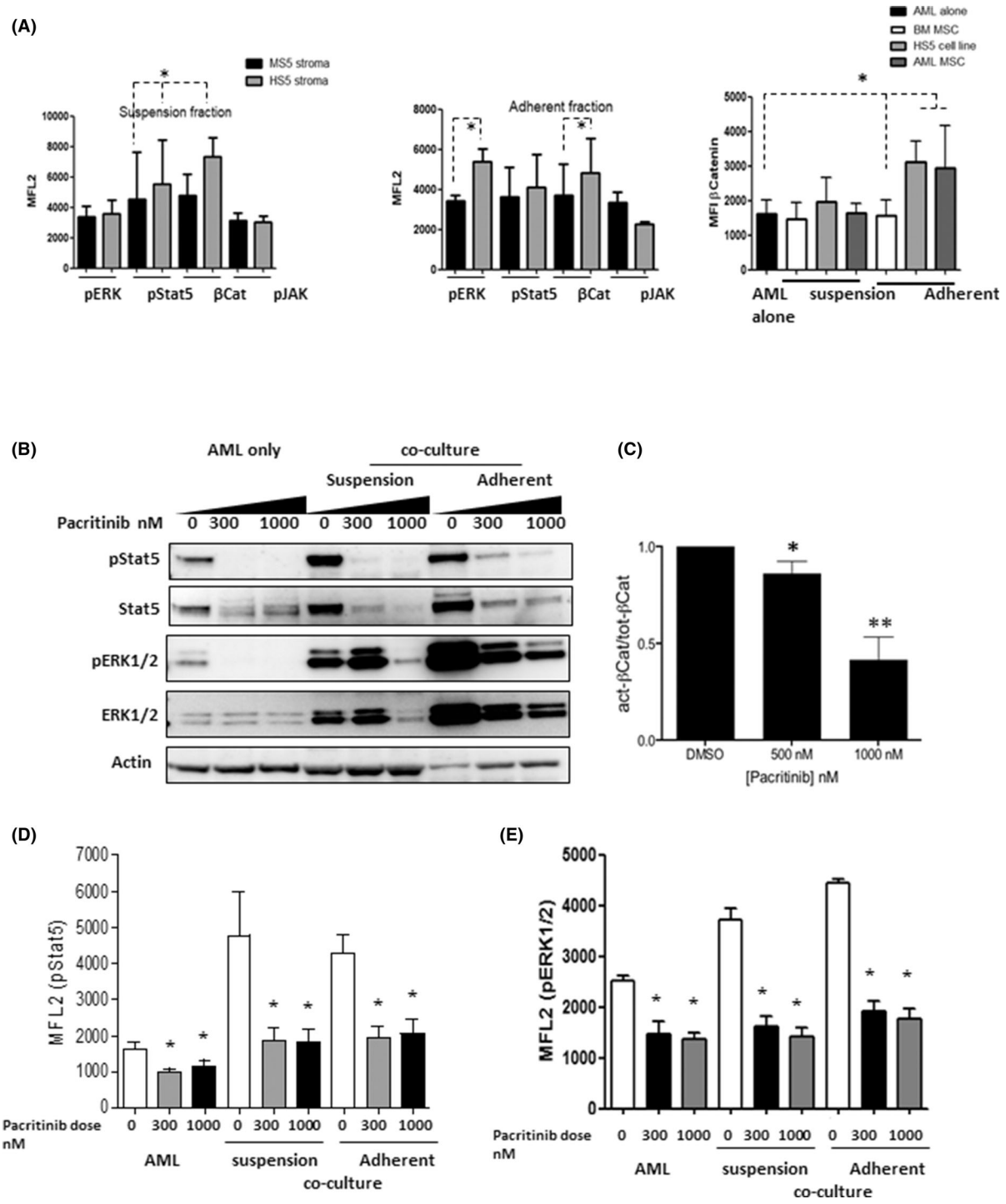

Figure 2. HS5 and AML-derived stroma drive maximal pro-AML effects. (A) Intracellular flow cytometry comparing MS5 and HS5 co-culture induction of AML survival signalling in suspension and adherent co-culture fractions. Comparison of active catenin induction in suspension and adherent fractions on HS5, AML-derived MSCs ($n = 4$) and NBM-derived MSCs ($n = 3$). (B) Representative Western blot of AML alone and stromal co-cultured AML blasts after 24 h with increasing doses of pacritinib ($n = 4$ AMLs blotted). (C) Reduction in active catenin intracellular flow levels after pacritinib drug treatment at 3 h (*$P < 0{\cdot}05$, **$P < 0{\cdot}01$, $n = 4$). Flow cytometry mean fluorescence of (D) pSTAT5 and (E) pERK1/2 in AML cell populations treated with pacritinib for 3 h compared to vehicle controls (*$P < 0{\cdot}05$, $n = 10$).

cytoprotection may enhance the efficacy of chemotherapy. This is particularly important as FLT3 inhibitors are currently used in intensive chemotherapy schedules and peri-transplantation.

In the present study, we investigated the ability of several TKIs, including the potent JAK2/FLT3 inhibitor pacritinib, to target stroma-adherent primary AML cells. This

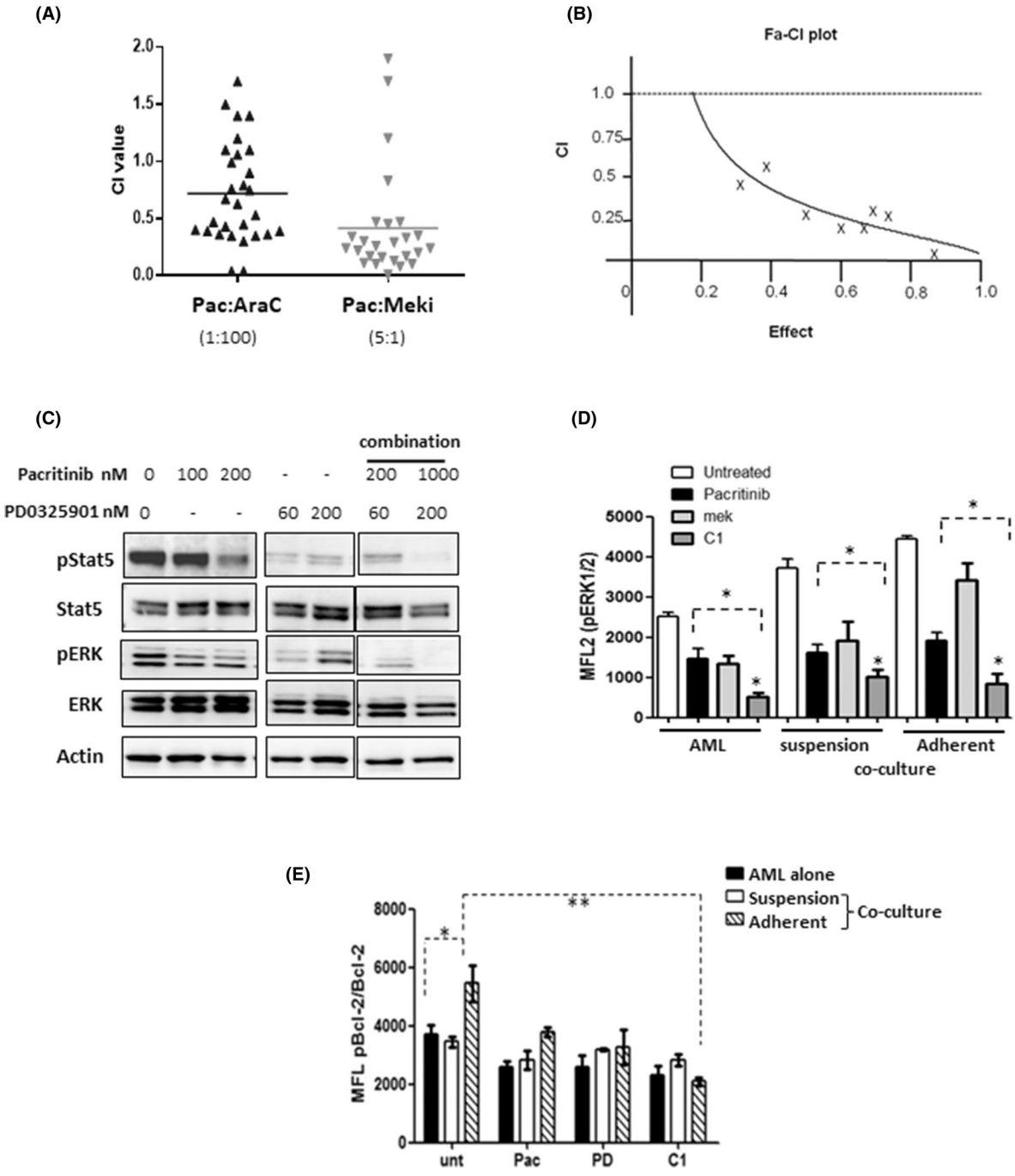

Figure 3. Combination treatment with pacritinib and the MEK inhibitor PD0325901. Primary *FLT3*-ITD AML samples were treated singly and in combination with pacritinib and Ara-C or the MEK inhibitor PD0325901 for 48 h. (A) Scatter graph of combination index (CI) values for primary AML cells treated with pacritinib and PD0325901 (fixed ratio 5:1, $n = 26$) compared to pacritinib and Ara-C (fixed ratio 1:100, $n = 29$), (median CI = 0·63 (+/−) for pacritinib:Ara-C, median CI = 0·25 (+/−) for pacritinib:PD0325901). (B) Example Isobologram analyses showing fraction affected at each CI of pacritinib: PD0325901 at a 5:1 fixed ratio. (C) Representative Western blot of synergistic knockdown of pSTAT5 and pERK1/2 in pacritinib and PD0325901 combination treated AML blasts (representative of three primary AMLs). (D) Flow cytometric analysis of pERK knockdown and (E) Bcl-2 reduction in single and co-culture assays at 48 h. Combination therapy produces significantly greater knockdown of target than single agent alone. ($n = 7$ primary AML, $P < 0·01$ MWU).

compound can suppress up-regulation of JAK2 in FLT3-inhibitor-resistant cells and reduce intrapulmonary leukaemia deposits in mice. It has demonstrated activity in a phase I trial in combination with cytarabine in *FLT3*-mutant AML[24]; however, its effects on stroma-adherent cells, which may drive relapse, has not been investigated. In the present study,

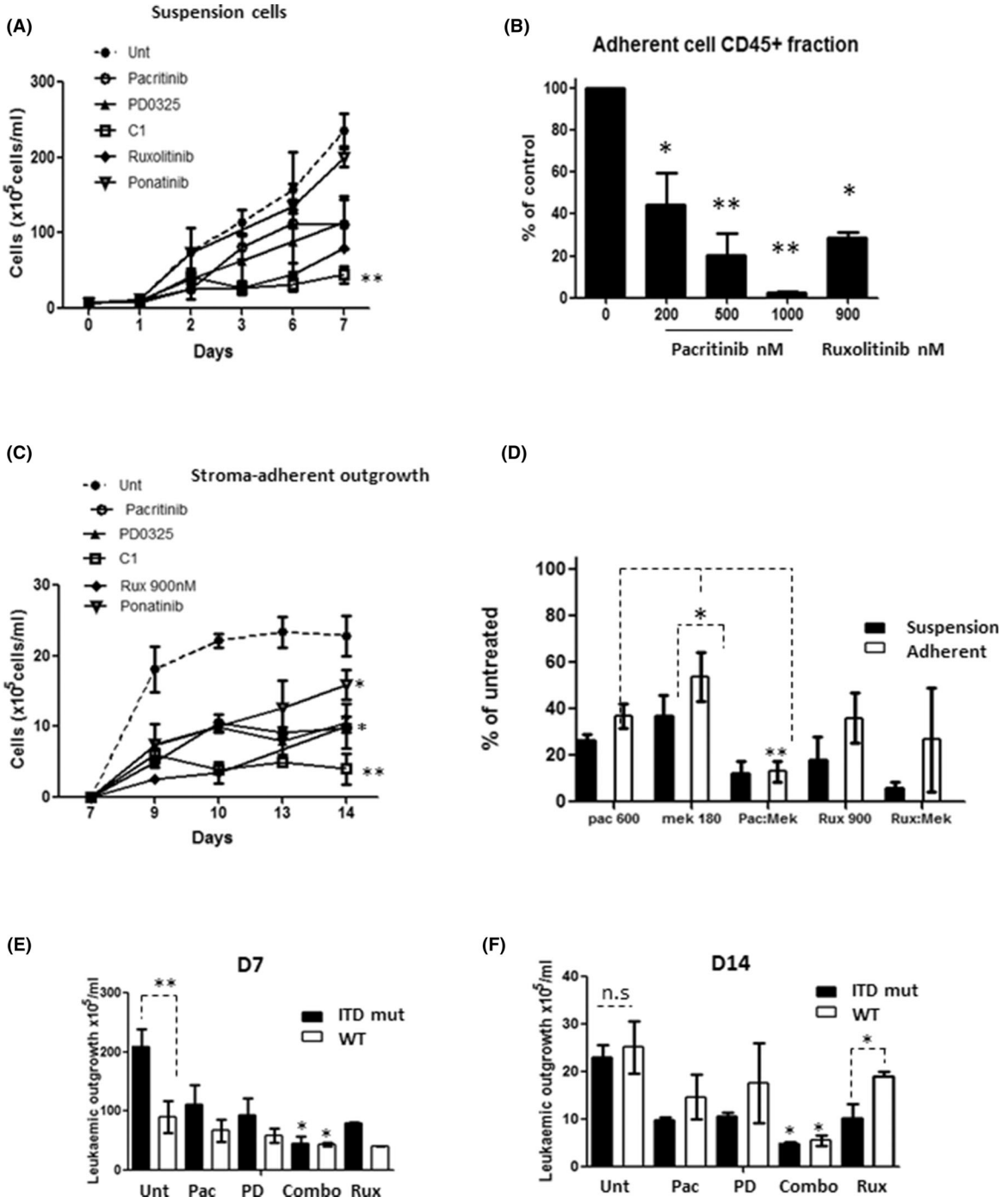

Figure 4. Dual FLT3/MEK targeting suppresses leukaemic out-growth of primary stroma-adherent AML cells. (A) Leukaemic blast growth in suspension fraction of HS5 co-culture over 7 days of single and combination agent treatment (n = 13, $^*P < 0{\cdot}01$). (B) Average co-culture adherent cells determined using vibrant adhesion assay ($^*P < 0{\cdot}01$, $^{**}P < 0{\cdot}001$). (C) Leukaemic blast outgrowth from adherent blast fraction on HS5 co-culture following washout of 7 day single and combination agent treatment (n = 13, $^*P < 0{\cdot}03$, $^{**}P < 0{\cdot}001$). (D) Relative cell counts in adherent and suspension fractions at day 14 of leukaemic outgrowth assays (n = 13, $^*P < 0{\cdot}01$, $^{**}P < 0{\cdot}001$ MWU). (E) Day 7 and (F) Day 14 adherent cell growth between ITD (n = 8) and WT (n = 5) after drug treatment ($^*P < 0{\cdot}01$ compared to single agents, $^{**}P < 0{\cdot}001$ MWU).

longer-term treatment with pacritinib reduced proliferation of non-adherent AML and adherent AML cells and suppressed re-growth of adherent leukaemia at clinically achievable drug doses (2 μmol/l, maximum clinical equivalent concentration). Pacritinib did not alter allelic ratios in residual stroma-adherent fractions and we surmise that adherent cells may have less dependency on *FLT3*-mutation status given the up-regulation of other pro-survival pathways

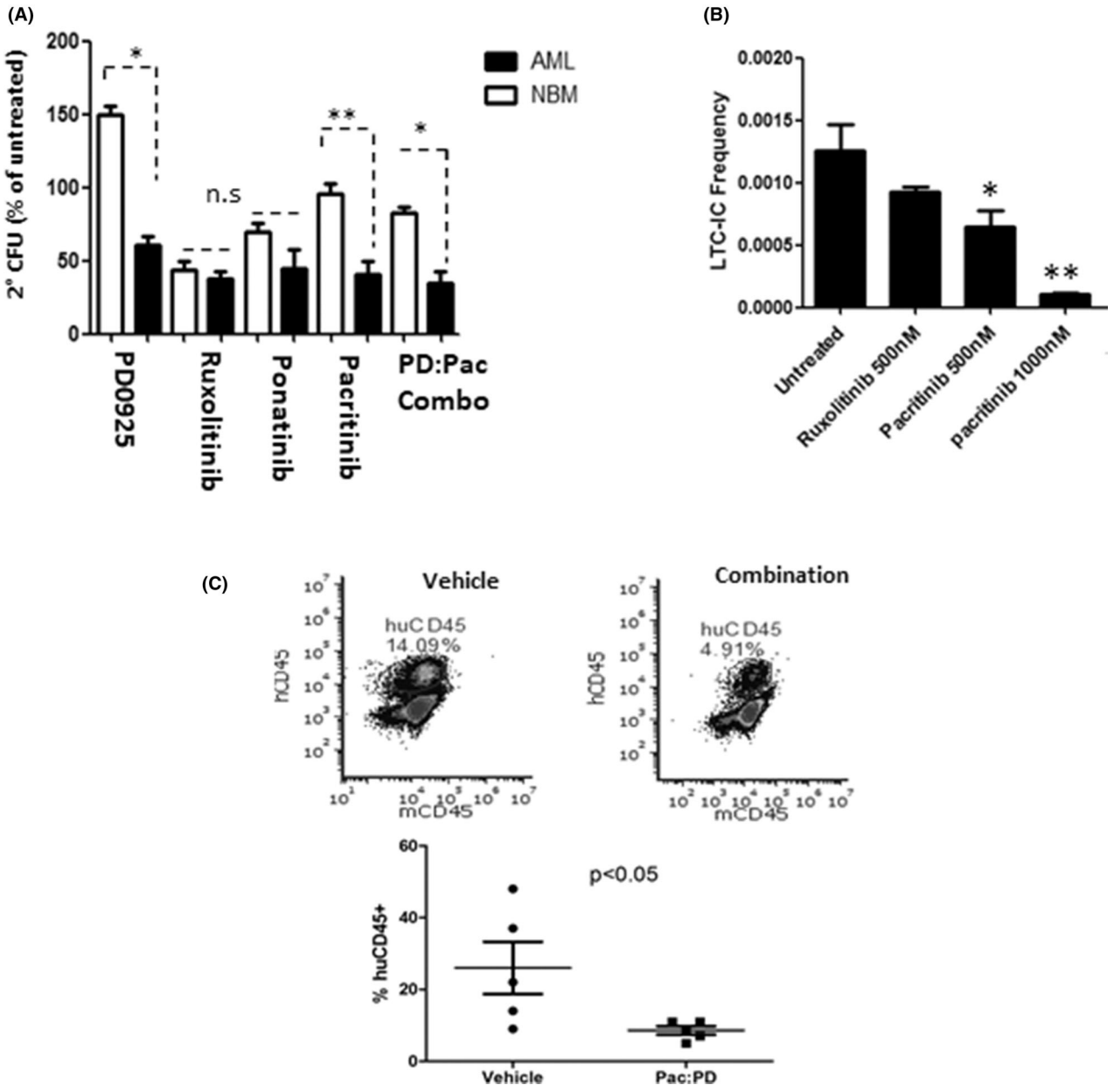

Figure 5. Dual FLT3/MEK targeting suppresses leukaemia-initiating cells *in vitro* and *in vivo*. (A) Histogram plot of CD34+CD38−ALDH^{int} fractions seeded by limiting dilution LTC-IC assay on HS5 layers. LTC-IC frequency determined by L-Calc (STEM CELL) at 5 weeks ($n = 7$, $^{*}P < 0{\cdot}01$,$^{**}P < 0{\cdot}001$ MWU). (B) Effects of pacritinib, ruxolitinib and combination treatment on 2° re-plated colony growth in AML blasts on HS5 stroma (black bars $n = 4$) and NBM (white bars $n = 3$) CD34+ cells ($^{*}P < 0{\cdot}01$, $^{**}P < 0{\cdot}001$). (C) *In vivo* analysis of combination efficacy on MV4;11 xenografts using 7 daily injections of fixed ratio pacritinib:PD0325901 (0·6:3·0 mg/kg respectively). Tumour load was assessed at 6 weeks after inoculation. Bone marrow harvests were analysed by FACS, with engraftment of human donor cells defined as hCD45+-positive ($^{*}P < 0{\cdot}05$).

observed. A subset of WT-adherent AML cells were also sensitive, arguing for a common pathway of inhibition on stroma-adherent populations, independent of mutational status. As expected, ruxolitinib showed little efficacy on primary AML cells cultured alone and no significant JAK2 activity in co-culture. However, ruxolitinib was effective in reducing suspension and adherent cells in leukaemic outgrowth assays, suggesting some activity on stroma-mediated protection of blasts. This is most likely due to non-specific drug effects at this dose, which is borne out by the significant toxicity to NBM colonies, limited efficacy on LTC-IC populations, and suggests targeting JAK2 alone may not resolve relapse from residual LTC-IC populations. In contrast, pacritinib showed a much wider therapeutic window due to reduced BM

toxicity even in combination with a MEK inhibitor that augmented its anti-AML efficacy. This combination showed significant suppression of leukaemic outgrowth on stroma, pro-survival ERK signalling and increase in apoptosis, suggesting benefit as maintenance therapy to prevent relapse. Kinase profiling of pacritinib has shown suppression of inhibitory cytokines, which may explain its reduced toxicity to normal haematopoietic cells.[25]

Both pSTAT5 and pERK were augmented in adherent AML cells consistent with reported ERK activation in stromal co-culture.[26] Pacritinib inhibited stroma-driven pSTAT5, but higher doses (1 μmol/l) were required to inhibit pERK in non-adherent cells. Residual pERK in adherent AML cells may explain the stroma-related resistance to apoptosis. Pacritinib and the MEK inhibitor, PD0325901, could be synergistically combined at lower doses to completely inhibit pERK. In contrast, there was no striking synergy between pacritinib and Ara-C, and TKI/MEK inhibitor combinations may be well placed following high-dose chemotherapy and in maintenance to suppress residual blasts with minimal toxicity to NBM.

LTC-ICs were reduced with pacritinib suggesting potential to inhibit disease recurrence. Griessinger *et al.*[18] have shown that engraftment potential can be maintained following *ex vivo* culture of primary AML cells on stroma, enabling the modelling of chemoresistance *ex vivo*. In addition, CD34+CD38−Lin− AML cells may show co-activation of pERK, pSTAT5 and β-catenin.[27] Secondary CFU assays support the efficacy of pacritinib on LIC populations in both mouse:human and in the human:human systems, the latter which we have found to have significantly higher pro-survival stimulation than cross-species models. This finding was replicated in our humanised NOD/SCID/IL2R$^{-/-}$ xenograft models confirming significant anti-leukaemic activity of the pacritinib/MEK combination treatment on human AML cells *in vivo*.

Wnt-β-catenin is essential for the survival of AML stem/progenitor cells[28,29] and is activated by *FLT3*-ITD through glycogen synthase kinase-3β inactivation and catenin stabilisation.[30] Active β-catenin was up-regulated in stroma-adherent cells, suggesting a role in AML-stromal interaction through direct binding, and of note integrin αvβ3 has been implicated in modulating sensitivity to sorafenib.[31] This observation was absent in adhesion to NBM-derived MSCs, suggesting a therapeutic window for the malignant *versus* healthy BM niche.

Taken together, these data suggest that pro-survival signalling is up-regulated in AML cells adherent to stroma and may account for residual disease detected following chemotherapy. The efficacy of pacritinib and other TKIs may be significantly enhanced by inhibition of stroma-driven ERK and catenin signalling through combination with a MEK inhibitor, providing a rationale strategy to reduce residual LICs in a human stromal context and prevent relapse in AML.

Author contributions

Gareth Edwards, Ceri A. Bygrave, Carol Guy, Michelle Lazenby, Lihui Zhuang and Nader Omidvar performed experiments and analysed data; Robert K. Hills performed statistical analyses, Steve Knapper provided patient samples, Alan K. Burnett reviewed the manuscript, Joanna Zabkiewicz and Caroline L. Alvares designed experiments, analysed data and wrote the manuscript.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig S1. Assessment of pacritinib efficacy in primary AML.

Fig S2. Intracellular flow detection of pro-survival proteins.

Fig S3. HS5 and AML derived stroma drive maximal pro-AML effects.

Fig S4. (A) Apoptotic induction in primary AML cells in response to single and combination treatment on HS5 stroma. C1 = 60:200pac, C2 = 200:1000pac, ($n = 7$, $^*P < 0.01$). (B) Cell cycle analysis using PI incorporation and flow analysis of MV4;11 cells +/− HS5 stromal layers. Drug treatments were 200 nmol/l pacritinib, 60 nmol/l PD0325, 1:5 combination ratio of PD0325:Pacritinib, $^*P < 0.01$ single to combination dose comparison. Cells were harvested labelled for CD45 and autoMACs separated prior to fixing.

Fig S5. Stroma adherent AML cells maintain leukemic markers during repopulating co-cultures.

Fig S6. LTC-IC assays- week 5 in untreated and pacritinib treated AML co-cultures.

Methods S1. Experimental methods.

References

1. Leung AY, Man CH, Kwong YL. FLT3 inhibition: a moving and evolving target in acute myeloid leukemia. *Leukemia*. 2013;**27**:260–8.
2. Schiller GJ, Tuttle P, Desai P. Allogeneic hematopoietic stem cell transplant in FLT3-ITD-positive AML: the role for FLT3 tyrosine kinase inhibitors post-transplant. *Biol Blood Marrow Transplant*. 2016;**22**:982–90.
3. Levis M, Ravandi F, Wang ES, Baer MR, Perl A, Coutre S, et al. Results from a randomized trial of salvage chemotherapy followed by lestaurtinib for patients with FLT3 mutant AML in first relapse. *Blood*. 2011;**117**:3294–301.
4. Fischer T, Stone RM, Deangelo DJ, Galinsky I, Estey E, Lanza C, et al. Phase IIB trial of oral midostaurin (PKC412), the FMS-like tyrosine kinase 3 receptor (FLT3) and multi-targeted kinase inhibitor, in patients with acute myeloid leukemia and high-risk myelodysplastic syndrome with either wild-type or mutated FLT3. *J Clin Oncol*. 2010;**28**:4339–45.
5. Rautenberg C, Nachtkamp K, Dienst A, Schmidt PV, Heyn C, Kondakci M, et al. Sorafenib and azacitidine as salvage therapy for relapse of FLT3-ITD mutated AML after allo-SCT. *Eur J Haematol*. 2017;**98**:348–54.
6. Zeng Z, Shi YX, Tsao T, Qiu Y, Kornblau SM, Baggerly KA, et al. Targeting of mTORC1/2 by the mTOR kinase inhibitor PP242 induces apoptosis in AML cells under conditions mimicking the bone marrow microenvironment. *Blood*. 2012;**120**:2679–89.
7. Konopleva MY, Jordan CT. Leukemia stem cells and microenvironment: biology and therapeutic targeting. *J Clin Oncol*. 2011;**29**:591–9.

8. Ghiaur G, Levis M. Mechanisms of Resistance to FLT3 Inhibitors and the Role of the Bone Marrow Microenvironment. *Hematol Oncol Clin North Am*. 2017;**31**:681–92.
9. Garz AK, Wolf S, Grath S, Gaidzik V, Habringer S, Vick B, *et al*. Azacitidine combined with the selective FLT3 kinase inhibitor crenolanib disrupts stromal protection and inhibits expansion of residual leukemia-initiating cells in *FLT3*-ITD AML with concurrent epigenetic mutations. *Oncotarget*. 2017;**8**:108738–59.
10. Kornblau SM, Womble M, Qiu YH, Jackson CE, Chen W, Konopleva M, *et al*. Simultaneous activation of multiple signal transduction pathways confers poor prognosis in acute myelogenous leukemia. *Blood*. 2006;**108**:2358–65.
11. Martelli AM, Nyåkern M, Tabellini G, Bortul R, Tazzari PL, Evangelisti C, et al. Phosphoinositide 3-kinase/Akt signaling pathway and its therapeutic implications for human acute myeloid leukemia. *Leukemia*. 2006;**20**:911–28.
12. Katsumi A, Kiyoi H, Abe A, Tanizaki R, Iwasaki T, Kobayashi M, *et al*. FLT3/ ITD regulates leukaemia cell adhesion through α4β1integrin and Pyk2 signalling. *Eur J Haematol*. 2011;**86**:191–8.
13. Weisberg E, Liu Q, Nelson E, Kung AL, Christie AL, Bronson R, *et al*. Using combination therapy to override stromal-mediated chemoresistance in mutant FLT3-positive AML: synergism between FLT3 inhibitors, dasatinib/multi-targeted inhibitors and JAK inhibitors. *Leukemia*. 2012;**26**:2233–44.
14. Hart S, Goh KC, Novotny-Diermayr V, Tan YC, Madan B, Amalini C, *et al*. Pacritinib (SB1518), a JAK2/FLT3 inhibitor for the treatment of acute myeloid leukemia. *Blood Cancer J*. 2011;**1**:e44.
15. Chen Y, Jacamo R, Shi YX, Wang RY, Battula VL, Konoplev S, *et al*. Human extramedullary bone marrow in mice: a novel in vivo model of genetically controlled hematopoietic microenvironment. *Blood*. 2012;**119**:4971–80.
16. Griessinger E, Anjos-Afonso F, Pizzitola I, Rouault-Pierre K, Vargaftig J, Taussig D, *et al*. A niche-like culture system allowing the maintenance of primary human acute myeloid leukemia-initiating cells: a new tool to decipher their chemoresistance and self-renewal mechanisms. *Stem Cells Transl Med*. 2014;**4**:520–9.
17. van Gosliga D, Schepers H, Rizo A, van der Kolk D, Vellenga E, Schuringa JJ. Establishing long-term cultures with self-renewing acute myeloid leukemia stem/progenitor cells. *Exp Hematol*. 2007;**35**:1538–49.
18. Griessinger E, Anjos-Afonso F, Vargaftig J, Taussig DC, Lassailly F, Prebet T, *et al*. Frequency and dynamics of leukemia-initiating cells during short-term ex vivo culture informs outcomes in acute myeloid leukemia patients. *Cancer Res*. 2016;**76**:2082–6.
19. Taxxbe Y, Jin L, Tsutsumi-Ishii Y, Xu Y, McQueen T, Priebe W, *et al*. Activation of integrin-linked kinase is a critical prosurvival pathway induced in leukemic cells by bone marrow-derived stromal cells. *Cancer Res*. 2007;**67**:684–94.
20. Jiang X, Mak PY, Mu H, Tao W, Mak D, Kornblau S, *et al*. Disruption of Wnt/β-catenin exerts anti-leukemia activity and synergizes with FLT3 inhibition in FLT3-mutant acute myeloid leukemia. *Clin Cancer Res*. 2018;**24**:2417–29.
21. Gerber JM, Smith BD, Ngwang B, Zhang H, Vala MS, Morsberger L. A clinically relevant population of leukemic CD34(+) CD38(-) cells in acute myeloid leukemia. *Blood*. 2012;**119**:3571–7.
22. Parmar A, Marz S, Rushton S, Holzwarth C, Lind K, Kayser S, *et al*. Stromal niche cells protect early leukemic FLT3-ITD+ progenitor cells against first-generation FLT3 tyrosine kinase inhibitors. *Cancer Res*. 2011;**71**:4696–706.
23. Alvares C, Schenk T, Hulkki S, Min T, Vijayaraghavan G, Yeung J, *et al*. Tyrosine kinase inhibitor insensitivity of non-cycling CD34+ human acute myeloid leukaemia cells with FMS-like tyrosine kinase 3 mutations. *Br J Haematol*. 2011;**154**:457–65.
24. Jeon JY, Zhao Q, Buelow DR, Phelps M, Walker AR, Mims AS, *et al*. Preclinical activity and a pilot phase I study of pacritinib, an oral JAK2/FLT3 inhibitor, and chemotherapy in FLT3-ITD-positive AML. *Invest New Drugs*. 2020;**38**:340–9.
25. Singer J, Al-Fayoumi S, Ma H, Komrokji R, Mesa R. Comprehensive kinase profile of pacritinib, a nonmyelosuppressive Janus kinase 2 inhibitor. *J Exp Pharmacol*. 2016;**8**:11–9.
26. Yang X, Sexauer A, Levis M. Bone marrow stroma-mediated resistance to FLT3 inhibitors in FLT3-ITD AML is mediated by persistent activation of extracellular regulated kinase. *Br J Haematol*. 2014;**164**:61–72.
27. Garg S, Shanmukhaiah C, Marathe S, Mishra P, Babu Rao V, Ghosh K, *et al*. Differential antigen expression and aberrant signaling via PI3/AKT, MAP/ERK, JAK/STAT, and Wnt/β catenin pathways in Lin−/CD38−/CD34+ cells in acute myeloid leukemia. *Eur J Haematol*. 2016;**96**:309–17.
28. Wang Y, Krivtsov A, Sinha A, North T, Goessling W, Feng Z, *et al*. The Wnt/beta-catenin pathway is required for the development of leukemia stem cells in AML. *Science*. 2010;**327**:1650–3.
29. Siapati E, Papadaki M, Kozaou Z, Rouka E, Michali E, Savvidou I, *et al*. Proliferation and bone marrow engraftment of AML blasts is dependent on β-catenin signalling. *Br J Haematol*. 2011;**152**:164–74.
30. Kajiguchi T, Chung E, Lee S, Stine A, Kiyoi H, Naoe T, *et al*. FLT3 regulates β-catenin tyrosine phosphorylation, nuclear localization, and transcriptional activity in acute myeloid leukemia cells. *Leukemia*. 2007;**21**:2476–84.
31. Yi H, Zeng D, Shen Z, Liao J, Wang X, Liu Y, *et al*. Integrin alphavbeta3 enhances β-catenin signaling in acute myeloid leukemia harboring Fms-like tyrosine kinase-3 internal tandem duplication mutations: implications for microenvironment influence on sorafenib sensitivity. *Oncotarget*. 2016;**7**:40387–97.