VEGF/KDR loop is a target of AG1296 in acute myeloid leukaemia showing FLT3-internal tandem duplications

The over activation of the receptor tyrosine kinase (RTK) signalling pathway has been shown to promote the growth, survival, and proliferation of malignant haematopoietic cells. In particular, mutations of *FLT3*, which result in constitutive kinase activity, are directly associated with the proliferation of acute myeloid leukaemia (AML) blast cells. Additionally, members of the vascular endothelial growth factor receptor (VEGFR) families contribute to tumoural progression through their ability to mediate angiogenesis and to enhance vascular permeability.

An internal tandem duplication (ITD) of the receptor FLT3 (FLT3/ITD) is the most frequent mutation in human adult AML, contributing to the constitutive activation of FLT3 itself and its downstream signal components and conferring a poor prognosis (Gayi *et al*, 2006; Hiyoi & Naoe, 2006). Pre-clinical studies have examined the effects of selective inhibitors of FLT3 on the FLT3 receptors and its signalling. Although such compounds are not yet used for the routine treatment for leukaemia, they hold promise as novel drugs against AML and as probes for understanding activation mechanisms and signalling pathways in the class III RTK family.

Recent evidence suggests that bone marrow angiogenesis plays an important role in the pathogenesis of AML (Padro *et al*, 2000). Several studies have shown that leukaemic cell lines and leukaemic blasts from patients with *de novo* AML produce vascular endothelial growth factor (VEGF) and express its receptor VEGFR-2 (KDR) (Padro *et al*, 2002; Barbarroja *et al*, 2007). Experimental models have shown that a paracrine feedback loop between AML blasts and endothelial cells involving VEGF is operational (Padro *et al*, 2002), so that internal and external autocrine VEGF/KDR loops regulate survival of AML blasts through distinct signalling pathways that sustain leukaemia migration and proliferation (Santos & Dias, 2004).

AG1296 is a compound of the tyrphostin class that is known to selectively inhibit the autophosphorylation of FLT3 ligand (FL)-stimulated wild type and constitutively activated FLT3/ITD, promoting a cytotoxic effect in primary AML cells (Tse *et al*, 2002). Previous works have demonstrated that the AG1296 inhibition of FLT3 selectively blocks the kinase activities of the FLT3, PDGF and KIT receptors but reported no effect on VEGF expression and KDR activity (Tse *et al*, 2002). Thus, the present study attempted to analyse this possibility in AML cell lines with or without FLT3/ITD mutation.

Our data showed for the first time that AG1296 suppressed angiogenesis in AML cell lines carrying *FLT3*/ITD, by inhibiting both the phosphorylation and nuclear translocation of KDR, and the secretion of VEGF.

Four AML cell lines were used in this study, THP-1 ($FLT3^{WT/WT}$), NB4 ($FLT3^{WT/WT}$), MOLM13 ($FLT3^{ITD/WT}$) and MV4-11 ($FLT3^{ITD/-}$) (DSMZ, Braunschweig, Germany).

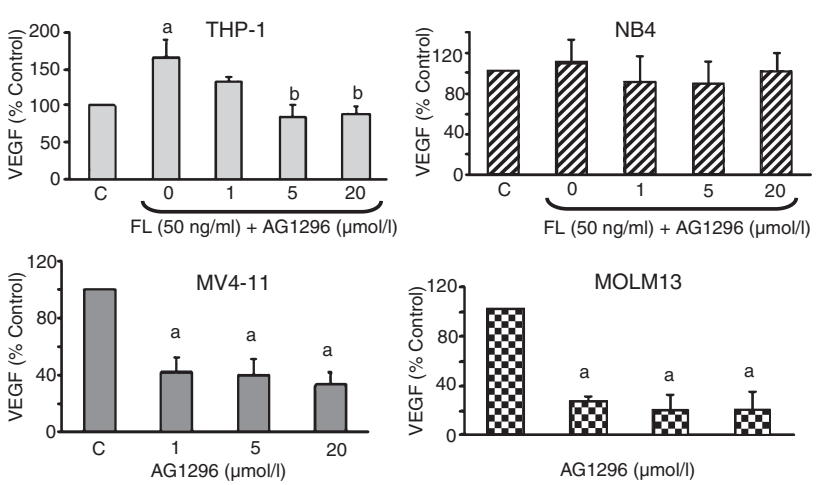

Fig 1. Inhibition of VEGF production by AG1296 in MV4-11 and MOLM13 cells. THP1 and NB4 cells were pre-treated with FL 50 ng/ml for 30 min. The four cell lines were incubated with AG1296 at different concentrations for 48 h. Levels of VEGF were analysed by ELISA. The bar graphs represent the percentage respect to control (the units are pg VEGF/mg of total protein). The means ± SD of three experiments are shown for each condition. $P < 0{\cdot}05$ (a) *versus* untreated cells, (b) *versus* cells treated with FL 50 ng/ml.

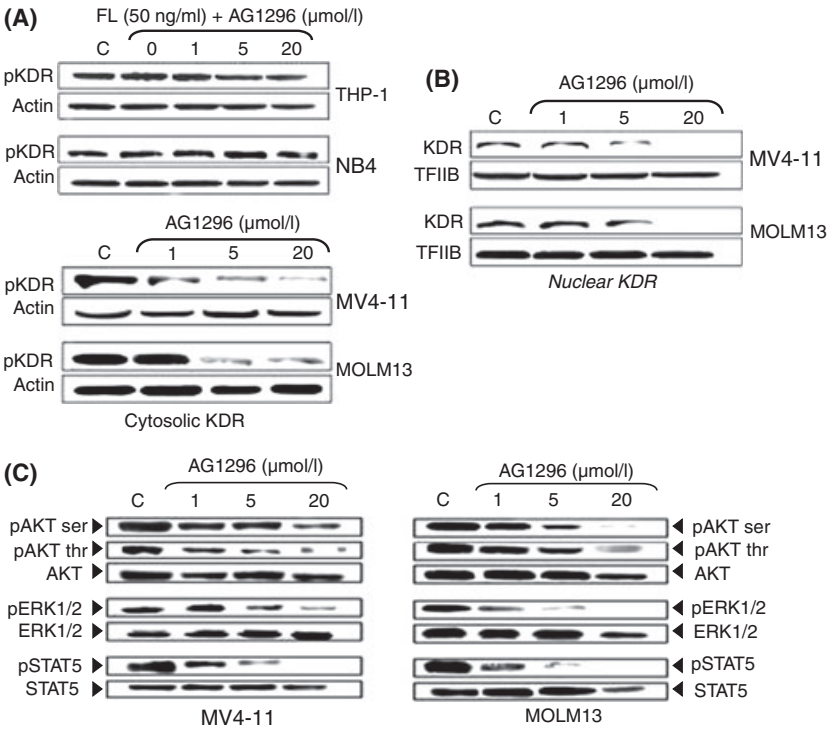

Fig 2. AG1296 inhibits the activation of KDR and several intracellular pathways in MV4-11 and MOLM13 cells. Cells were incubated with AG1296 at different concentrations for 48 h. NB4 and THP-1 were treated with FL (50 ng/ml) 30 min prior to the addition of AG1296. (A) KDR phosphorylation: 1 mg of proteins was immunoprecipitated with human 2 μg anti-KDR antibodies, electrophoresed on 7% SDS/PAGE gels, and then transferred to nitrocellulose membranes. The filters were probed with anti-phospho-tyr. The panels show one representative experiment of three independent experiments with similar results. (B) Nuclear KDR localization: Western blot analysis of nuclear protein extracts from FLT3/ITD cells (MV4-11 and MOLM13), showing the reduction of the presence of KDR in the nucleus with the AG1296 treatment. TFIIB protein represents the nuclear control. (C) Cell lysates were prepared and total proteins (50 μg/lane) were electrophoresed on a 10% and 6% SDS/PAGE gels and then transferred to nitrocellulose membranes. The membranes were probed with anti-phospho-ERK, anti-phospho-STAT5 and anti-phospho-Akt polyclonal antibodies. To demonstrate equal protein loading, the immunoblots were stripped and reprobed with anti-ERK, anti-STAT5 and anti-Akt antibodies. The panels show one representative experiment of three independent experiments with similar results.

The cell lines were treated with AG1296 (1–20 μmol/l) (Calbiochem, La Jolla, CA, USA) for 48 h. In FLT3/WT cells, FL (50 ng/ml) (Sigma-Aldrich, St Louis, MO, USA) were added 30 min prior to the addition of AG1296.

For Western blot analysis, cell cytosolic lysates (50 μg) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblots were probed with the following antibodies: human anti-pSTAT5 a/b, anti-phospho-ERK 1/2, anti-phospho-Akt (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The immunoblots were reprobed with human anti-STAT5, anti-ERK 1/2, anti-Akt and anti-actin from the same manufacturer. Immunocomplexes were complexed with the appropriate horseradish peroxidase-conjugated secondary antibody and detected by enhanced chemiluminescence (ECL) from GE Healthcare (Little Chalfont, UK). Similarly, the nuclear fractions were immunoblotted and probed with antibodies anti-KDR and anti-TFIIB (Santa Cruz Biotechnology) and equally detected.

To detect KDR phosphorylation, whole cells lysates (1 mg) were incubated with 2 μg of antibodies to human anti-VEGFR2 (Santa Cruz Biotechnology) for 4 h at 4°C, followed by incubation with 20 μl of protein A-Agarose overnight at 4°C with continuous mixing. The immunoprecipitates were separated by SDS–PAGE and immunoblotted by incubation with mouse monoclonal anti-phosphotyrosine antibody to human phospho-Tyr (Santa Cruz Biotechnology).

VEGF concentrations were determined using a commercially available enzyme-linked immunoassay (ELISA) (Quantikine; R&D Systems Europe, UK).

All experiments were assayed in triplicate and the data are expressed as mean ± standard error of the mean (SEM). Statistical analyses were carried out with the SIGMASTAT software package (Systat, Point Richmond, CA, USA). Before comparing two data groups, a normality test and an equal variance test were performed. If data groups passed both tests, a comparison was made by a parametric approach (paired Student's *t*-test). Differences were considered statistically significant at $P < 0{\cdot}05$.

To assess the effect of AG1296 on the VEGF production, we firstly measured VEGF levels in tissue culture supernatants from the four cell lines included in this study, which was normalized for the total protein amount. All cell lines produced constitutively significant levels of VEGF; treatment

with AG1296 reduced, in a dose-dependent manner, VEGF production in FLT3/ITD cells (MOLM13 and MV4-11) with respect to control (Fig 1). In THP1 cells, the addition of FLT3-ligand increased VEGF secretion, and this effect was slightly inhibited by AG1296 (Fig 1). Similar results were obtained by O'Farrell *et al* (2003), when they treated AML cells with SU11248. They found that FLT3 signalling lead to secretion of VEGF *in vitro*, most notably in FLT3/ITD cell lines.

In our hands, all AML cells lines showed relevant expression of the phosphorylated KDR form. However, AG1296 treatment inhibited the phosphorylation of KDR only in FLT3/ITD cells (MOLM13 and MV4-11) (Fig 2A). As KDR internalisation requires the phosphorylation of the receptor (Santos & Dias, 2004), AG1296 blocked the KDR nuclear translocation in FLT3/ITD cells (Fig 2B).

VEGF and FLT3 signalling in AML are likely to reflect activation of a number of downstream effector pathways, such as the RAF/MEK/ERK, PI3K/Akt and STAT (List *et al*, 2004; Santos & Dias, 2004; Choudhary *et al*, 2007). Our results showed a parallel VEGF and Akt, ERK and STAT5 inhibition after AG1296 treatment in FLT3/ITD cells (MOLM13 and MV4-11) (Fig 2C), suggesting that FLT3/ITD signalling seems to converge in some point with the VEGF production.

Taken together, our data show *in vitro* suppression of VEGF production, inhibition of KDR activity, and inhibition of key signal transduction RTK intermediates (such as Akt, ERK and STAT5), after treatment of *FLT3*/ITD cell lines with AG1296. VEGF is known to act by paracrine and autocrine mechanisms to stimulate leukaemogenesis, and FLT3 mediates key survival and proliferation signals in AML blasts. The present data provides evidence of the anti-angiogenic activity of AG1296, specifically on AML cells whose proliferation is dependent on mutant kinases, such as FLT3.

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Disclosures

All authors (LAT, NB, GD, FV and CLP) gave substantial contributions to the conception and design of the study, analysis and interpretation of data, drafting and revising the article critically for important intellectual content, and gave the final approval of the present version of the manuscript. The authors reported no conflict of interest.

Luis Arístides Torres[1]*
Nuria Barbarroja[1]*
Gabriel Dorado[2]
Francisco Velasco[3]
Chary López-Pedrera[1]

[1]*Unidad de Investigación, Hospital Universitario Reina Sofía, Córdoba,* [2]*Department Bioquímica y Biología Molecular, Universidad de Córdoba, Córdoba, and* [3]*Servicio de Hematología, Hospital Universitario Reina Sofía, Córdoba, Spain.*

E-mail: nuria.barbarroja.exts@juntadeandalucia.es

**L.A. Torres and N. Barbarroja contributed equally to this work.*

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