with a KDR-specific inhibitor (that acts internally), and analyzed KDR localization by western blotting and immunofluorescence. As shown in Figure 1D, KDR nuclear localization is also reduced by treatment with the KDR-specific inhibitor, although the effect is not as striking as with the VEGF neutralizing antibody. Taken together, these results suggest the external and interned VEGF/KDR autocrine loops regulate the localization of KDR, the former having a stronger effect.

Having demonstrated KDR internal and external autocrine loops operate on leukemia cells, we asked whether this was a general phenomenon, and the other VEGF receptor present on these cells, FLT-1, could be regulated in a similar fashion. In contrast to what wa seen for KDR, FLT-1 expression on HEL cells remains largely cytosolic, in all experimental conditions tested (FLT-1 staining of HEL cells treated with the Ab 4.6.1 is shown in Fig 1 E).

SIGNALING PATHWAYS ACTIVATED AUTOCRINE VEGF

We took advantage of the VEGF neutralizing antibody 4.6.1 (which acts only externally) and of the KDR inhibitor (internal), shown above to regulate KDR localization and activation, respectively, and used them to dissect the signaling pathways activated by autocrine VEGF on leukemia cells. Since VEGF stimulation of KDR-positive endothelial cells was shown to activate the PI 3-kinase and the MAP kinase pathways, we decided to investigate a putative role for these pathways in regulating the survival of KDR-positive leukemias, in the context of autocrine VEGF stimulation of KDR-positive leukemias.

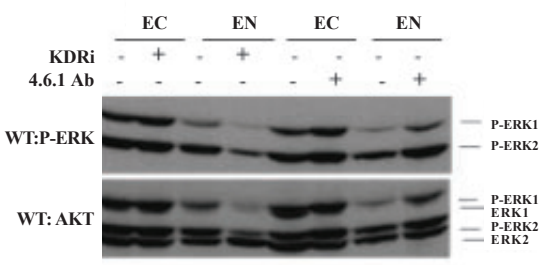
Cells treated with the internal inhibitor showed a clear decrease in phosphorylated Erk 1/2 (Fig 2A) and AKT (Fig 2B) levels, this effect being particularly evident in nuclear protein fractions. Total Erk 1/2 levels remained unchanged throughout. In contrast, treatment with the external blocker had little effect on either the MAP kinase or the AKT pathways. As shown in Fig 2A and B, the levels of phosphorylated Erk 1/2, in nuclear or cytosolic protein fractions, showed little variation after HEL cell treatment with the VEGF neutralizing antibody.

These results were confirmed on HL-60 cells and also 3 primary leukemias (Fig. 2C and Fig. 2D). As shown by FACS staining against phosphorylated proteins, the levels of P-Erk 1/2 and P-AKT decreased after treatment with the KDR internal inhibitor, while the Ab 4.6.1 showed little effect (results for the HL-60 cell line are shown in Fig 2G; Table 1 (Fig 2D) summarizes the results obtained for the other leukemia samples).

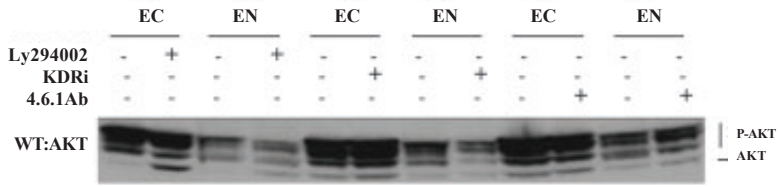
These results show primarily that external and internal VEGF/KDR loops act via distinct mechanisms. In addition, they also demonstrate that the blockade of internal VEGF signaling results in decreased constitutive activation of MAP and AKT kinase pathways.

Next, we investigated the effects of blocking the internal or external VEGF loop on the NF-kB pathway.

A)



B)



C)

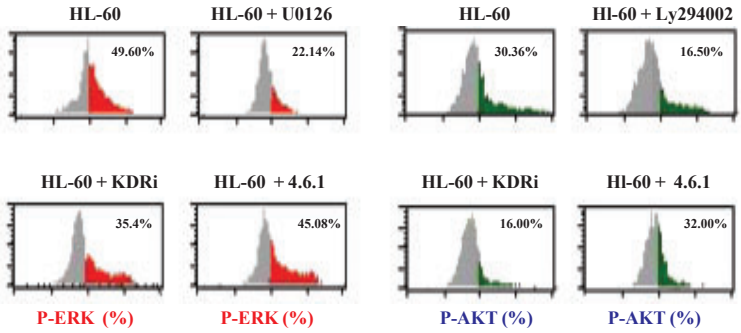


Fig. 2

D)

Cell line	Treatment	P-ERK (%)	P-AKT (%)
HEL	-	43.72	31.22
	KDR1	24.22	12.50
	4.6.1	42.00	30.94
	U0126	12.78	-
	Ly294002	-	10.18
AML#1	-	51.12	15.09
	KDR1	35.78	0
	4.6.1	52.40	16.07
	U0126	20.17	-
	Ly294002	-	0
	-	32.00	21.00
AML#3	KDR1	19.00	4.50
	4.6.1	34.00	22.08
	U0126	2.26	-
	Ly294002	-	7.00

Fig. 2: Table 1