IN VIVO CHARACTERIZATION AND MECHANISM OF ACTION OF ORALLY ACTIVE AURORA KINASE / VEGFR INHIBITORS

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ABSTRACT

Aurora kinases are Ser/Thr kinases that play an important role in chromosome alignment, segregation and cytokinesis during mitosis. Over-expression of Aurora kinases has been demonstrated in both solid tumors and leukemia and is considered to be associated with aneuploidy and carcinogenesis. Inhibition of Aurora kinase activity induces growth arrest and apoptosis in cells and leads to tumor-growth inhibition in xenograft models of human cancer. We identified a novel class of selective equipotent Aurora kinase and VEGFR kinase inhibitors with IC_{50}s less than 10 nM. The lead molecules inhibited histone H3 phosphorylation and induced growth inhibition in a panel of nine human cancer cell lines. These compounds also inhibited VEGF-dependent proliferation of human umbilical endothelial cells (HUVEC) with IC_{50}s less than 100 nM. The compounds induced growth arrest and polyploidy in the human colon cancer cell line HCT116. The compounds induced the accumulation of cells with >4N DNA content followed by apoptosis, as demonstrated by cell cycle analysis and activation of caspase-3/7 in the human promyelocytic leukemia cell line HL60. The compounds induced primarily G2/M arrest in A549 lung cancer cells which have a functional p53-dependent post-mitotic checkpoint, and they induced polyploidy in HCT116 cells with a defective checkpoint. More than 80% tumor-growth inhibition was observed following a once-daily oral dosing schedule in an HL60 xenograft model. A single oral dose in mice resulted in 6-13 μM concentration in blood. Analysis of tumor samples for phosphorylated histone H3 and endothelial cell marker CD31 revealed that histone H3 phosphorylation was inhibited and blood vessel formation was reduced in tumors from compound-treated animals, demonstrating inhibition of Aurora kinase and VEGFR activity *in vivo*, respectively. These data suggest that this class of Aurora kinase/VEGFR kinase inhibitors has therapeutic potential in both hematologic and solid malignancies.

INTRODUCTION

Aurora A and B are important mitotic kinases

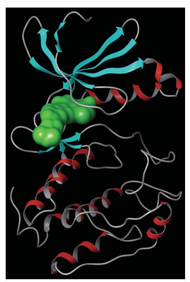
- Key regulators of entry into mitosis & chromosomal segregation
- Over-expressed & amplified in a variety of tumors
- Inhibitors will arrest cell division and promote apoptosis
- Potential targets for cancer treatment (multiple on-going phase 2 clinical trials)

Neovascularization is important for tumor growth

- VEGF and VEGF receptors are key factors for angiogenesis
- Agents targeting VEGF or VEGF receptors are FDA-approved cancer therapies

We have reported the identification of a novel class of small molecules that are nanomolar inhibitors of Aurora and VEGFR kinases (*Abstract #C206, 2007 AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics, San Francisco, CA)*. In this study, the compounds are further characterized for their effects on cancer cell growth in cell-based assays. The oral anti-tumor efficacies of the compounds are demonstrated in human tumor xenograft models.

FIGURE 1: 2.5 Å Crystal Structure of the Complex of Aurora A and EAB7



The kinase domain of Aurora A (residues 122-403) was crystallized in the presence of EAB7 and structure of the complex was solved to 2.5 Å resolution by X-ray diffraction (a service provided by Structure Based Design, Inc., San Diego, CA). The co-crystal structure indicated that the molecule occupied the ATP binding pocket in Aurora A.

TABLE 1: Inhibition of Aurora / VEGFR2 Kinases

Telik Compound	Enzymatic Assay (nM) IC_{50}			Cell-based Assay (μM)		
	Aur A	Aur B	VEGFR	H3 Phosphorylation IC_{50}	Cell Cycle Polyploidy (8N)	HUVEC Proliferation IC_{50}
EAB7	6.3	13	13	0.40	1.0	0.028
EBF0	6.4	4.4	3.6	0.15	0.5	0.15
EBF4	10	11	23	0.16	0.5	0.080
ED60	18	2.2	28	0.093	0.2	0.012
EDC7	2.2	4.2	13	0.27	0.3	0.091
EDC8	3.9	6.5	27	0.18	0.5	0.080

Enzymatic Assays

- The IC_{50}s against Aurora and VEGFR2 kinases were determined in fluorescence polarization-based kinase activity assays using a peptide substrate.
- Additional kinases inhibited include FLT-3, Ret, PDGFRβ and CHK1.

Cell-based Assays

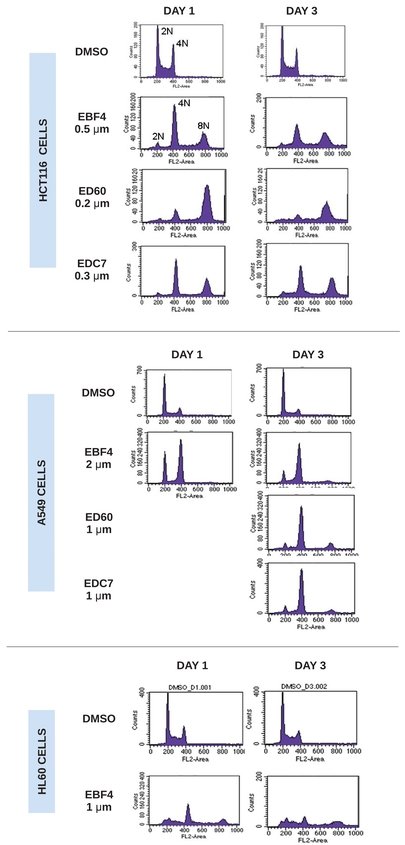
- Histone H3 phosphorylation was determined in HCT116 cells using an immuno-dot blot assay. The HCT116 cells were treated with compounds for 4 h in duplicate.
- Under cell cycle analysis, the numbers reported were the lowest concentrations required to induce polyploidy in HCT116 cells following one day incubation with compounds.
- HUVEC proliferation was measured by BrdU incorporation (Roche).

TABLE 2: Inhibition of Cancer Cell Growth

	Telik Compound (μM)	EBF0	EBF4	ED60	EDC7	EDC8
Solid Tumor						
A549	lung	1.30	0.43	0.56	0.39	0.44
BxPC-3	pancreas		0.45	4.41	0.52	0.72
HCT116	colon	0.70	0.30	0.05	0.25	0.26
HT29	colon		0.41	1.55	0.54	0.68
MiaPaca-2	pancreas	1.54	0.26	0.79	0.27	0.55
NCI460	lung		0.28	0.32	0.26	0.32
PC-3	prostate		0.43	3.85	0.65	0.91
Hematologic						
HL60	promyelocytic	0.11	0.50	0.04	0.23	0.29
IM9	B lymphoblastic	0.04	0.09	0.05	0.12	0.08
Jurkat	T lymphoblastic		0.18	0.50	0.17	0.21
K562	erythroleukemia		0.54	1.20	0.38	0.34
Reh	lymphoblastic	0.12	0.19	0.05	0.17	0.20
RPMI1788	B lymphoblastic	0.39	0.39	1.39	0.37	0.33
U937	promonocytic		0.15	0.07	0.15	0.18

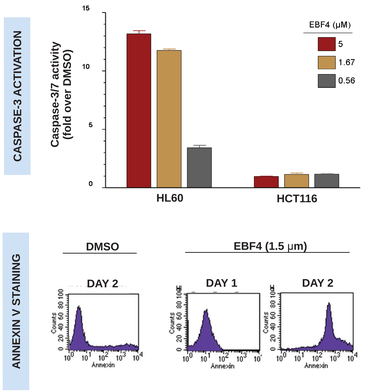
Proliferating cells were incubated with compound in appropriate growth media for 3 or 4 days (~3 doublings). Cell viability at the end of incubation was measured using the CellTiterGlo reagent (Promega). The compound treatment was carried out in triplicate.

FIGURE 2: Induction of Cell Cycle Arrest in Tumor Cells



Cells were incubated with compounds at indicated concentrations in appropriate growth media. Cells were fixed and stained with propidium iodide after 1, 2 or 3 day compound treatment. Cell cycle profiles were analyzed by FACS.

FIGURE 3: Induction of Apoptosis in HL60 Cells



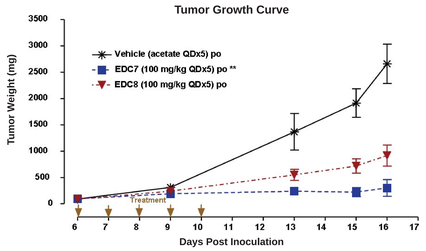
Caspase-3/7 Activation

HL60 or HCT116 cells were incubated with EBF4 at indicated concentrations in triplicate for 6 h. Caspase-3/7 activity was measured using Caspase-Glo 3/7 Assay Kit from Promega.

Annexin V Staining

HL60 cells were incubated in growth media with 0.1% DMSO or 1.5 μM EBF4 for 1 or 2 days. Annexin V staining was performed using a kit from BD Biosciences.

FIGURE 4: Anti-Tumor Efficacy in HL60 Leukemia Xenograft Model



Nude mice were implanted with HL60 cells at $1x10^7$ cells/mouse on Day 0. Compound treatment began when the average tumor weight reached ~100 mg. Animals (n = 10 per group) were administered orally once a day with vehicle (0.1 M sodium acetate, pH 5.0) or 100 mg/kg compounds dissolved in vehicle on Days 6 – 10. One death occurred in each of the compound treatment groups and was attributed to tumor ulceration or bleeding.

**P < 0.01, compared to vehicle group using Repeated Measures ANOVA followed by Tukey's Multiple Comparison Test.

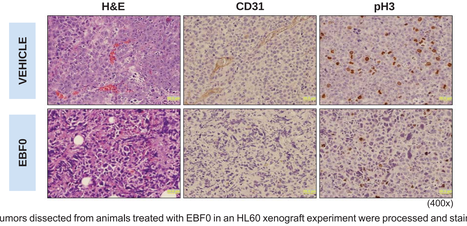
TABLE 3: Blood Concentration and Tumor Growth Inhibition Following Oral Administration of Compounds in Mice

Telik Compound	Swiss Mice (*fasted, single dose*) Dose (mg/kg)	C_{max} (μm)	Nude Mice Xenograft (*QD x 5*) Dose (mg/kg)	Tumor Growth Inhibition HL60	Tumor Growth Inhibition HCT116
EBF4	100 (rats)	1.7	100	38%**	
			150	64%***	46%*
ED60	40	6.2	35	45%**	
			40	65%***	46%*
			50	62%**	
EDC7	150	12.5	50	45%**	
			100	89%***	
				95%**	
EDC8	150	11.5	50	35%**	
			100	65%**	
				64%**	

Compounds were administered orally as solution in 0.1 M sodium acetate, pH 5.0. The *C*max was estimated from PK screens using Swiss mice (n = 5) or CD rats (n = 6). Tumor growth inhibition was calculated based on actual weight of tumors removed on Day 9 or 10 post treatment initiation in the HL60 xenograft experiments or Day 18 post treatment initiation in the HCT116 xenograft experiments.

*P < 0.05, **P < 0.01 and ***P < 0.001, compared to vehicle group using One-way ANOVA followed by Tukey's Multiple Comparison Test.

FIGURE 5: Immuno-histochemistry Staining on HL60 Tumor Samples



Tumors dissected from animals treated with EBF0 in an HL60 xenograft experiment were processed and stained for endothelial cell marker CD31 and phosphorylated histone H3. Compared to tumors from vehicle-treated animals, the data showed a reduction in the number of CD31+ and pH3+ cells, indicating a reduction of blood vessel formation and inhibition of Aurora kinase, respectively.

SUMMARY

- The nanomolar inhibitors of Aurora and VEGFR kinases were also potent inhibitors of Aurora kinase and VEGFR kinase activities in cells. The compounds inhibited the growth of a broad spectrum of human cancer cells.
- The compounds induced polyploidy in HCT116 colon cancer cells and arrested A549 lung cancer cells at 4N DNA content. The compounds also induced cell cycle arrest, followed by apoptosis, in HL60 leukemia cells.
- High resolution X-ray crystal structure revealed that this novel class of Aurora/VEGFR kinase inhibitors binds to the ATP binding pocket in Aurora A with specific molecular interactions.
- The Aurora/VEGFR kinase inhibitors significantly inhibited tumor growth in two human tumor xenograft models. Immuno-histochemistry staining of tumor samples was consistent with the inhibition of Aurora and VEGFR kinase *in vivo*.
- This class of Aurora/VEGFR kinase inhibitors may have therapeutic potential in both hematologic and solid malignancies.