LB-298

AP26113, a potent ALK inhibitor, overcomes mutations in EML4-ALK that confer resistance to PF-02341066

Sen Zhang, Frank Wang, Jeffrey Keats, Yaoyu Ning, Scott D. Wardwell, Lauren Moran, Qurish K. Mohemmad, Emily Ye, Rana Anjum, Yihan Wang, Xiaotian Zhu, Juan J. Miret, David Dalgarno, Narayana I. Narasimhan, Tim Clackson, William C. Shakespeare, Victor M. Rivera

ARIAD Pharmaceuticals, Inc., Cambridge, MA, USA

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Background

Anaplastic Lymphoma Kinase (ALK)

- A receptor tyrosine kinase in the insulin receptor superfamily
- Activating fusions detected in up to 70% of ALCL (NPM-ALK) and 3-7% of NSCLC (EML4-ALK) patients
- Oncogenic mutations in 10-15% of neuroblastoma patients

PF-02341066

- A dual cMet/ALK inhibitor
- Promising clinical activity against NSCLC carrying ALK gene fusions in a Phase I trial
- Phase III trial in NSCLC initiated

AP26113

- A potent and highly selective ALK inhibitor
- Inhibits cell growth and signaling both *in vitro* and *in vivo* in ALCL and NSCLC models
- Orally bioavailable and well-tolerated at efficacious drug levels

Tyrosine Kinase Inhibitor (TKI) - resistance

- Development of resistance to targeted agents is common, e.g. imatinib in CML and erlotinib in NSCLC
- Resistance to TKIs commonly due to mutations in kinase domain that impair inhibitor binding
- Mutations in patients that confer resistance to TKIs (e.g. targeting BCR-ABL and KIT) have been successfully predicted by an accelerated *in vitro* mutagenesis screen in Ba/F3 cells

Here, the Ba/F3 system was used to identify mutations in ALK that confer resistance to PF-02341066 or AP26113

Methods

Reagents: AP26113 was synthesized by ARIAD Pharmaceuticals. PF-02341066 was either synthesized by ARIAD Pharmaceuticals or purchased from Wuxi Howfond Biopharma, Co., Ltd.

***In vitro* mutagenesis screen:** Ba/F3 cells expressing native EML4-ALK were treated overnight with 100 μg/ml N-Ethyl-N-nitrosourea (ENU, Sigma-Aldrich), pelleted, resuspended in fresh media, and distributed into 96-well plates in 200 μl media supplemented with graded concentrations of PF-02341066 or AP26113. The wells were observed for media color change and cell growth. The contents of outgrown wells were expanded in 24-well plates in media supplemented with PF-02341066 or AP26113 at the same concentration as in the initial 96-well plate. At confluence, cells were collected by centrifuge for DNA extraction and for further characterization. Genomic DNA was extracted from cell pellets using DNEasy 96 Blood & Tissue kit (Qiagen). The ALK kinase region was amplified using primer: 5'-AGCGATGCAGATGGAATTGCAGAG-3' and 5'-CAATAGGCAGCGCCGTGTTGATTA-3'. PCR products were purified by QIAquick PCR purification kit (Qiagen) and sequenced by MGH DNA sequencing Core.

Model of PF-02341066-bound ALK: A homology model of ALK was built based on the crystal structure of activated insulin kinase (PDB code: 3irk) with Prime (Schrodinger software package). PF-02341066 was docked into ALK by using Glide SP with post docking minimization and the top scoring pose was chosen for further analysis.

Re-introduction of mutations into Ba/F3 cells: Plasmids encoding EML4-ALK mutations were generated with QuickChange site-directed mutagenesis (Stratagene) according to manufacturer's instructions using pMSCVneo encoding native EML4-ALK as a template. Each mutation was confirmed by DNA sequencing. Ba/F3 cells expressing EML4-ALK mutants were generated by infecting Ba/F3 parental cells (supplemented with IL-3 in media) with retrovirus encoding EML4-ALK mutations followed by selection with G418 in the absence of IL-3.

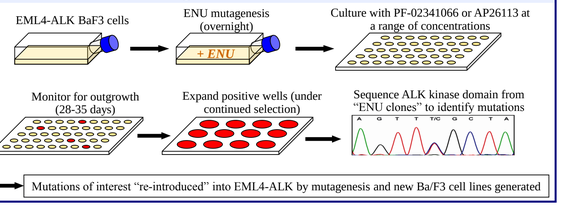
***In vitro* viability assay:** Ba/F3 parental and Ba/F3 cells expressing EML4-ALK mutants were plated in 96-well plates and treated with compounds for 72 h. Cell viability (IC50) was assessed using CellTiter 96 AQueous One (Promega) and plotted as percent viable relative to vehicle treated cells using XLfit.

Inhibition of ALK phosphorylation: EML4-ALK expressing Ba/F3 cells were treated with increasing concentrations of PF-02341066 or AP26113 for 2 hours. Cell lysates were prepared and analyzed by PathScan Sandwich ELISA kit (Cell Signaling) for p-ALK and for total ALK. IC50 was determined as the concentration of compound required to decrease ALK phosphorylation by 50% compared to vehicle control.

***In vivo* efficacy:** Ba/F3 cells expressing native or mutant EML4-ALK were implanted into the right flank of SCID Beige mice (10 x 10^6 cells/mice). When the average tumor volume reached ~200 mm^3, either vehicle or compound was administered by once daily oral gavage for 8-12 days. Mean tumor volume (± SE) was calculated for each group.

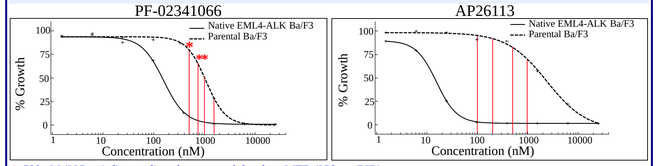
Pharmacodynamics/Pharmacokinetics: For pharmacodynamic analysis, 6 or 24 hours after treating tumor-bearing mice with a single dose of vehicle or compound, tumors were collected, homogenized and analyzed by ELISA. Inhibitor concentrations in plasma were determined by an LC/MS/MS method. Calibration standards were prepared in blank mouse plasma. Internal standard was added to all plasma samples and the samples were deproteinized before analysis.

Accelerated *in vitro* mutagenesis scheme



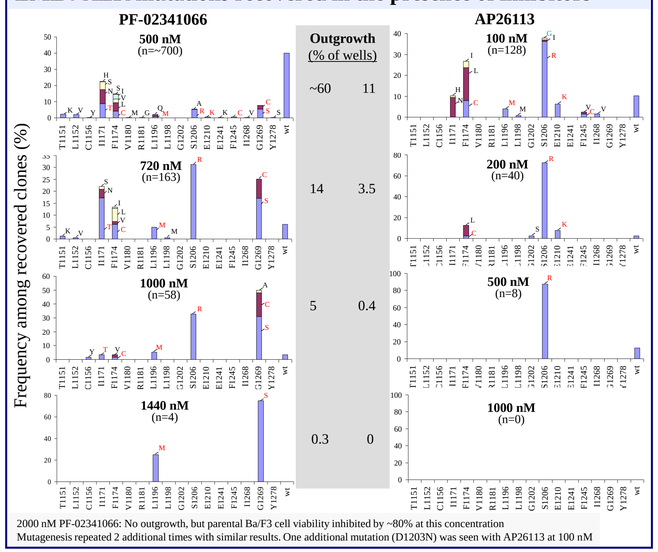
Results

Concentrations used for selection; relative to therapeutic index



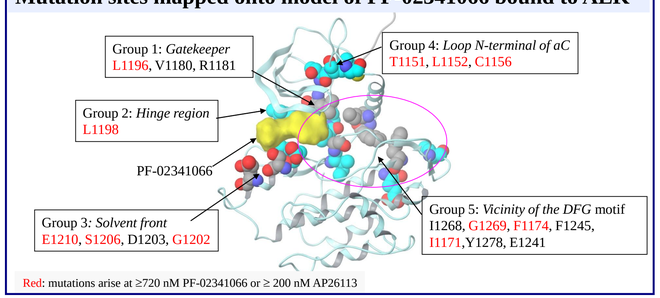
* 500 nM (225 ng/ml): ~median plasma trough levels at MTD (250 mg BID)
**720 nM (325 ng/ml): ~median plasma trough levels beyond MTD (300 mg/BID)
Kwak ASCO 2009: #3509

EML4-ALK mutations recovered in the presence of inhibitors



2000 nM PF-02341066: No outgrowth, but parental Ba/F3 cell viability inhibited by ~80% at this concentration
Mutagenesis repeated 2 additional times with similar results. One additional mutation (D1203N) was seen with AP26113 at 100 nM

Mutation sites mapped onto model of PF-02341066 bound to ALK



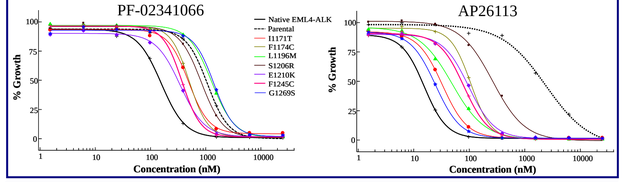
Red: mutations arise at ≥720 nM PF-02341066 or ≥ 200 nM AP26113

IC50 values for AP26113 and PF-02341066 against selected EML4-ALK mutants in Ba/F3 viability assays

	PF-02341066			AP26113		
Ba/F3 line	**IC50s (nM)**		**TI****	**IC50s (nM)**		**TI****
Parental*	1210 ± 334		1	2189 ± 410		1
Native EML4-ALK	139 ± 30		9	11 ± 4		205
EML4-ALK Mutants:	***ENU-clone***	***Re-introduced***		***ENU-clone***	***Re-introduced***	
I1171T	395 ± 199	413 ± 62	3	22 ± 11	18 ± 4	102
F1174C	478 ± 31	319 ± 90	3	68 ± 31	51 ± 14	32
L1196M	1131 ± 296	1215 ± 708	1	45 ± 23	40 ± 19	49
S1206R	711 ± 250	728 ± 362	2	194 ± 84	158 ± 86	11
E1210K	315 ± 124	297 ± 92	4	64 ± 33	86 ± 21	34
F1245C	413 ± 90	269 ± 194	3	44 ± 29	34 ± 28	50
G1269S	1141 ± 527	1196 ± 649	1	16 ± 6	16 ± 5	141

*Parental Ba/F3 cells lack EML4-ALK and therefore require IL-3 for growth (n=5)
**Therapeutic Index = Parental IC50 / EML4-ALK IC50

Representative proliferation curves

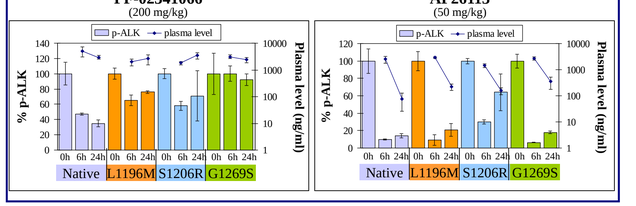


AP26113 inhibits ALK phosphorylation in Ba/F3 cell lines expressing selected EML4-ALK mutants

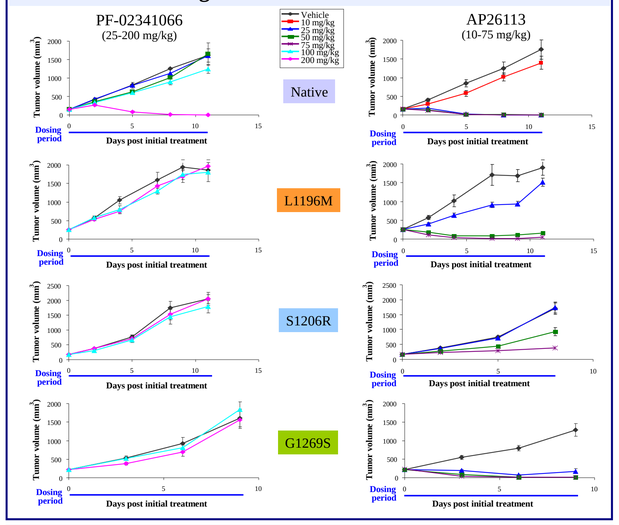
	PF-02341066		AP26113	
EML4-ALK	**IC50s (nM)**		**IC50s (nM)**	
Native	74 ± 48		5.3 ± 3.8	
Mutants:	***ENU-clone***	***Re-introduced***	***ENU-clone***	***Re-introduced***
F1174C	696 ± 514		12 ± 4	
L1196M	1291 ± 704	817 ± 324	15 ± 8	7.6 ± 0.2
S1206R	1012 ± 347	172 ± 71	170 ± 135	17 ± 2
E1210K	335 ± 106		76 ± 25	
F1245C	364 ± 223		7 ± 4	
G1269S	1353 ± 733	1005 ± 24	17 ± 5	8.6 ± 0.1

(n=2 or 3)

AP26113 inhibits ALK phosphorylation in EML4-ALK mutant tumors *in vivo*



AP26113 is efficacious in PF-02341066-resistant EML4-ALK mutant mouse xenograft models



Conclusions

- **EML4-ALK accelerated mutagenesis screen identified mutations at 18 amino acids**
  - 8 common to PF-02341066 and AP26113
  - 8 unique for PF-02341066; 2 unique for AP26113 (rare and only appeared at low concentrations)
  - 4 occur at amino acids mutated in neuroblastoma: T1151, I1171, F1174, F1245
- **Multiple mutations identified that confer strong resistance to PF-02341066 both *in vitro* and *in vivo***
  - Resistant mutations identified at concentrations up to 1440 nM
    - Parental Ba/F3 cell proliferation inhibited by > 70% at this concentration
  - L1196M (gate keeper), S1206R and G1269S conferred strongest resistance
    - *In vitro* viability assay: IC50s similar to parental Ba/F3 cells (711-1141 nM)
    - *In vivo* xenograph model: no anti-tumor activity at daily doses up to 200 mg/kg
  - I1171T, F1174C, E1210K and F1245C: *in vitro* IC50s 315-478 nM
- **AP26113 is able to overcome all mutations that confer resistance to PF-02341066**
  - Completely suppressed the emergence of resistance at 1000 nM
    - Only one mutation recovered at 500 nM: S1206R
  - L1196M and G1269S
    - *In vitro* viability assay: IC50s of 45 and 16 nM
    - *In vivo* xenograph model: tumor regression at 50 and 25 mg/kg
  - I1171T, F1174C, E1210K and F1245C: *in vitro* IC50 < 70 nM
  - S1206R: potent *in vitro* (IC50: 194 nM) and *in vivo* (75 mg/kg) activity against mutant that confers greatest resistance to AP26113
- **Clear potential for clinical resistance to PF-02341066 based on its modest potency, narrow therapeutic index and reported clinically achievable plasma levels**

These results identify several mutations that may confer resistance to PF-02341066 in patients and suggest that more potent compounds such as AP26113 may be able to overcome such resistance