#794. CG'806, a first-in-class pan-FLT3/pan-BTK inhibitor, targets multiple pathways to kill diverse subtypes of acute myeloid leukemia and B-cell malignancy *in vitro*.

Hongying Zhang, MD, PhD[1], Andrea Local, PhD[1], Khalid Benbatoul[1], Peter Folger[1], Susan Sheng, PhD[1], Cheng-Yu Tsai PhD,[2], Stephen B. Howell MD,[2] and William G. Rice, PhD[1]

[1]Aptose Biosciences Inc., San Diego, CA; [2]University of California San Diego, San Diego CA.

Abstract

Small molecules targeting the FLT3 kinase with an internal tandem duplication mutation (FLT3-ITD) and the Bruton's Tyrosine Kinase (BTK) have shown great potential to treat hematologic malignancies. However, acquired mutations have emerged in resistant/relapsed patients treated with the FLT3 inhibitor Quizartinib (Smith et al., 2012) or the BTK inhibitor Ibrutinib (Woyach et al., 2017), respectively. This study explored the potency and molecular mechanism of CG'806, a pan-FLT3/pan-BTK inhibitor, in hematologic malignancies relative to other FLT3 or BTK inhibitors commercialized or in development.

In FLT3-ITD AML cells, CG'806 induced apoptosis through inhibition of FLT3 signaling (decreased phospho-FLT3, -STAT5 and -ERK) and promotion of G_0/G_1 cell cycle arrest determined by immunoblotting and flow cytometry, and CG'806 was approximately 10-fold more potent than Quizartinib. Although FLT3-ITD is found in 25-30% of AML patients, most AML patients express wild type (WT) FLT3. CG'806 had an IC_{50} = 11 nM against FLT3 WT-transfected Ba/F3 cells and was superior to Quizartinib, Gilteritinib, and Crenolanib FLT3 inhibitors (1,956, 500 and 2,617 nM, respectively). In FLT3-WT AML cell lines, or Ba/F3 cells transfected with FLT3-WT, D835Y, ITD+D835Y, or ITD+F691L, CG'806 markedly decreased phosphorylation of BTK, Aurora kinases (AURK) and phospho-H3S10, resulting in G_2/M arrest or polyploidy, and apoptosis with less or no effect on FLT3-WT activity. In contrast, Quizartinib did not affect BTK or AURK signaling, and CG'806 was >2,000-fold more effective than Quizartinib on FLT3-WT AML cells in MTS based cell proliferation assays.

In B-cell malignancies, BTK signaling plays a pivotal pathogenic role. CG'806 decreased BTK phosphorylation in all malignant B cell lines tested (n =10) and inhibited cell proliferation and colony formation 50-6,000 times more potently than ibrutinib, an effect which could not be explained by the exclusive inhibition of BTK signaling. Further analysis revealed CG'806 effectively inhibited AURK signaling and caused polyploidy and apoptosis in B-cell lines, sequelae which were not induced by ibrutinib. BTK-C481S is the most common mutation induced by treatment with ibrutinib and is associated with ibrutinib resistance in the clinic. CG'806 equivalently inhibited BTK-WT and BTK-C481S in HEK293 transfected cells, whereas ibrutinib was much less potent against the BTK-C481S mutant.

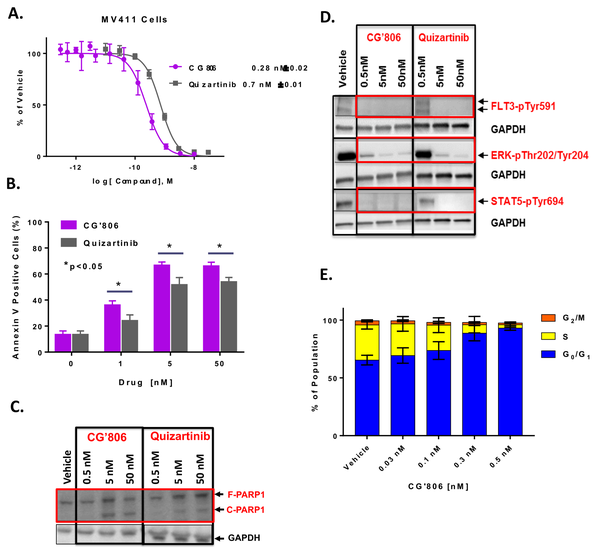
The ability of CG'806 to target all WT and mutant forms of FLT3 and BTK, and to inhibit multiple signaling pathways, produces killing of diverse subtypes of hematologic malignancies driven by different genomic aberrations. Considering the efficacy of CG'806 in the absence of observed toxicity in murine AML models, CG'806 appears superior to other FLT3 or BTK inhibitors and is suitable for development in patients with either AML or B-cell malignancy.

Materials and Methods

- Cell lines – CG'806 activity was assayed in human and mouse cell lines with FLT3-ITD, WT FLT3, FLT3 mutants, and WT BTK.
- Cytotoxicity assay - Cell viability was measured by MTS assay and graphed as percent vehicle control.
- Apoptosis was assayed via flow cytometry with Annexin V / PI staining and cell cycle (polyploidy) via flow cytometry with EdU / PI staining.
- Immunoblotting – Whole cell lysates were collected from cells after treatment with either Vehicle (DMSO) or CG'806 at the concentrations and for the times listed in each figure. The total and phospho levels of the indicated proteins were measured by Western Blotting. For AURK pathway analysis cells were synchronized with Nocodozole for 16 h prior to CG'806 treatment.
- Colony forming assay – Number of colonies present after 16 days growth in Methocult™ media with Vehicle, CG'806, or Ibrutinib at the concentration listed was calculated as percent vehicle control.

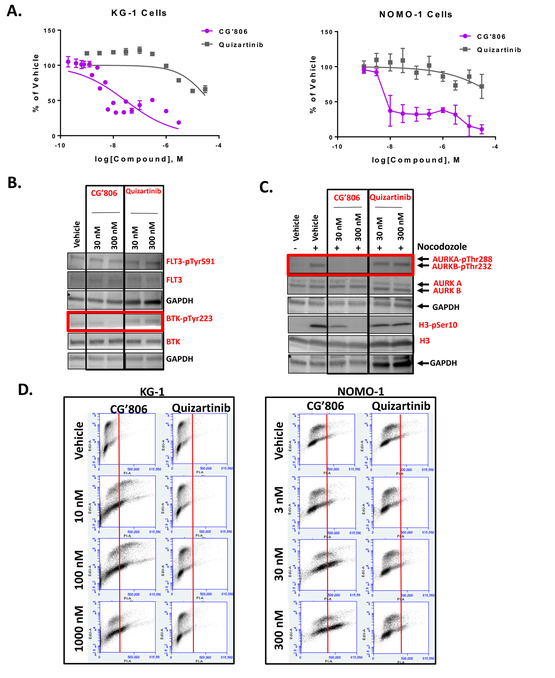
Disclosures: Zhang: Aptose Biosciences Inc.: Employment. Local: Aptose Biosciences Inc.: Employment. Benbatoul: Aptose Biosciences Inc.: Employment. Folger: Aptose Biosciences Inc.: Employment. Sheng: Aptose Biosciences Inc.: Employment. Rice: Aptose Biosciences Inc.: Employment.

CG'806 inhibits FLT3-ITD signaling more effectively than Quizartinib



CG'806 inhibits FLT3-ITD signaling and induces apoptosis more effectively than Quizartinib, and causes G0/G1 cell cycle arrest in FLT3-ITD MV4-11 AML cells. A) Cytotoxic activity of CG'806 or Quizartinib was assessed in FLT3-ITD cell line MV4-11 by MTS assay. B) Apoptosis was assayed via flow cytometry with Annexin V / PI staining after treatment with CG'806 or Quizartinib at the concentrations listed. C) Apoptosis was measured by appearance of cleaved-PARP1 (c-PARP) in MV4-11 cells treated with vehicle DMSO, CG'806, or Quizartinib at the concentrations listed. D) Lysates from MV4-11 cells treated with CG'806 or Quizartinib were assayed by immunoblotting. E) Cell cycle was analyzed by flow cytometry in MV4-11 cells at the concentrations of CG'806 listed.

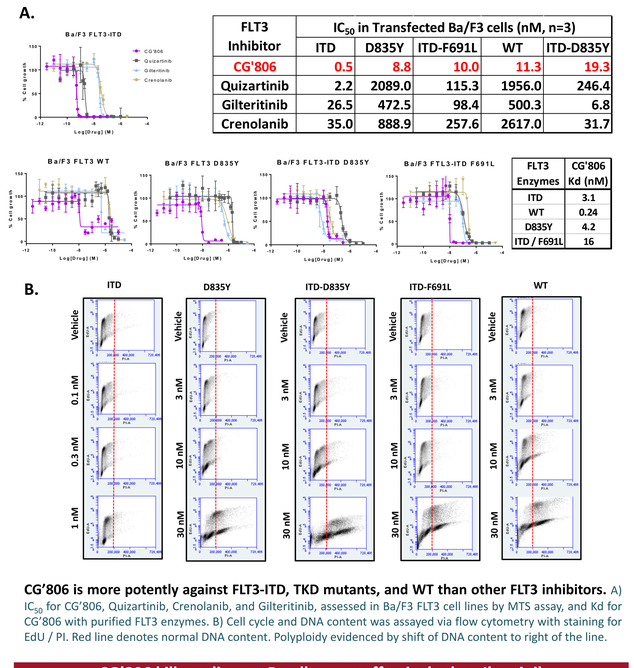
CG'806 inhibits BTK, AURK and downstream signals in FLT3-WT AML cells



CG'806 potently inhibits BTK and AURK in FLT3-WT AML cell lines. A) Cytotoxic activity of CG'806 or Quizartinib was assessed in FLT3 WT cell lines by MTS assay. B) Lysates from KG-1 cells treated with CG'806 or Quizartinib were assayed by immunoblotting for FLT3, BTK total and phospho proteins. C) After synchronizing with Nocodozole, KG-1 cells were treated with CG'806 or Quizartinib at the concentrations listed and lysates immunoblotted for AURK and H3 Ser10 total and phospho proteins. D) Cell cycle and DNA content was assayed via flow cytometry with staining for EdU / PI. Red line denotes normal DNA content. Polyploidy evidenced by shift of DNA content to right of the line.

References
1. Smith CC, et al., Nature. 2012. 485(7397):260-3.
2. Woyach JA, et al., J. Clin. Oncol. 2017. 1;35(13):1437-43

CG'806 potently targets WT and mutant (ITD, TKD, gatekeeper) forms of FLT3

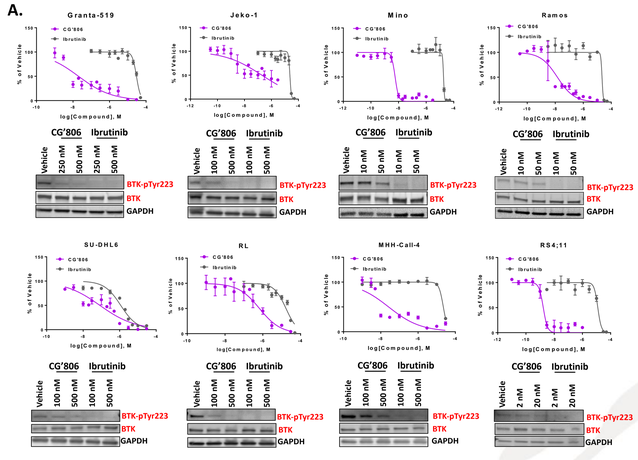


FLT3 Inhibitor	IC_{50} in Transfected Ba/F3 cells (nM, n=3)				
	ITD	D835Y	ITD-F691L	WT	ITD-D835Y
CG'806	0.5	8.8	10.0	11.3	19.3
Quizartinib	2.2	2089.0	115.3	1956.0	246.4
Gilteritinib	26.5	472.5	98.4	500.3	6.8
Crenolanib	35.0	888.9	257.6	2617.0	31.7

FLT3 Enzymes	CG'806 Kd (nM)
ITD	3.1
WT	0.24
D835Y	4.2
ITD / F691L	16

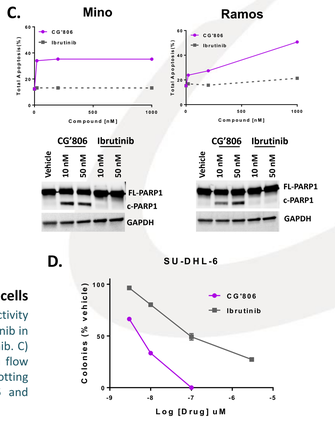
CG'806 is more potently against FLT3-ITD, TKD mutants, and WT than other FLT3 inhibitors. A) IC_{50} for CG'806, Quizartinib, Crenolanib, and Gilteritinib, assessed in Ba/F3 FLT3 cell lines by MTS assay, and Kd for CG'806 with purified FLT3 enzymes. B) Cell cycle and DNA content was assayed via flow cytometry with staining for EdU / PI. Red line denotes normal DNA content. Polyploidy evidenced by shift of DNA content to right of the line.

CG'806 kills malignant B-cells more effectively than Ibrutinib

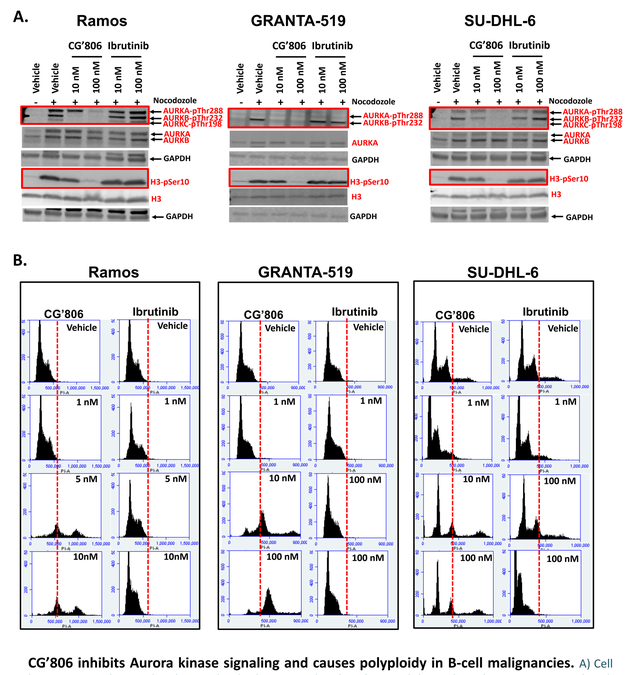


Disease Type	Cell Line	IC_{50} (μM)		Fold difference
		CG'806	Ibrutinib	
MCL	Mino	0.006	18.2	3033
	Granta-519	0.020	29.9	1495
	Jeko-1	0.197	23.6	120
B-ALL	RS411	0.002	12.4	6200
	MHH-Call4	0.026	22.8	877
Burkitt's	Ramos	0.014	22.0	1571
	Daudi	0.236	23.9	101
ABC-DLBCL	OCI-Ly3	0.830	29.0	35
	U2932	0.632	8.9	14
	SU-DHL2	0.744	27.0	36
GCB-DLBCL	BJAB	0.882	1.8	2
	SU-DHL6	0.083	1.2	14
	RL	0.667	17.0	25
FL	DOHH2	0.003	0.441	147

CG'806 is more potent at killing malignant B-cells than Ibrutinib. A) Comparison of cytotoxic activity (sigmoid curve) and BTK inhibition for CG'806 and Ibrutinib in B-cell malignancies. B) IC_{50} values CG'806 versus Ibrutinib. C) Apoptosis of Mino and Ramos cells was assayed via flow cytometry with Annexin V / PI staining and immunoblotting for c-PARP1. D) Colony formation assay for CG'806 and Ibrutinib treated SU-DHL-6 cells.

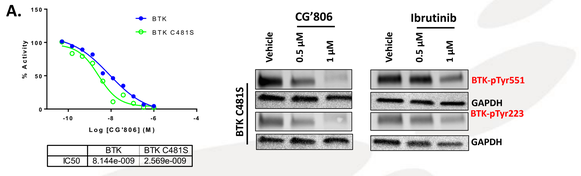


CG'806 inhibits AURK and induces polyploidy in malignant B-cell lines



CG'806 inhibits Aurora kinase signaling and causes polyploidy in B-cell malignancies. A) Cell lines were synchronized with Nocodozole then treated with CG'806 and Ibrutinib at the concentrations listed. Lysates were immunoblotted for total and phospho AURKs and H3Ser10. B) Cell cycle and DNA content was analyzed via flow cytometry with EdU / PI staining in cells treated with either CG'806 or Ibrutinib at the concentrations listed. Red line denotes normal DNA content. Polyploidy evidenced by shift of DNA content to right of the line.

CG'806 is equally potent against WT and C481S mutant BTK



	BTK	BTK C481S
IC50	8.144e-009	2.569e-009

CG'806 maintains activity against Ibrutinib-resistant BTK-C481S. A) BTK and BTK-C481S inhibition assessed by biochemical enzymatic activity assay. B) Immunoblotting of HEK293 cells transfected with C481S mutant BTK, comparing inhibitory activity of CG'806 and ibrutinib.

Conclusions

- **CG'806 exerts superior cell killing relative to other FLT3 inhibitors against AML cells with WT or mutant (ITD, TKD, gatekeeper) FLT3.**
- **In AML cells carrying FLT3-ITD, CG'806 inhibits FLT3 signaling, and induces G0/G1 cell cycle arrest and apoptosis.**
- **In AML cells carrying FLT3-WT or FLT3-TKD/gatekeeper plus ITD mutants, CG'806 inhibits BTK/FLT3/AURK signaling and induces polyploidy.**
- **CG'806 on average is approx. 1000-fold more potent than ibrutinib at inducing apoptosis and directly killing malignant B-cells.**
- **In malignant B-cells, CG'806 inhibits BTK and AURK signaling.**
- **CG'806 inhibits BTK-C481S mutant and BTK-WT equivalently.**
- **CG'806, as a potent pan-FLT3/pan-BTK inhibitor that is well tolerated in murine xenograft models, is suitable for development in patients with AML or certain B-cell malignancies.**