Efficacy of EGFR and GAK Inhibitor Compounds on Chordoma *in vitro*

<u>Aim:</u> To characterize potency of a set of compounds designed to inhibit the kinases EGFR and/or GAK (cyclin-G associated kinase) in chordoma cells in vitro. Many published EGFR inhibitors also demonstrate some affinity for GAK, and inhibition of GAK may play a role in EGFR activation[1,2]. Because EGFR is a therapeutic target for many chordomas and EGFR inhibitors with chordoma activity such as gefitinib and erlotinib also have affinity for GAK, we seek to clarify the roles of EGFR and GAK in chordoma.

<u>Methods:</u>

- Cell lines were cultured as per the protocols established by the Chordoma Foundation.
- Chordoma cell lines U-CH1 and U-CH2 were plated in clear, gelatin-coated 96-well plates (Corning) at a density of 2.5×10^3 cells/well. This population size was selected based on observed growth rates so that untreated cells would be near confluence at the end of the assay.
- After growth overnight at 37°C with 5% CO_2, cell media was changed and drugs were administered. Vehicle controls were 1% DMSO and drug concentrations ranged from 1 nM-100 µM in DMSO. All drug treatments were performed in triplicate for each replicate sample. All pipetting of media and drug solutions automated by Tecan Freedom Evo liquid handling system.
  - Some compounds fell out of solution at 10 µM or higher; the data points containing insoluble compound were removed from analyses.
- Cells were treated for 72 hours. This time point was based on a time course series performed to assess cell viability at 24, 48, 72, and 96 hours (Figure 1) which revealed activity of candidate compounds at 72 hours.

Compound EC50 over timecourse (µM)						
	CA75			Erlotinib		
Time (hr)	U-CH1	U-CH2		Time (hr)	U-CH1	U-CH2
24	>50	>50		24	>50	4.5
48	20.3	15.6		48	4.2	18.6
72	11.7	16.5		72	1.4	40.4
96	7.3	18.2		96	0.33	>50

Figure 1: Time course of EGFR & GAK Inhibitor Potency
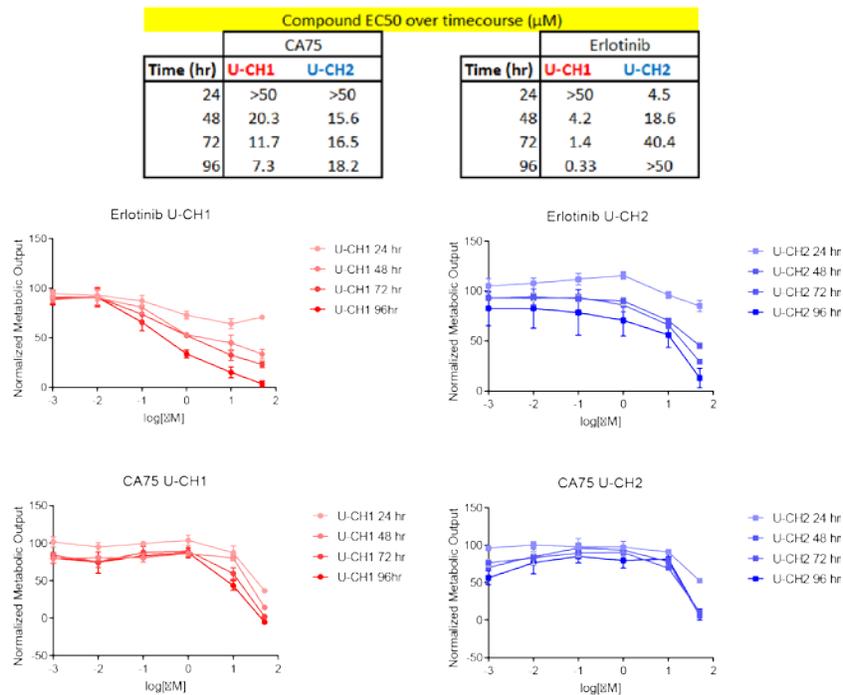
- After 72 hours, cell metabolic activity was measured using Alamar Blue Reagent (Invitrogen) as a proxy for cell viability. Fluorescence of Alamar metabolite was read on a Tecan Infinite 200 automated plate reader using 535 nm excitation and 595 nm emission wavelengths.
- EC_{50} concentrations for each compound and dose-response curves were generated using GraphPad PRISM 7.03.
- General lethality of the compounds was assessed by screening on WS1 normal human dermal fibroblasts (ATCC) grown in DMEM with 10% Fetal Bovine Serum. Cells were plated at a density of 2.0×10^3 cells/well to allow for untreated wells to be nearly confluent at end of assay. All other parameters were identical to the above protocol for Chordoma cells.

Results

Initial compound screening of a set of literature EGFR inhibitors established a baseline of potency (Table 1). These data indicate that U-CH1 is more responsive to EGFR inhibitors than U-CH2, confirming previously published observations[3] and that many of these inhibitors have EC_{50} values < 1 µM.

	EC50 (µM)	
	U-CH1	U-CH2
BMS 690514	0.53	>50
AZD8931/Sapitinib	0.42	5.71
Poziotinib	0.11	8.12
Dacomitinib	0.05	2.65
Canertinib	0.08	16.6
Cabozantinib	*	*
Bosutinib	14.6	35.9
Neratinib	5.5	2.4
Saracatinib	4.3	28.1
Pelitnib	0.09	1.6
Tesevatinib (XL-647)	0.15	1.1
Vendetanib	1.04	4.2
Afatinib	0.10	6.43
Gefitinib	1.59	8.81

Table 1: EC_{50} Concentrations for Published EGFR Inhibitors
Cabozantinib was out of solution at concentrations >1 µM; at concentrations <1 µM, the compound showed no efficacy.

EGFR and GAK inhibitor compounds were generated by Chris Asquith (Structural Genomics Consortium, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill) around a 4-anilino-quinoline/quinazoline/3-cyanoquinoline scaffold (Figure 2) similar to that of EGFR inhibitors erlotinib, gefinitinib, and lapatinib.
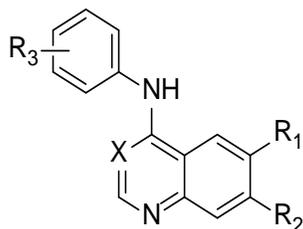
Figure 2: 4-Anilino-quinoline/quinazoline/3-cyanoquinoline scaffold for EGFR/GAK inhibitors

First, affinity of each compound for EGFR and GAK was observed both *in vitro* and with in-cell assays. These results reveal that some compounds that were highly specific to one of the two kinases and some compounds are likely to bind both kinases (Table 2).

Cmpd	R_1	R_2	X	R_3	*In vitro* binding		In-cell binding	
					EGFR K_d (nM)	GAK K_d (nM)	ProQinase EGFR IC50 (nM)	NanoBRET GAK IC_{50} (nM)
CA62	CF_3	H	CH	Trimethoxy	<50% 1µM	31	> 50000	170
CA72	CF_3	H	N	Trimethoxy	6100	190	> 50000	4500
CA93.0	Br	H	CH	Trimethoxy	<50% 1µM	1.9	> 50000	86
CA75	OMe	OMe	CH	Trimethoxy	410	2.9	27000	26
CA172	OMe	OMe	N	Trimethoxy	22	10	5400	349
CA249	OMe	OMe	CN	Trimethoxy	7.4	0.42	7100	6
CA156	OMe	OMe	CH	3-Acetylene	0.27	7.6	42	512
CA176	OMe	OMe	N	3-Acetylene	0.55	6.8	19	79
CA252	OMe	OMe	CN	3-Acetylene	1.8	2.6	61	40
CA239	6,7-diOCH$_2$CH$_2$OMe		N	Trimethoxy	53	150	2400	3000
Erlotinib	6,7-diOCH$_2$CH$_2$OMe		N	3-Acetylene	0.7	3.1	30	884
CA175	6,7-diOCH$_2$CH$_2$OMe		CH	3-Acetylene	390	1200	340	>5000
CA128	OMe	OMe	CH	3-Bromo	58	240	170	522
CA336	OMe	OMe	N	3-Bromo	0.32	29	< 15	282
CA335	OMe	OMe	CN	3-Bromo	2.4	12	90	112
CA313	OMe	OMe	CH	Lapatinib Aniline	32	>10000	1700	>5000
CA314	OMe	OMe	N	Lapatinib Aniline	1.3	5000	< 15	>5000
Lapatinib	-		CN	Lapatinib Aniline	10.8	<50% 1µM	21	>5000
GSK RIPK2	-		CH	-	<50% 1µM	<50% 1µM	> 50000	>5000
CA71	CF_3	H	CH	-	<50% 1µM	7.1 µM	2200	>5000
CA74	CF_3	H	CH	-	<50% 1µM	<50% 1µM	> 50000	>5000
Gefitinib							< 15	

Table 2: EGFR and GAK Binding Data for Inhibitor Compounds
In vitro and in-cell binding assays were used to assess affinity of compounds for EGFR and GAK built around scaffold shown in Figure 2. GSK RIPK2 inhibitor, having no GAK activity, was included as a control, as some GAK inhibitor compounds show activity against RIPK2 as well.

Potencies of the EGFR-GAK compound series in U-CH1, U-CH2, and WS1 are shown in Table 3 as EC_{50} concentrations from Alamar Blue assays. Each run included three biological replicates. With a few notable exceptions, most compounds were ineffective in the EGFR inhibitor-resistant U-CH2 chordoma cell line.

	U-CH1			U-CH2			WS1	
	Run 1	Run 2	Run 3	EC50	EC50	EC50	EC50	EC50
CA62	24.6	>50		37.1	31.4	29.9	>50	20.6
CA72	>50	49.9		>50	>50	>50	>50	44.49
CA93.0	>50	>50		>50	28.4	>50	16.5	4.52
CA75	11.2	10.0	12.2	27	14.4	36.9	16.4	11.5
CA172	10.7	7.34	3.74	>10	>10	>10	>10	27.9
CA249	>10	10.6	11.1	>10	>10	>10	>10	>10
CA156	1.7	0.52	4.00	>50	>50	11.6	5.8	9.63
CA176	1.5	0.58	0.69	>50	>50	>50	5.0	3.81
CA252	28.1	9.04	12.3	>50	>50	>50	>50	>50
CA239	50.1	11.2	13.6	>50	>50	>50	>50	>50
Erlotinib	14.2	0.37	0.58	>10	17.8	>10	>10	8.56
CA175	10.2	2.96	9.49	17.5	11.0	17.9	13.2	11.3
CA128	3.53	5.4	1.95	10.1	5.72	>10	>10	2.93
CA336	0.53	0.38	1.07	12.4	17.9	48.5	8.4	11.0
CA335	16.2	6.29	3.96	>50	>50	>50	?50	14.1
CA313	3.47	3.85	0.58	4.05	3.6	1.18	8.63	>1
CA314	21.9	13.7	1.91	>10	>10	>10	>10	>10
Lapatinib	3.91	3.19	1.19	>10	>10	>10	>10	13.3
GSK RIPK2	>50	14.3	>50	45.7	13.27	>50	>50	>50
CA71	5.1	>50	>50	7.2	>50	>50	>50	>50
CA74	27.0	>50	46.6	>50	>50	>50	>50	49.3
Gefitinib	1.82	0.88	1.41	15.5	15.0	38.3	27.5	18.7

Table 3: EC$_{50}$ concentrations (µM) for EGFR-GAK Inhibitors
EC$_{50}$ concentrations (µM) are pseudocolored green-red with increasing potency. White cells indicate no observed changes in cell viability at the indicated concentration (maximal concentration)

Conclusions

The series of compounds synthesized are useful in discerning the roles of EGFR and GAK in chordoma. A number of compounds are effective against chordoma – and intriguingly, a few show activity in U-CH2, a cell line resistant to EGFR inhibition. However, most of these compounds with U-CH2 activity also show strong activity in normal human fibroblasts (WS1), indicating potential toxicity of the compound or a vital role of GAK in normal cellular processes. Further investigation of this mechanism is warranted. Of the compounds with high affinity for GAK and low affinity for EGFR, CA75 is the primary compound to show potency in U-CH1 and U-CH2. CA156, CA176, CA313, and CA336 have EC$_{50}$ values comparable to those of published EGFR inhibitors and can be further pursued. Because we used Alamar Blue as a readout for cell viability, though, follow-up experiments are needed to discern if the compounds are arresting cell growth or causing cell death.

The activity of CA75 as well as EGFR inhibitors gefitinib and erlotinib (which also have affinity for GAK) leave open the opportunity to pursue GAK as a target for chordoma. We next plan to assess the roles of GAK in chordoma through further characterization of a subset of these compounds and shRNA knockdown of GAK in U-CH1 and U-CH2.

References

1   Sakurai MA, Ozaki Y, Okuzaki D, *et al.* Gefitinib and luteolin cause growth arrest of human prostate cancer PC-3 cells via inhibition of cyclin G-associated kinase and induction of miR-630. *PLoS ONE* 2014; **9**: e100124.

2   Brehmer D, Greff Z, Godl K, *et al.* Cellular targets of gefitinib. *Cancer Res* 2005; **65**: 379–382.

3   Scheipl S, Barnard M, Cottone L, *et al.* EGFR inhibitors identified as a potential treatment for chordoma in a focused compound screen. *J Pathol* 2016; **239**: 320–334.