Identification of Novel GANT61 Analogs with Activity in Hedgehog Functional Assays and Gli1-Dependent Cancer Cells

Dina Abu Rabe [1,*], Lhoucine Chdid [2], David R. Lamson [2], Christopher P. Laudeman [2], Michael Tarpley [2], Naglaa Elsayed [2], Ginger Smith [2], Weifan Zheng [2,3], Maria S. Dixon [2,*], Kevin P. Williams [2,3,*]

[1] INBS PhD Program, [2] Biomanufacturing Research Institute and Technology Enterprise, [3] Department of Pharmaceutical Sciences, North Carolina Central University, Durham, NC 27707, USA

[*]Authors to whom correspondence should be addressed.

Table of Contents for Supplemental Material

Figure S1: Activity of Hh pathway agonist and antagonist controls in Hh-responsive cell lines C3H10T1/2 and *Gli*-reporter NIH3T3 cellular assays.

Figure S2: Screening of GANT61 analog set in C3H10T1/2 cells using AP absorbance assay.

Figure S3: Effects of selected GANT61 analogs in C3H10T1/2 stimulated by different Smo agonists.

Figure S4: Time course of *Gli1* mRNA expression in C3H10T1/2 cells.

Figure S5: Quantification of *GLI1* and *GLI2* mRNA expression in tumor cells.

Figure S6: Molecular docking prediction for the binding mode of GANT61 to GLI1.

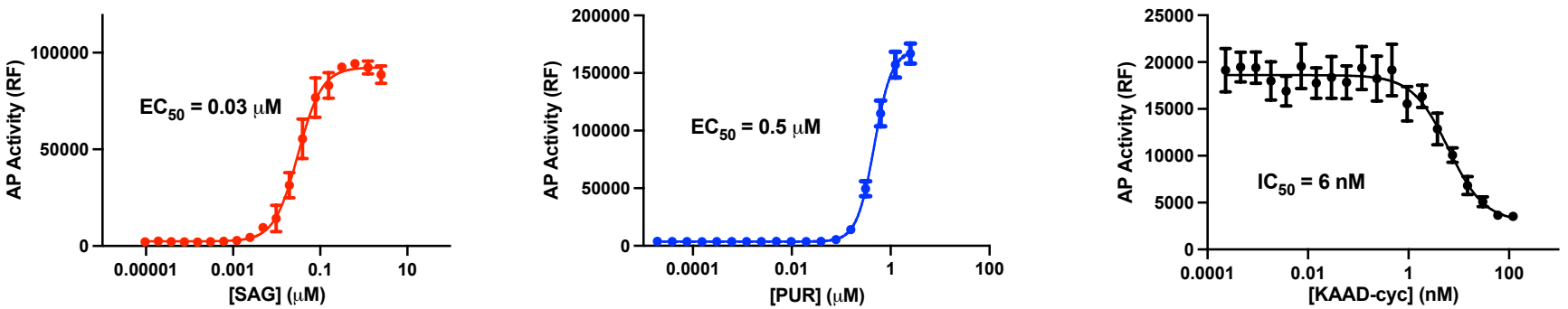
Table S1: Compound ID and chemical structures for the GANT61 analog compound set used in this study.

Table S2: Top 12 predicted poses for unbiased docking of BAS 07019774 to GLI1-ZF.

Table S3: Table of Top 15 predicted poses for biased docking of BAS 07019774 to GLI1-ZF at Glu119/Glu167.

Figure S1

(A)


C3H10T1/2 AP Fluorescence Assay
AP Activity (RF)
100000
50000
0
EC50 = 0.03 μM
0.00001
0.001
0.1
10
[SAG] (μM)
AP Activity (RF)
200000
150000
100000
50000
0
EC50 = 0.5 μM
0.0001
0.01
1
100
[PUR] (μM)
AP Activity (RF)
25000
20000
15000
10000
5000
0
IC50 = 6 nM
0.0001
0.01
1
100
[KAAD-cyc] (nM)

(B)

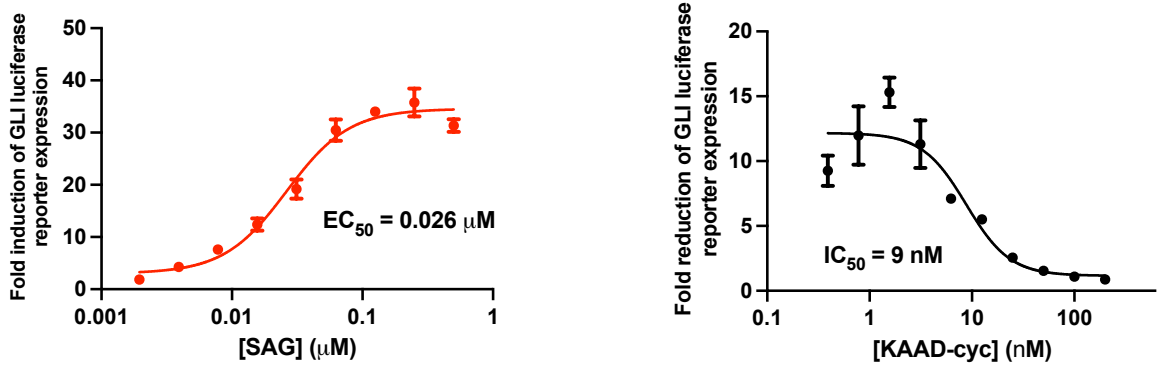
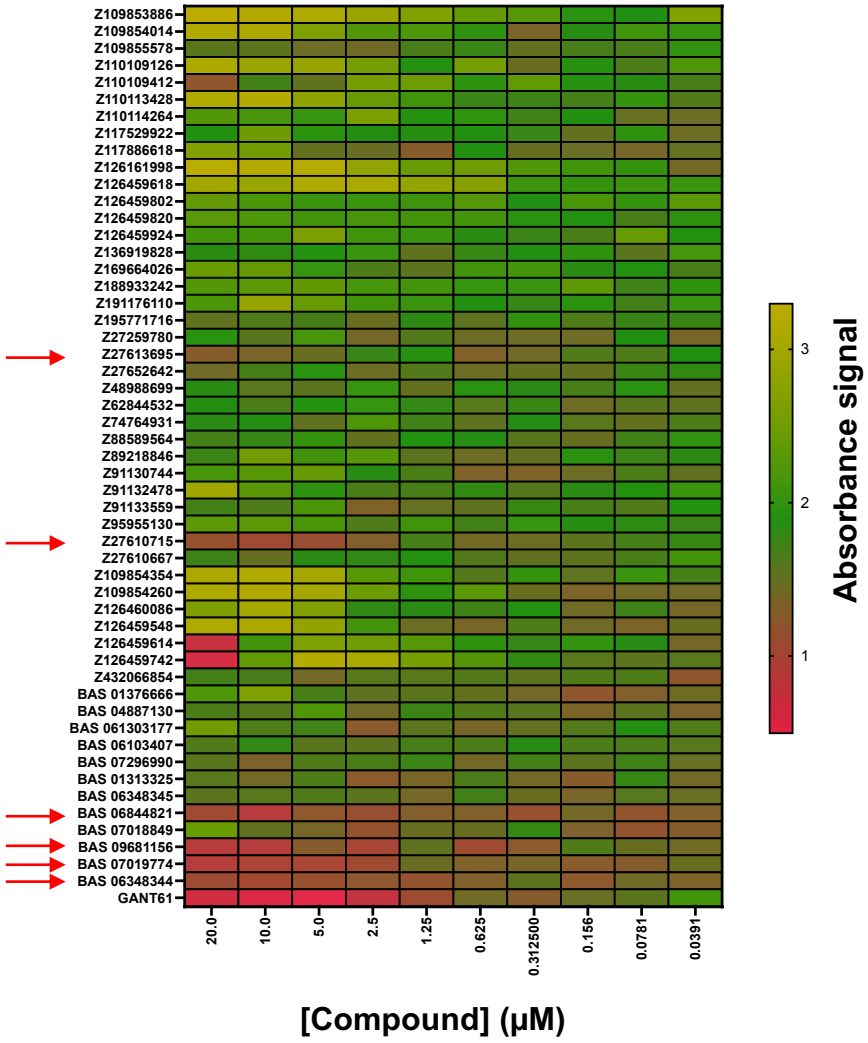

Gli-Reporter NIH3T3 Luciferase Assay
Fold induction of GLI luciferase reporter expression
50
40
30
20
10
0
EC50 = 0.026 μM
0.001
0.01
0.1
1
[SAG] (μM)
Fold reduction of GLI luciferase reporter expression
20
15
10
5
0
IC50 = 9 nM
0.1
1
10
100
[KAAD-cyc] (nM)

Figure S2

(A)


C3H10T1/2 AP Absorbance Assay
Z109853886
Z109854014
Z109855578
Z110109126
Z110109412
Z110113428
Z110114264
Z117529922
Z117886618
Z126161998
Z126459618
Z126459802
Z126459820
Z126459924
Z136919828
Z169664026
Z188933242
Z191176110
Z195771716
Z27259780
Z27613695
Z27652642
Z48988699
Z62844532
Z74764931
Z88589564
Z89218846
Z91130744
Z91132478
Z91133559
Z95955130
Z27610715
Z27610667
Z109854354
Z109854260
Z126460086
Z126459548
Z126459614
Z126459742
Z432066854
BAS 01376666
BAS 04887130
BAS 061303177
BAS 06103407
BAS 07296990
BAS 01313325
BAS 06348345
BAS 06844821
BAS 07018849
BAS 09681156
BAS 07019774
BAS 06348344
GANT61
20.0
10.0
5.0
2.5
1.25
0.625
0.312500
0.156
0.0781
0.0391
[Compound] (μM)
Absorbance signal
3
2
1

(B)

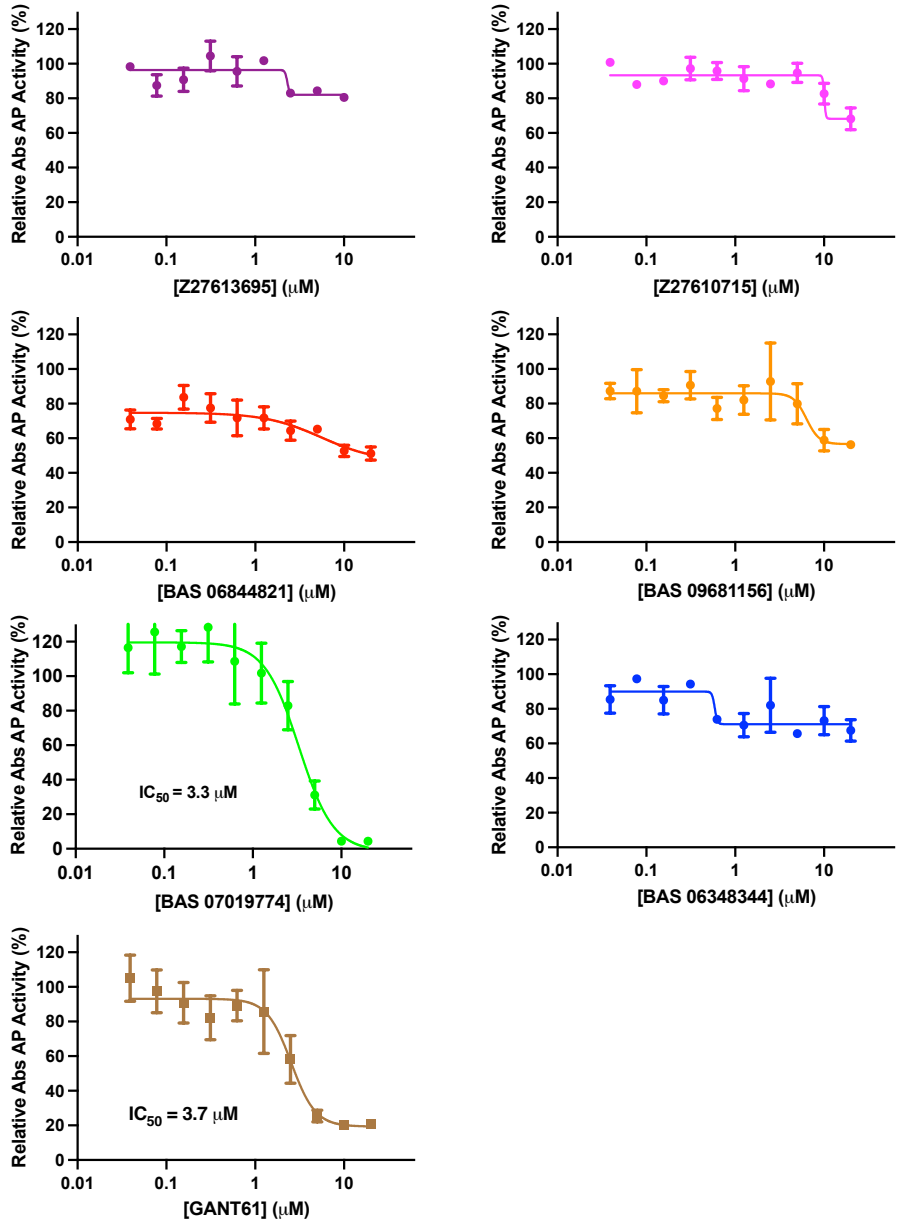

Relative Abs AP Activity (%)
[Z27613695] (μM)
[Z27610715] (μM)
[BAS 06844821] (μM)
[BAS 09681156] (μM)
IC50 = 3.3 μM
[BAS 07019774] (μM)
[BAS 06348344] (μM)
IC50 = 3.7 μM
[GANT61] (μM)

Figure S3

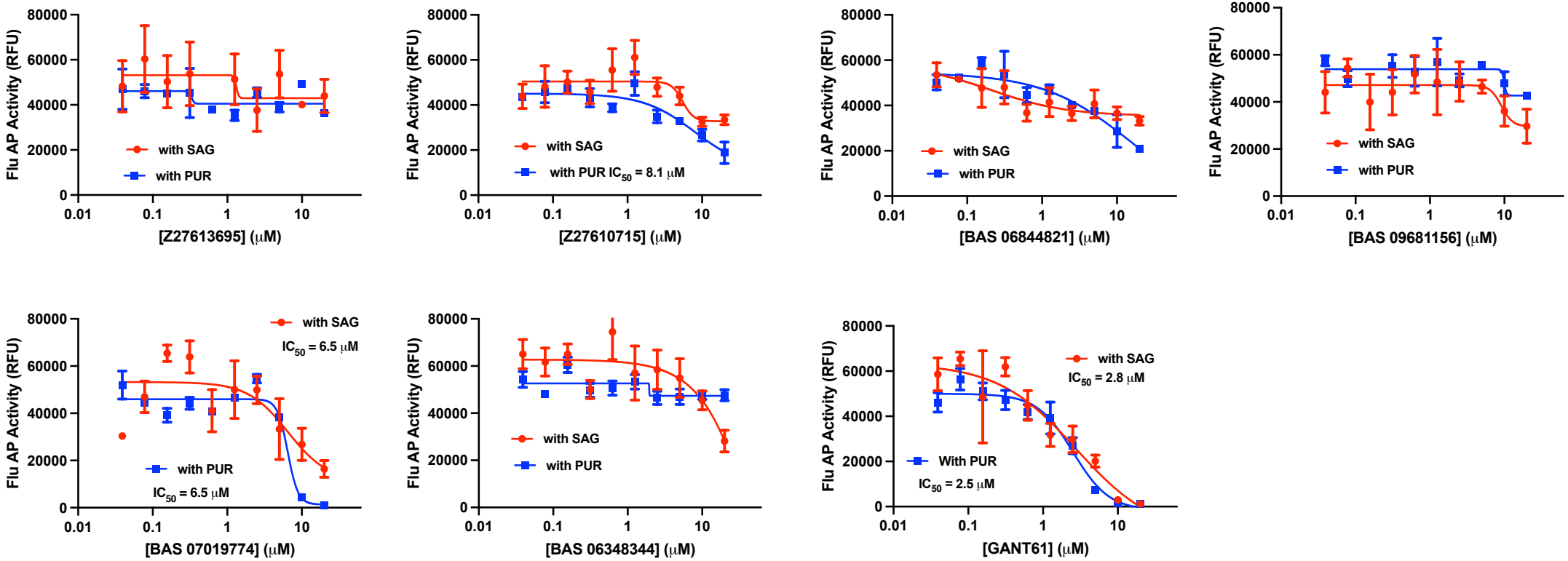
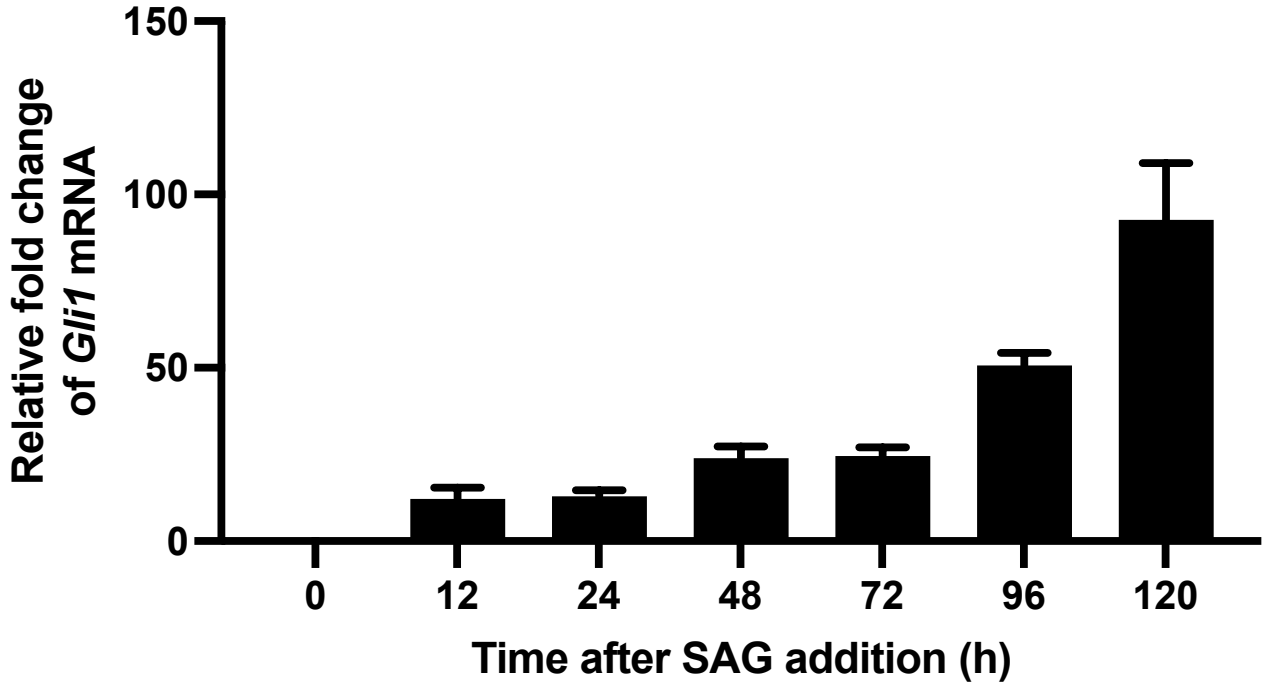
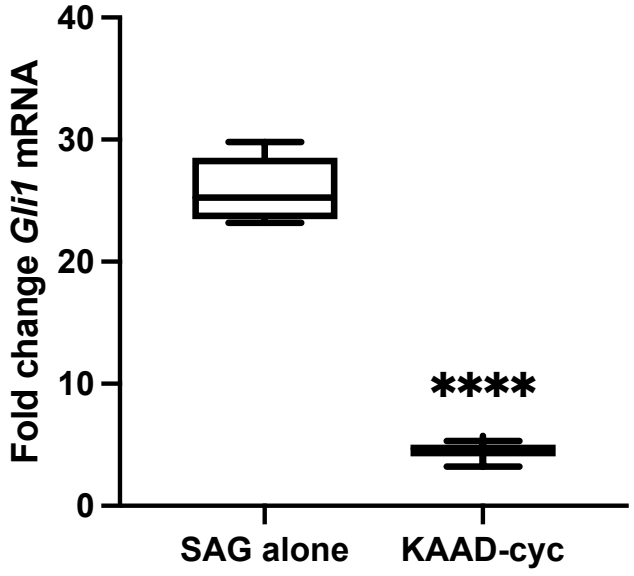

Flu AP Activity (RFU)
80000
60000
40000
20000
0
0.01
0.1
1
10
with SAG
with PUR
[Z27613695] (μM)
with PUR IC50 = 8.1 μM
[Z27610715] (μM)
[BAS 06844821] (μM)
[BAS 09681156] (μM)
IC50 = 6.5 μM
[BAS 07019774] (μM)
[BAS 06348344] (μM)
IC50 = 2.8 μM
With PUR
IC50 = 2.5 μM
[GANT61] (μM)

Figure S4

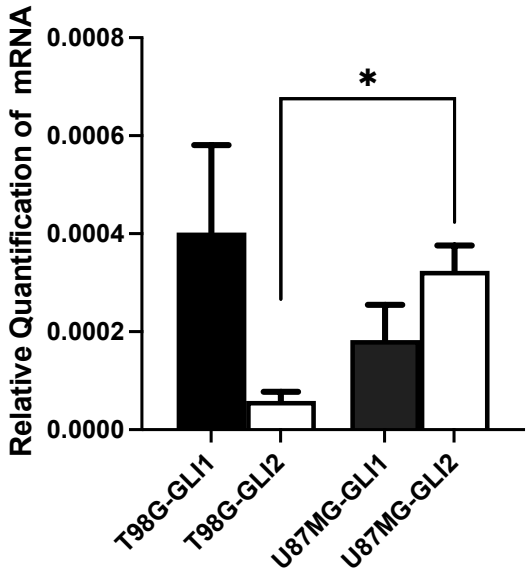

Relative fold change of *Gli1* mRNA
150
100
50
0
0
12
24
48
72
96
120
Time after SAG addition (h)

(A)

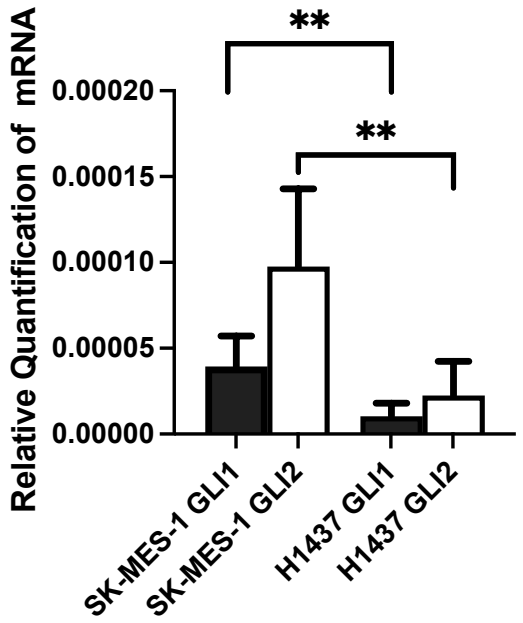

Fold change *Gli1* mRNA
40
30
20
10
0
****
SAG alone
KAAD-cyc

(B)

Figure S5


Relative Quantification of mRNA
0.0008
0.0006
0.0004
0.0002
0.0000
*
T98G-GLI1
T98G-GLI2
U87MG-GLI1
U87MG-GLI2

(A)


Relative Quantification of mRNA
0.00020
0.00015
0.00010
0.00005
0.00000
**
**
SK-MES-1 GLI1
SK-MES-1 GLI2
H1437 GLI1
H1437 GLI2

(B)

Figure S6

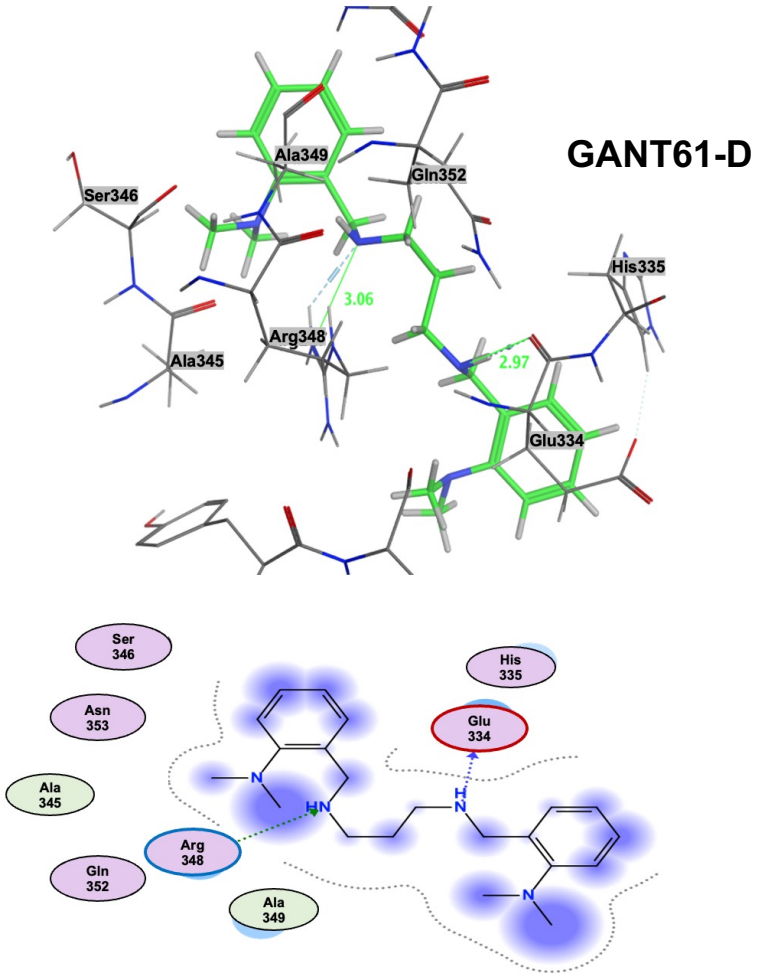

GANT61-D
Ala349
Gln352
Ser346
His335
3.06
Arg348
Ala345
2.97
Glu334
Ser 346
His 335
Asn 353
Glu 334
Ala 345
Arg 348
Gln 352
Ala 349

Figure S1: Activity of Hh pathway agonist and antagonist controls in Hh-responsive cell lines C3H10T1/2 and *Gli*-reporter NIH3T3 cellular assays. (**A**) For agonist dose response curves, SAG or PUR were added to C3H10T1/2 cells at the indicated concentrations and cells incubated for a further 5 days. For antagonist dose response curve, C3H10T1/2 cells were stimulated with SAG at its EC_{50} value (30 nM) and KAAD-cyc added at the indicated concentrations. AP activity was measured using a fluorescent substrate as described in Methods. All experiments conducted as a minimum of three independent experiments. (B) For agonist dose response curves, SAG was added to *Gli*-reporter NIH3T3 cells at the indicated concentrations and cells incubated for a further 30 h. For antagonist dose response curve, *Gli*-reporter NIH3T3 cells were stimulated with SAG at its EC_{50} value (26 nM) and KAAD-cyc added at the indicated concentrations. One-step luciferase assay was performed as described in Methods. Data are presented as the mean of fold change of GLI luciferase reporter expression ± SD of three replicates. EC_{50} and IC_{50} values were determined by non-linear regression in GraphPad Prism 9.

Figure S2: Screening of GANT61 analog set in C3H10T1/2 cells using AP absorbance assay. (**A**) Representative heat map profile for Hh-pathway inhibitory effects of the GANT61 analog set in C3H10T1/2 cells. C3H10T1/2 cells were stimulated with SAG at its EC_{50} value (30 nM), GANT61 analogs added in dose response at the indicated concentrations and after 5 days, AP activity was measured at 405 nm using *p*NPP substrate. Each row represents dose response data for a single GANT61 analog. The heat map key indicates red for maximum inhibition through to pale green for minimal inhibition. Red arrows indicate GANT61 analogs selected for further study. (**B**) Dose response curves for selected GANT61 analogs in C3H10T1/2 cells using colorimetric readout as in A. Data were normalized to SAG-stimulated cells and displayed as the mean of % activity (n =

3 independent experiments). Dose response curves were generated using non-linear regression and IC_{50} values determined in GraphPad Prism 9.

Figure S3: Effects of selected GANT61 analogs in C3H10T1/2 stimulated by different Smo agonists. C3H10T1/2 cells were treated with a fixed concentration of either SAG (30 nM) or PUR (500 nM) and GANT61 analogs added at the indicated concentrations. After 5 days, AP activity was measured using the fluorescence substrate and readout. Dose response curves were generated using non-linear regression and IC_{50} values determined in GraphPad Prism 9.

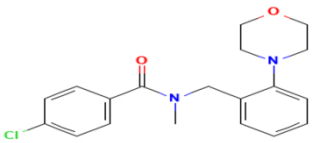
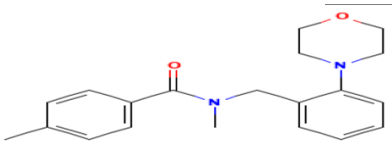
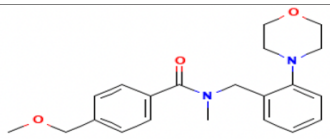
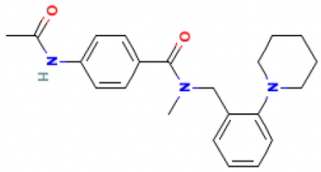
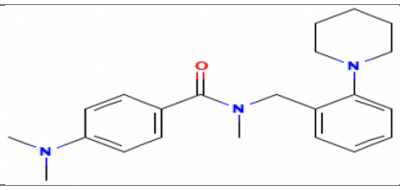
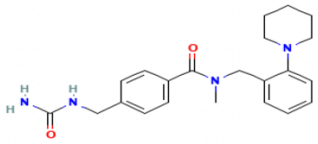
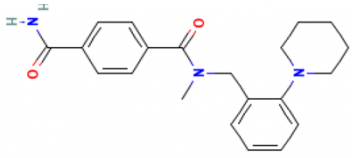
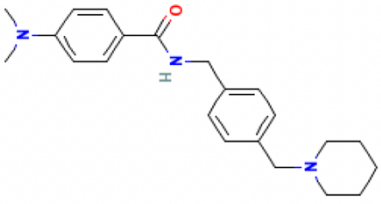
Figure S4: Time course of *Gli1* mRNA expression in C3H10T1/2 cells. (**A**) SAG induction of *Gli1* mRNA expression at different time points. C3H10T1/2 cells were treated with SAG at its EC_{50} (30 nM), cells collected at the indicated time points for RNA extraction and cDNA synthesis. *Gli1* mRNA expression was determined using TaqMan qRT-PCR. (**B**) KAAD-cyc inhibition of SAG-induced *Gli1* mRNA at 48 h in C3H10T1/2 cells. Cells were treated with 30 nM SAG with and without KAAD-cyc at its IC_{90} value (40 nM). Data was normalized to the housekeeping gene β-actin and displayed as the mean of fold change of *Gli1* mRNA expression ± SD of three independent experiments. Two-tailed t-test and Mann-Whitney test represent SAG + KAAD-cyc significantly different (****p-value <0.0001) from SAG alone.

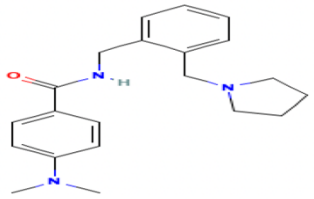
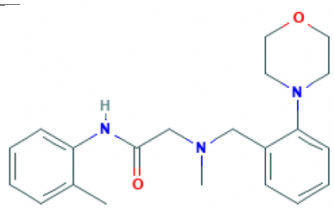
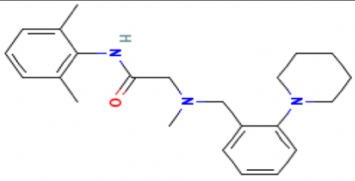
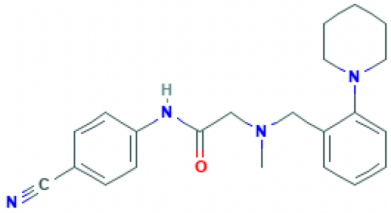
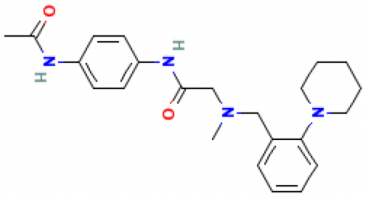
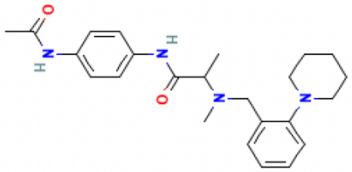
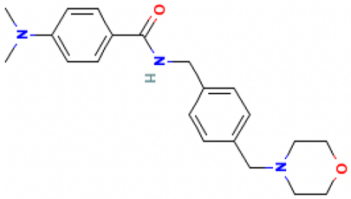
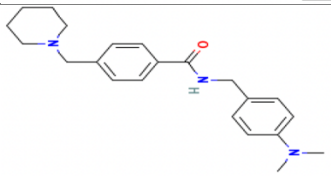
Figure S5: Quantification of *GLI1* and *GLI2* mRNA expression in tumor cells. (**A**) Glioblastoma U87MG and T98G. (**B**) Lung cancer SK-MES-1 and H1437. *GLI1* and *GLI2* mRNA was quantified using TaqMan RT-PCR. The housekeeping gene *GAPDH* was used for normalization and to determine fold change using the $2^{-\Delta\Delta Ct}$ method. Data presented as the mean of the relative

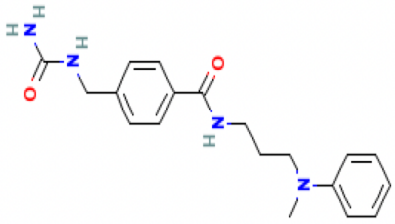
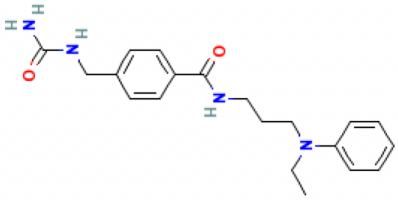
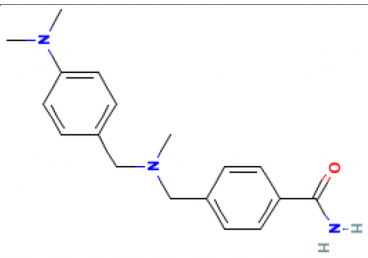
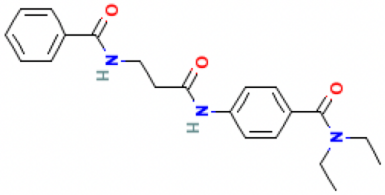
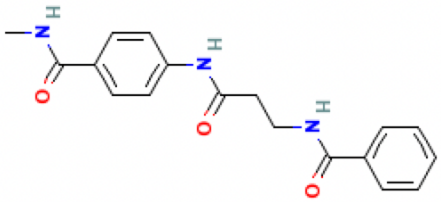
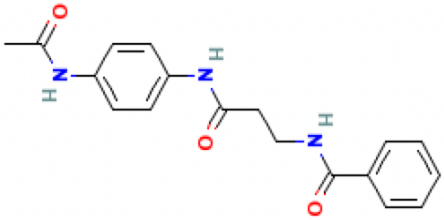
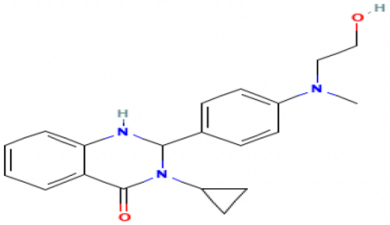
quantification of mRNA ± SD of three replicates.–One-way ANOVA and Tukey's multiple comparisons show significantly different (* and **p-value < 0.05) expression of GLI1 and GLI2 mRNA between cell lines.

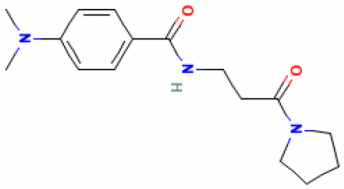
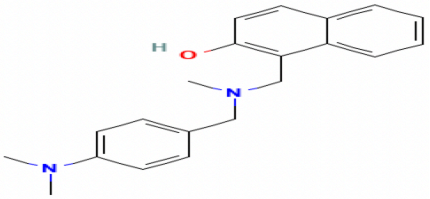
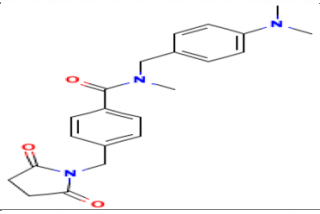
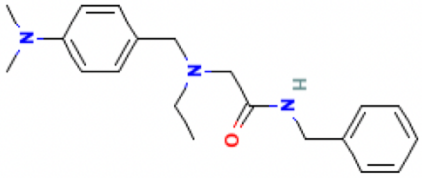
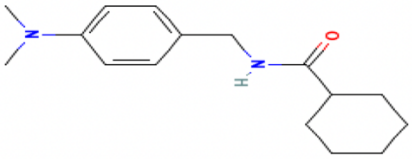
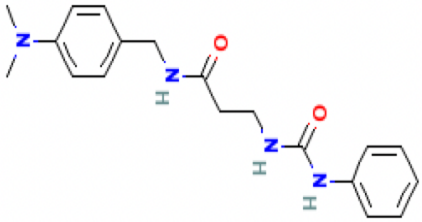
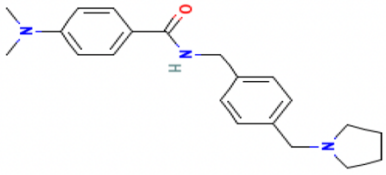
Figure S6: Molecular docking to predict the binding mode of GANT61 to GLI1 using the crystal structure of the five-zinc finger domain (PDB ID: 2GLI1). Best-ranked docking pose predicted binding of GANT61 to GLI1-ZF domain at amino acid residues Glu334 and Arg348. Ligand interactions were generated in MOE software.

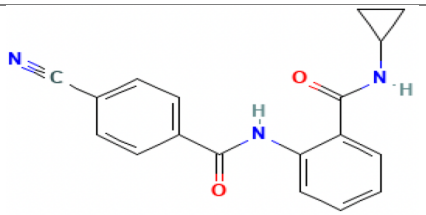
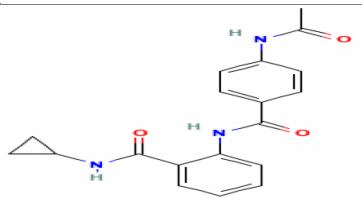
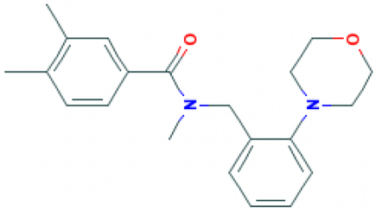
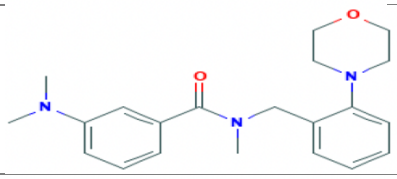
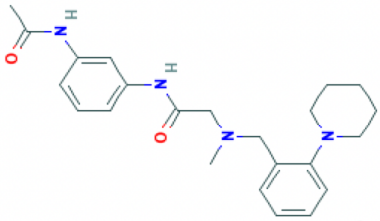
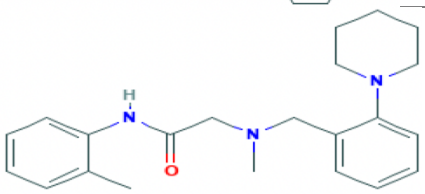
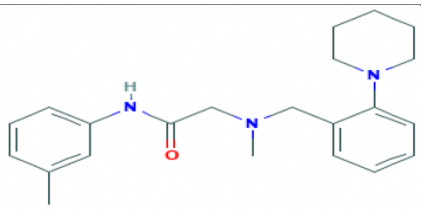
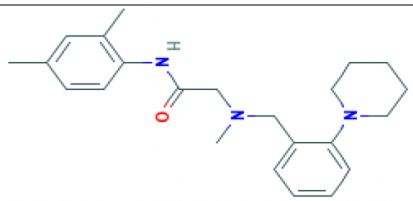
Supplemental Table S1. Compound ID and chemical structures for the GANT61 analog compounds used in this study.

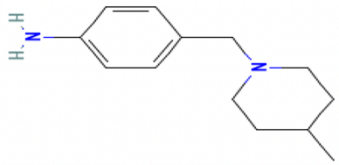
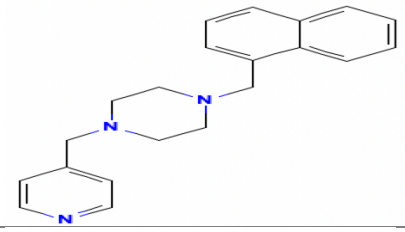
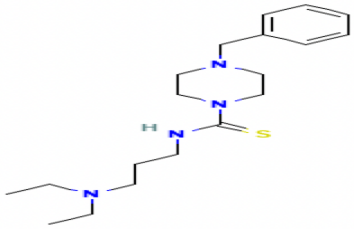
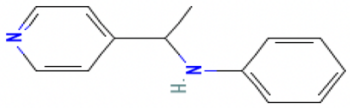
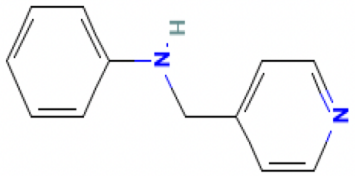
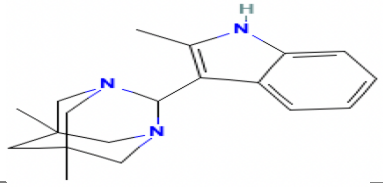
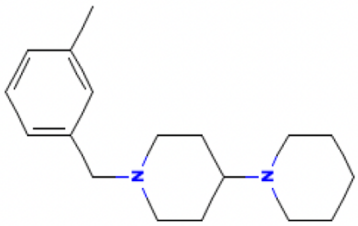
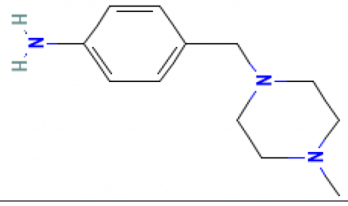
Compound Vendor ID	Compound Structure
Z109853886	
Z109854014	
Z109855578	
Z110109126	
Z110109412	
Z110113428	
Z110114264	
Z117529922	

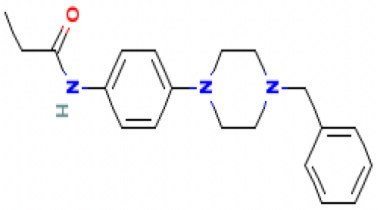
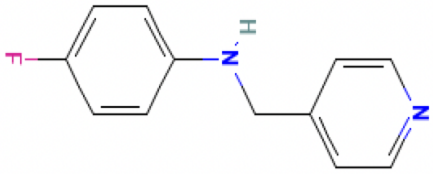
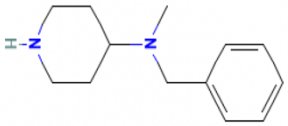
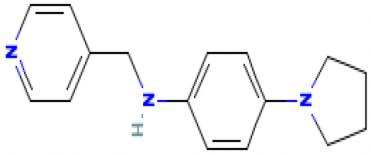
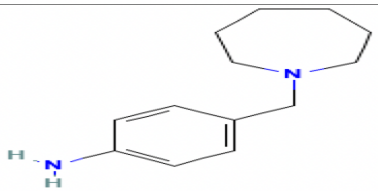
Z117886618	
Z126161998	
Z126459618	
Z126459802	
Z126459820	
Z126459924	
Z136919828	
Z169664026	

Z188933242	
Z191176110	
Z195771716	
Z27259780	
Z27613695	
Z27652642	
Z48988699	

Z62844532	
Z74764931	
Z88589564	
Z89218846	
Z91130744	
Z91132478	
Z91133559	
Z95955130	

Z27610715	
Z27610667	
Z109854354	
Z109854260	
Z126460086	
Z126459548	
Z126459614	
Z126459742	

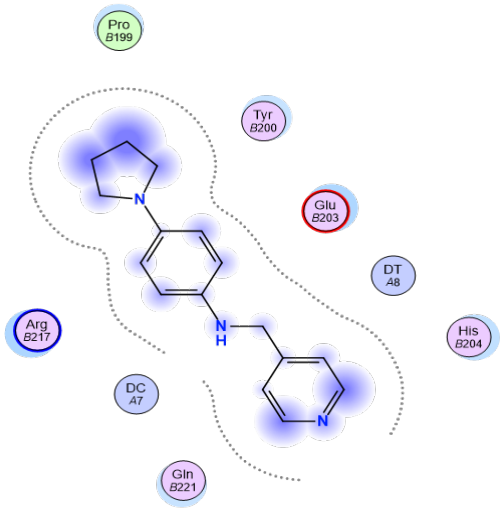
Z432066854	
BAS 01376666	
ASN 04887130	
BAS 06103177	
BAS 06103407	
BAS 07296990	
BAS 01313325	
BAS 06348345	

BAS 06844821	
BAS 07018849	
BAS 09681156	
BAS 07019774	
BAS 06348344	

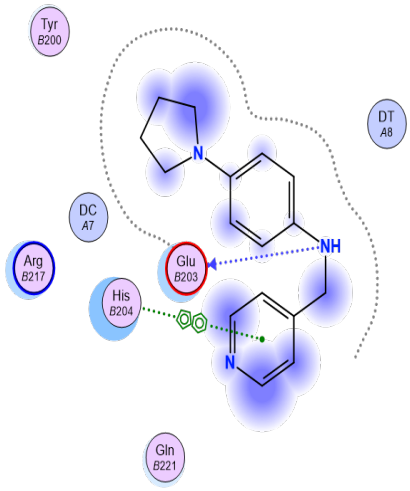
Supplemental Table S2. Top 12 predicted poses for unbiased docking of BAS 07019774 to GLI1 ZF.

Pose	Number of interactions	Residues[a]	Types of interactions	Energy	Distance A	Score
1	0	NA	NA	NA		-4.5157
2	2	Glu 334 His335	H-bond Pi-Pi interaction	- 1.6 Kcal	2.99 3.61	-4.4947
3	1	Glu334	H-bond	-0.8 Kcal	3.42	-4.2653
4	1	His335	Pi-Pi interaction	-0.0 Kcal	3.96	-4.2068
5	1	Glu334	H-bond	-1.7 Kcal	3.11	-4.1595
6	1	DT A8	H-donor	-1.3Kcal	3.30	-4.0843
7	**3**	**Arg348 Glu334 His335**	**H-bond Pi-H interaction Pi-H interaction**	**-1.8 Kcal**	**3.31 3.72 3.30**	**-4.0129**
8	2	Arg 348 His 335	H-bond H-pi interaction	-2.0 Kcal	3.06 3.91	-3.9755
9	0	NA	NA	NA		-3.9617
10	1	DC A7	Pi-H interaction	-0.9 Kcal	3.53	-3.9410
11	2	Arg 348 DT A8	H-bond H-bond	-2.5 Kcal	3.16 3.5	-3.9080
12	1	His335	Pi-H interaction	-1.2 Kcal	3.58	-3.8048

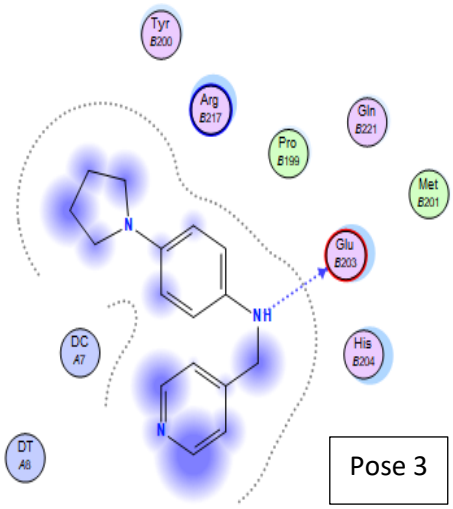
[a] Residue numbering is for full length GLI1 (UNIPROY P08151). Residue numbering in poses below is as found in the Protein Data Bank for 2GLI. To convert to numbering for full length GLI1 (UNIPROY P08151) add 131.



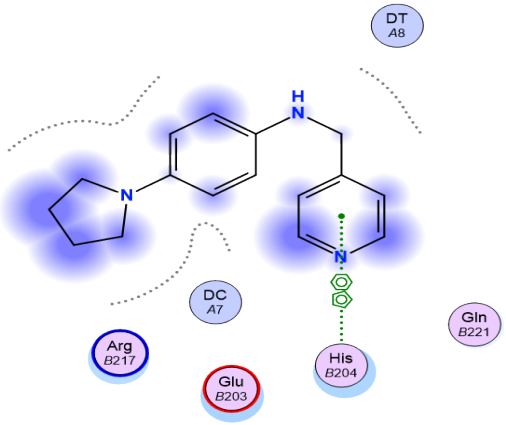
Pose 1



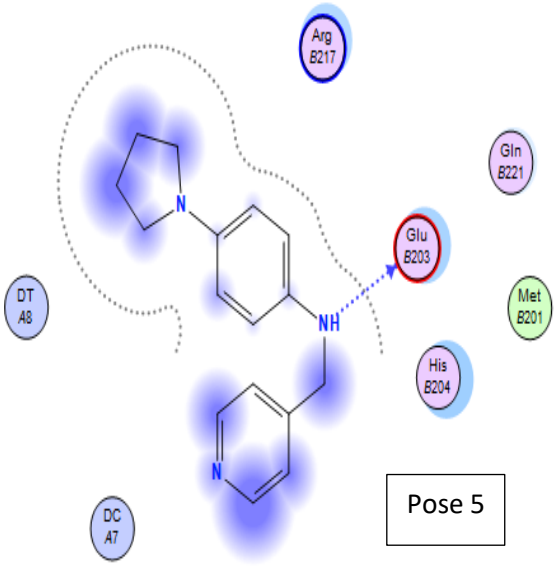
Pose 2



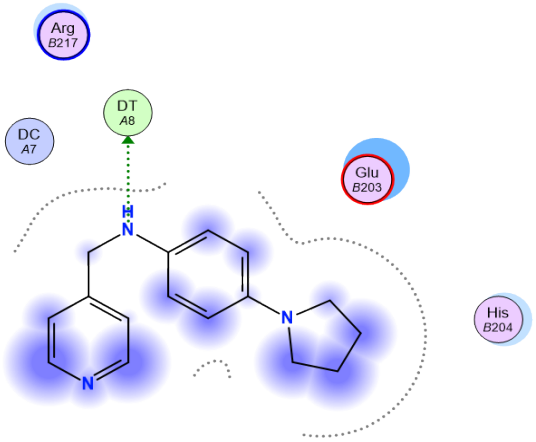
Pose 3



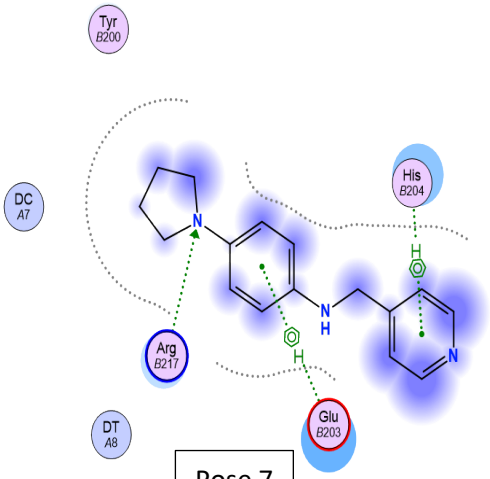
Pose 4



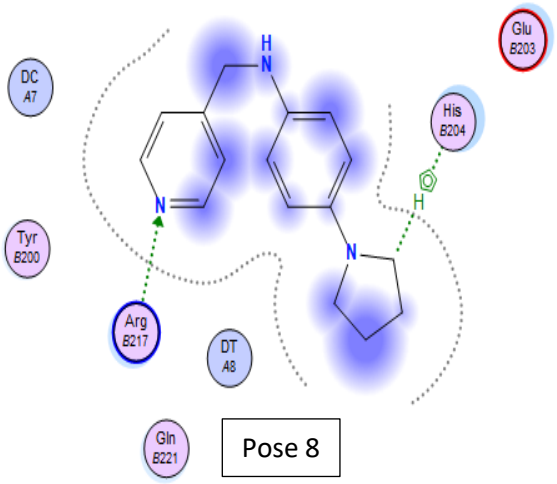
Pose 5



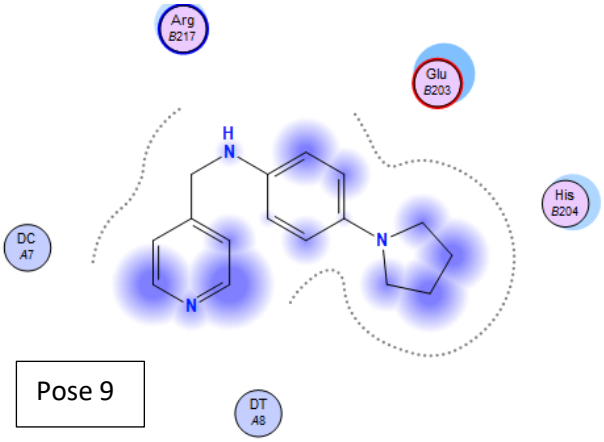
Pose 6



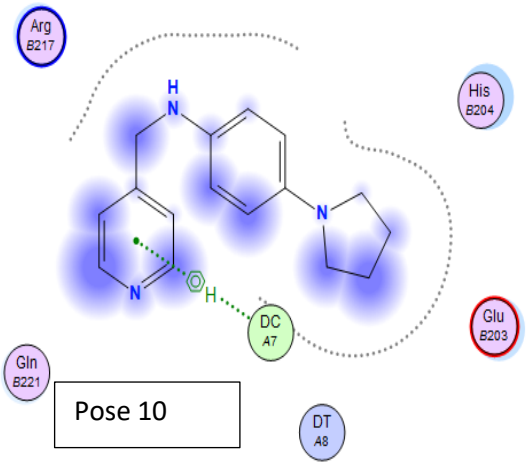
Pose 7



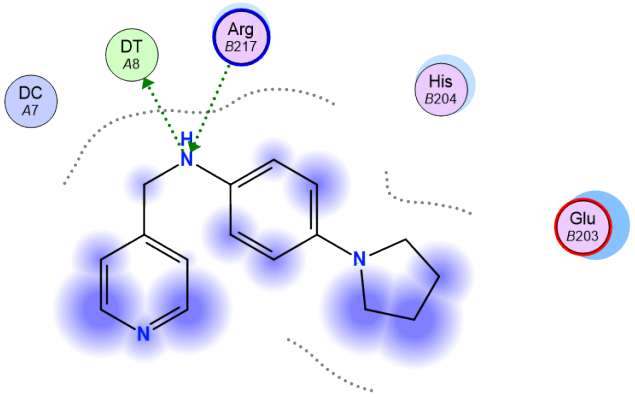
Pose 8



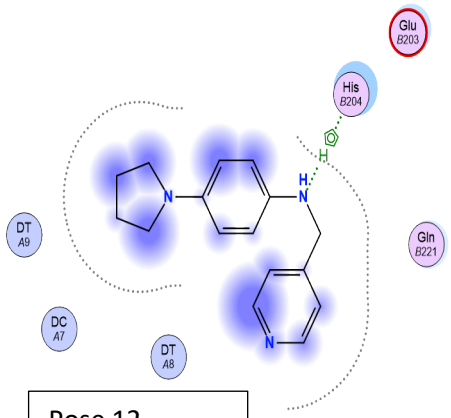
Pose 9



Pose 10



Pose 11



Pose 12

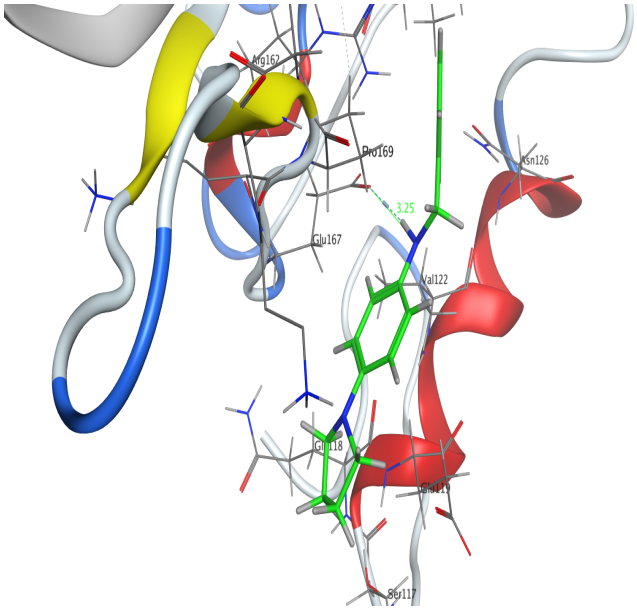
Supplemental Table S3. Top 15 predicted poses for biased docking of BAS 07019774 to GLI1 ZF at Glu119/Glu167.

(A)

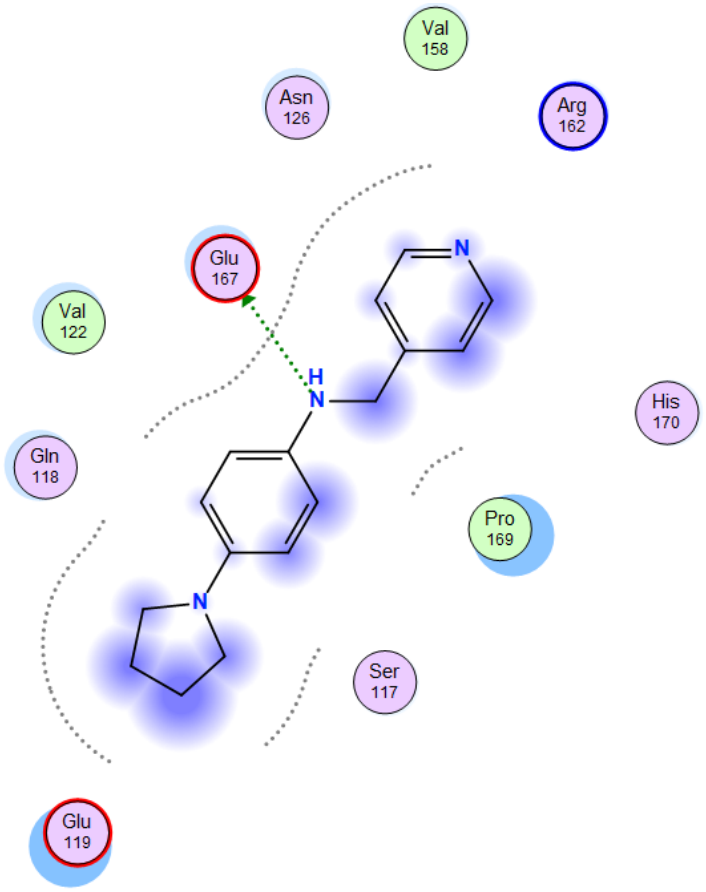
File Edit Display Compute Window Help SVL DBV MOE Cancel

	mol	rseq	mseq	S	rmsd	rmsd_refine	E_conf	E_place	E_sc
1	1087685	1	1	-5.2245	31.8559	2.0207	-21.2643	-52.4070	-7
2	1087685	1	1	-5.2229	31.8953	1.4026	-21.3303	-56.0397	-7
3	1087685	1	1	-5.1398	30.0399	1.2291	-20.9146	-47.0204	-7
4	1087685	1	1	-5.1094	31.9854	1.1513	-19.1667	-56.5847	-8
5	1087685	1	1	-5.1079	32.9078	1.6559	-19.7336	-53.7915	-7
6	1087685	1	1	-5.0130	30.7462	1.1695	-19.6123	-51.3568	-7
7	1087685	1	1	-4.9935	34.0132	1.8567	-15.8517	-55.5493	-8
8	1087685	1	1	-4.9813	32.6022	1.3722	-20.3066	-56.7351	-7
9	1087685	1	1	-4.9761	31.4409	2.3126	-20.1758	-50.8227	-7
10	1087685	1	1	-4.9564	31.6166	2.0501	-20.5946	-46.8090	-7
11	1087685	1	1	-4.9324	31.2390	2.0174	-21.4409	-46.9732	-7
12	1087685	1	1	-4.8921	30.8040	2.2614	-17.3607	-50.7861	-7
13	1087685	1	1	-4.8878	31.9322	0.7939	-19.8852	-60.2349	-7
14	1087685	1	1	-4.8399	33.6236	2.3172	-21.4713	-59.1381	-7
15	1087685	1	1	-4.7815	31.6037	1.6934	-20.0873	-58.1580	-7

(B)



(C)



For docking in MOE, a grid was set at Glu119/Glu167. (B) 3D and (C) 2D best pose for of BAS 07019774 interacting at Glu119/Glu167 site.

Residue numbering is as found in the Protein Data Bank for 2GLI. To convert to numbering for full length GLI1 (UNIPROY P08151) add 131.