Novel PROTACs Demonstrate Dual Kinase Inhibition and Degradation of HCK and BTK, and are Highly Active in MYD88 Mutated WM and ABC DLBCL cells

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INTRODUCTION

Activating mutations in MYD88 are prevalent in many B-cell malignancies, including Waldenström Macroglobulinemia (95-97%), primary CNS lymphoma (70-80%), ABC DLBCL (40%), marginal zone lymphoma (5-10%), and CLL (5-15%) (1, 2). Mutated MYD88 transcriptionally upregulates the SRC family member HCK, which in turn serves as a master signal for triggering multiple pro-survival cascades in mutated MYD88 lymphoma cells including BTK/NFKB, SYK, and ERK (3-5). In previous work, we developed and characterized a novel HCK kinase inhibitor, KIN-8194 (**Fig. 1A**) (6). KIN-8194 blocked both HCK and BTK and was active both in vitro and in vivo in MYD88 mutated xenograft lymphoma models, including BTKCys481Ser ibrutinib resistant models. Proteolysis targeting chimeras (PROTACs) represent a novel approach for blocking kinase based signaling and may provide an advantage over kinase inhibitors with greater selectivity and sustained target inhibition. We therefore initiated a medicinal chemistry campaign to develop highly potent, selective, and bio-available HCK/BTK targeting PROTACS for the treatment of MYD88 driven lymphomas (**Fig. 1B**).

AIM

To develop PROTACs targeting HCK/BTK for the treatment of MYD88 Mutated malignancies.

METHOD

We developed and characterized a large series of PROTACs. We rationally designed linkers to facilitate E3 ubiquitin ligase binding and improve physical properties leading to degraders with ideal oral pharmacokinetic properties and bioavailability. We characterized their kinome profiling by performing a KINOMEscan assay against a panel of 468 kinases (Fig. 2). Subsequently, we conducted rigorous biological efficacy screenings using the ATP-based Cell Titer-Glo Luminescent Cell Viability Assay to identify the most potent compounds. Compounds with IC50 scores lower than KIN-8194 were selected for further validation through flow cytometry based quantitative assays that verified apoptotic and cytotoxic effects on WM and MYD88-mutated lymphoma cell lines, as well as primary human peripheral blood mononuclear cells (PBMCs), utilizing Annexin V and Propidium iodide (PI) staining.

RESULTS

We identified and characterized several novel PROTACs that exhibited potent dual inhibition of HCK and BTK kinase activity at 1 nM, as well as marked degradation of HCK and BTK by western blot analysis at dose levels as low as 100 nM in MYD88 mutated WM and ABC DLBCL cell lines. Enhanced cell killing due to degradation of HCK and BTK versus inhibition alone was confirmed using respective PROTAC analogues with modified IMID warheads that do not bind cereblon. We observed suppression of downstream pro-survival ERK signaling in response to the dual HCK/BTK PROTACs (**Fig.1C**). Moreover, several HCK/BTK PROTACs (DFCI-002-05, -06) demonstrated remarkable low IC50 values (1-100 nM) in proliferation assays of MYD88 mutated BCWM.1 WM and TMD-8 ABC DLBCL cells (**Fig.1D**). KINOMEscan profiling showed greater selectivity for novel PROTACs (**Fig. 2**). Importantly, the dual HCK/BTK PROTACs elicited higher levels of apoptosis in TMD-8 ABC DLBCL cells in comparison to the native KIN-8194 kinase inhibitor (**Fig. 3**) and showed sparing of healthy donor B- and T-cells akin to KIN-8194. Finally, murine PK studies showed that the HCK/BTK PROTACs DFCI-005 and -006 were highly bioavailable with F=40-42%, and CMax of 700-2700 nM at single oral dose ranges of 10-60 mg/kg (**Fig.4**).

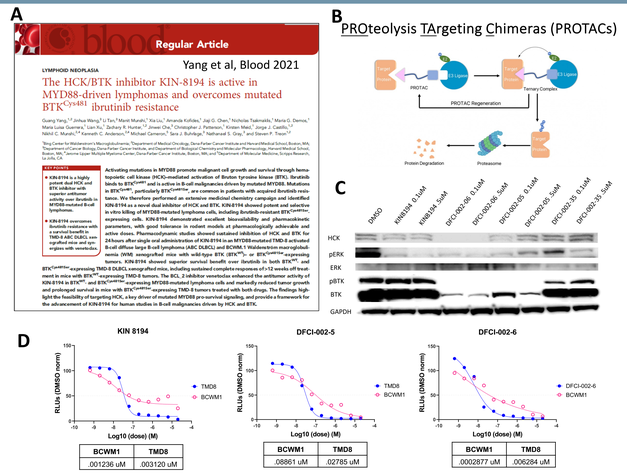

*Figure 1. Identification of PROTACs (DFCI-002-05, -06) targeting HCK and BTK. KIN-8194, which is a reported dual inhibitor of HCK and BTK (**A**) was used as activity reference. (**B**) Illustrate the design of PROTACs. (**C**) Degradation of HCK and BTK by PROTACs (DFCI-002-05, -06) as compared to control (DFCI-002-35). (**D**) IC50 of PROTACs compounds measured by CellTiter-Glo® 2.0 Cell Viability Assay.*

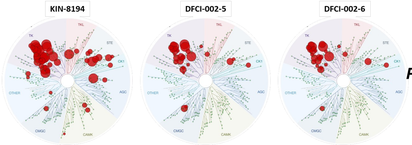

Figure 2. Kinome selectivity profile for KIN-8194, and HCK/BTK dual PROTACs DFCI-002-5 and -6. *Kinome selectivity was assessed against 468 kinases at 1 μM*

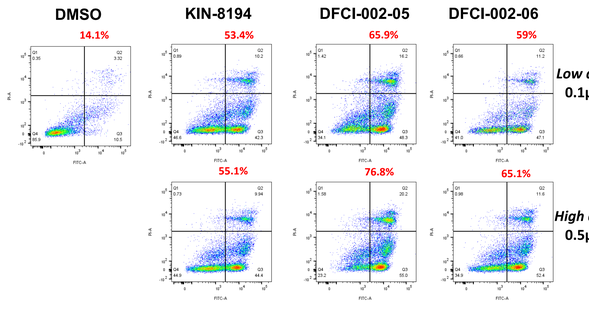

Figure 3. Novel HCK/BTK dual degraders induce increased apoptosis/death in TMD8 cells. *Cells were treated with low (0.1μM) and high (0.5μM) concentrations of PROTACs DFCI-002-05, DFCI-002-06, or KIN-8194 for 20 hours. Induced cell death and apoptosis were evaluated by Flow Cytometry with Annexin V-FITC and PI Staining.*

Individual and mean plasma concentration-time data of DFCI-002-5-02 after an IV dose at 2 mg/kg in male C57BL/6 mice

Dose (mg/kg)	Dose route	Sampling time (hr)	Concentration (ng/mL) Individual			Mean (ng/mL)	SD	CV(%)
2	IV	Pre-dose	BQL	BQL	BQL	**BQL**	NA	NA
		0.083	595	320	519	**481**	137	28.5
		0.25	801	196	386	**394**	203	51.4
		0.5	435	342	350	**375**	51.4	13.7
		1	419	248	303	**323**	87.4	27.0
		2	207	176	275	**219**	49.4	22.6
		4	207	104	276	**162**	93.6	57.8
		8	70.1	24.9	176	**90.8**	76.3	84.0
		24	29.7	39.0	54.9	**54.8**	15.3	28.1

PK parameters	Unit	Estimated Value
Req_adjusted		0.975
CL	L/hr/kg	0.446
V_{ss}	L/kg	6.34
$T_{1/2}$	hr	9.96
Regression Points	hr	3
AUC_{last}	hr*ng/mL	3761
AUC_{inf}	hr*ng/mL	4466
MRT_{last}	hr	7.23
MRT_{inf}	hr	11.9
Ct	ng/mL	531

Individual and mean plasma concentration-time data of DFCI-002-5-02 after a PO dose at 2 mg/kg in male C57BL/6 mice

Dose (mg/kg)	Dose route	Sampling time (hr)	Concentration (ng/mL) Individual			Mean (ng/mL)	SD	CV(%)
10	PO	Pre-dose	BQL	BQL	BQL	**BQL**	NA	NA
		0.083	5.73	19.5	40.3	**21.7**	17.3	79.4
		0.25	68.6	84.3	40.1	**64.3**	22.4	34.9
		0.5	173	120	175	**156**	35.4	16.6
		1	168	243	163	**191**	44.7	23.4
		2	205	518	509	**411**	179	43.6
		4	333	616	559	**502**	143	28.4
		8	540	540	548	**543**	4.58	0.84
		24	136	194	102	**144**	46.5	32.2

PK parameters	Unit	Estimated Value
Req_adjusted		0.999
T_{max}	hr	4.00
C_{max}	ng/mL	551.0 (0.00 (ub))
$T_{1/2}$	hr	15.2
Regression Points	hr	3
AUC_{last}	hr*ng/mL	7868
AUC_{inf}	hr*ng/mL	9991
MRT_{last}	hr	8.94
MRT_{inf}	hr	15.3
F	%	21.5

Individual and mean plasma concentration-time data of DFCI-002-5-02 after an PO dose at 30 mg/kg in male C57BL/6 mice

Dose (mg/kg)	Dose route	Sampling time (hr)	Concentration (ng/mL) Individual			Mean (ng/mL)	SD	CV(%)
30	PO	Pre-dose	BQL	BQL	BQL	**BQL**	NA	NA
		0.083	12.0	24.1	24.8	**20.9**	7.22	35.5
		0.25	133	67.8	94.1	**98.4**	33.0	33.5
		0.5	215	308	240	**254**	48.3	19.0
		1	292	325	382	**333**	45.3	13.6
		2	963	518	718	**733**	224	30.6
		4	956	1200	1360	**1170**	148	12.6
		8	1170	1100	1500	**1260**	206	16.4
		24	504	510	324	**413**	124	29.9

PK parameters	Unit	Estimated Value
Req_adjusted		0.999
T_{max}	hr	4.00
C_{max}	ng/mL	1800.4 (13.9 (ub))
$T_{1/2}$	hr	9
Regression Points	hr	3
AUC_{last}	hr*ng/mL	17664
AUC_{inf}	hr*ng/mL	29133
MRT_{last}	hr	10.1
MRT_{inf}	hr	24.7
F	%	41.8

Individual and mean plasma concentration-time data of DFCI-002-5-02 after a PO dose at 60 mg/kg in male C57BL/6 mice

Dose (mg/kg)	Dose route	Sampling time (hr)	Concentration (ng/mL) Individual			Mean (ng/mL)	SD	CV(%)
60	PO	Pre-dose	BQL	BQL	BQL	**BQL**	NA	NA
		0.083	13.8	10.5	24.8	**16.5**	7.07	44.8
		0.25	106	84.9	93.3	**94.8**	10.7	11.3
		0.5	247	194	184	**208**	36.3	17.7
		1	367	320	362	**350**	25.6	7.32
		2	813	640	638	**697**	100	14.4
		4	1163	723	505	**797**	346	43.1
		8	1170	1200	1500	**1290**	182	14.1
		24	470	398	561	**477**	81.5	17.1

PK parameters	Unit	Estimated Value
Req_adjusted		0.999
T_{max}	hr	4.00
C_{max}	ng/mL	915.0(0.00 (ub))
$T_{1/2}$	hr	NA
Regression Points	hr	NA
AUC_{last}	hr*ng/mL	16798
AUC_{inf}	hr	NA
MRT_{last}	hr	10.5
MRT_{inf}	hr	NA
F	%	NA

Individual and mean plasma concentration-time data of DFCI-002-6-02 after an IV dose at 2.61 mg/kg in male C57BL/6 mice

Dose (mg/kg)	Dose route	Sampling time (hr)	Concentration (ng/mL) Individual			Mean (ng/mL)	SD	CV(%)
2.61	IV	Pre-dose	BQL	BQL	BQL	**BQL**	NA	NA
		0.083	1380	1380	781	**1174**	357	30.4
		0.25	1410	1520	1300	**1410**	110	7.80
		0.5	1270	1170	1300	**1240**	69.3	5.59
		1	1250	1120	1300	**1220**	282	23.5
		2	1160	1360	1200	**1240**	100	7.94
		4	1170	920	941	**1017**	134	13.2
		8	826	717	188	**577**	341	59.0
		24	242	248	238	**243**	3.5	4.35

PK parameters	Unit	Estimated Value
Req_adjusted		0.943
Cl	L/hr/kg	0.140
V_{ss}	L/kg	1.97
$T_{1/2}$	hr	9.70
Regression Points	hr	3
AUC_{last}	hr*ng/mL	14421
AUC_{inf}	hr*ng/mL	17888
MRT_{last}	hr	7.66
MRT_{inf}	hr	13.5
Ct0	ng/mL	1174

Individual and mean plasma concentration-time data of DFCI-002-6-02 after a PO dose at 10 mg/kg in male C57BL/6 mice

Dose (mg/kg)	Dose route	Sampling time (hr)	Concentration (ng/mL) Individual			Mean (ng/mL)	SD	CV(%)
10	PO	Pre-dose	BQL	BQL	BQL	**BQL**	NA	NA
		0.083	34.1	40.0	79.5	**51.7**	22.9	42.7
		0.25	129	343	205	**226**	108	48.1
		0.5	341	1080	1050	**824**	412	50.0
		1	640	470	296	**535**	180	25.2
		2	347	1500	900	**916**	577	63.0
		4	1180	325	1300	**935**	532	56.9
		8	1450	978	1270	**1233**	238	19.3
		24	310	963	499	**924**	326	35.6

PK parameters	Unit	Estimated Value
Req_adjusted		NA
T_{max}	hr	8.00
C_{max}	ng/mL	1232.6(67.1(0.55 (ub))
$T_{1/2}$	hr	NA
Regression Points	hr	3
AUC_{last}	hr*ng/mL	7300
AUC_{inf}	hr*ng/mL	NA
MRT_{last}	hr	10.2
MRT_{inf}	hr	NA
F	%	39.1

Individual and mean plasma concentration-time data of DFCI-002-6-02 after an PO dose at 30 mg/kg in male C57BL/6 mice

Dose (mg/kg)	Dose route	Sampling time (hr)	Concentration (ng/mL) Individual			Mean (ng/mL)	SD	CV(%)
30	PO	Pre-dose	BQL	BQL	BQL	**BQL**	NA	NA
		0.083	41.6	15.5	70.5	**42.5**	27.5	64.7
		0.25	385	218	296	**297**	41.6	15.0
		0.5	427	479	251	**386**	119	31.0
		1	925	1020	971	**989**	27.2	2.75
		2	1900	1570	1470	**1647**	225	13.7
		4	1510	1500	1050	**1353**	263	19.4
		8	2390	1610	2360	**2120**	442	20.8
		24	1430	1980	1000	**1470**	345	23.4

PK parameters	Unit	Estimated Value
Req_adjusted		NA
T_{max}	hr	8.00
C_{max}	ng/mL	2129.2 (0.00 (ub))
$T_{1/2}$	hr	NA
Regression Points	hr	3
AUC_{last}	hr*ng/mL	41212
AUC_{inf}	hr*ng/mL	NA
MRT_{last}	hr	11.9
MRT_{inf}	hr	NA
F	%	24.9

Individual and mean plasma concentration-time data of DFCI-002-6-02 after a PO dose at 60 mg/kg in male C57BL/6 mice

Dose (mg/kg)	Dose route	Sampling time (hr)	Concentration (ng/mL) Individual			Mean (ng/mL)	SD	CV(%)
60	PO	Pre-dose	BQL	BQL	BQL	**BQL**	NA	NA
		0.083	239	227	118	**195**	67.1	34.3
		0.25	304	494	533	**444**	123	27.6
		0.5	941	1080	1050	**1017**	73.4	6.92
		1	1530	1450	543	**1174**	549	46.7
		2	2000	2000	1540	**1847**	290	14.4
		4	1900	2300	2190	**2130**	207	9.70
		8	1900	3000	2100	**2333**	588	25.3

PK parameters	Unit	Estimated Value
Req_adjusted		NA
T_{max}	hr	8.00
C_{max}	ng/mL	2130 (2.00 (ub))
$T_{1/2}$	hr	NA
Regression Points	hr	3
AUC_{last}	hr*ng/mL	43422
AUC_{inf}	hr*ng/mL	NA
MRT_{last}	hr	11.9
MRT_{inf}	hr	NA
F	%	52.6

Figure 4. Pharmacokinetics of PROTACs DFCI-002-05 (upper panel), and DFCI-002-06 (bottom panel).

CONCLUSIONS

In these studies, we demonstrated the development and characterization of novel, dual HCK/BTK PROTACs that demonstrate potent and selective kinase inhibition and protein degradation of HCK and BTK. Importantly, the novel PROTACs showed enhanced apoptosis of MYD88 mutated malignant cells over the native kinase inhibitor KIN-8194. Lastly, novel HCK/BTK PROTACs exhibited high levels of bioavailability. Our studies therefore provide a framework for the advancement of dual HCK/BTK PROTACs for the treatment of MYD88 mutated lymphomas.

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