ARQ 531, a Novel, Oral, Non-Covalent Inhibitor of Wild Type and C481S Mutant BTK with Potent Anti-Tumor Activity

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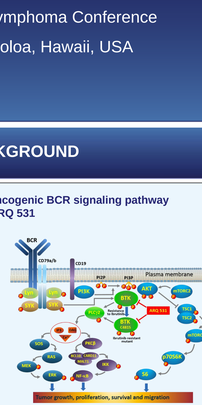
BACKGROUND

Targeting ibrutinib resistant oncogenic BCR signaling pathway by ARQ 531

Bruton tyrosine kinase (BTK) is a key component of the B-cell receptor (BCR) signaling cascade and has emerged as a critical target in the treatment of B-cell malignancies. Although ibrutinib has demonstrated great response in patients with elevated BCR signaling, resistance has been observed and the BTK-C481S mutation that prevents covalent binding of ibrutinib to BTK appears to be a predominant resistance mechanism. An alternative mechanism of resistance can be via activation of the the PI3K/AKT pathway[1,2]. Here we report development and characterization of a potent reversible inhibitor, ARQ 531, capable of inhibiting activation of both wild type and the C481S mutant of BTK kinase with similar potency



MATERIALS AND METHODS

BTK biochemical assay
Biochemical inhibition assay was measured using full length BTK constructs of wild type or C481S mutant (Reaction Biology). Kinase profiling was performed by Millipore and Carna Biosciences. Profiling on Millipore's 236 kinases identified 45 kinases with >50% inhibition at 200 nM concentration of ARQ 531. Subsequently, the potency of this ATP competitive inhibitor was determined on such kinases at the physiological 1mM ATP concentration

Cell culture
TMD8 cells were obtained from Tohda at the Tokyo Medical and Dental University (TMDU). Cells were grown in RPMI-1640 supplemented with 10% FBS and maintained at 37°C in a humidified atmosphere at 5 % CO_2

Cell growth assay
Cells were seeded in 96-well tissue-culture treated plates and treated with compounds or vehicle for 72-hours. CellTiter-Glo reagent (Promega) was added, and plates were incubated with gentle shaking for 2 minutes. The reaction was allowed to proceed for 10 additional minutes, and then plates were read on a Victor® microplate reader (Perkin Elmer). GI_{50} values were determined using a four-parameter logistic curve fit

Cell cycle analysis
TMD8 cells ($1x10^6$) were seeded into 6-well plate O/N and added with compounds at designated concentrations for 24 and 48 hours. Cells were processed based according to the instruction from Cycletest Plus Reagent kit (BD Biosciences) and cell cycle was analyzed in FACS Calibur Flow Cytomter

Reverse phase protein assay (RPPA)
TMD8 cells were grown in 6-well plates, and treated with 0.3 μM or 3 μM ARQ 531 or DMSO vehicle for 2 hrs. Cells were then lysed following the RPPA procedure provided by Carna Bio Science, and protein concentration was determined by Bradford. Samples were shipped to Carna for analysis of phospho-protein expression. Data were normalized to the control samples and relative values for individual targets were grouped in their assigned pathways

Animal care
Six week old female CB-17 SCID mice were purchased from Taconic Farms, Germantown, NY and allowed to acclimate 1-2 weeks. Mice were housed in sterile micro isolator cages, five mice per cage and receive food and *water ad libitum*. All experimental procedures were approved in accordance with ArQule's Institutional Animal Care and Use Committee (IACUC)

Formulations
Dosing volume for all groups was 10ml/kg or 0.1ml/10g body weight, thus if animal weighs 22g it would be administered 220ul of dosing solution

Tumor model
Femal SCID mice were implanted subcutaneously with $8x10^6$ TMD8 cells in 0.2ml sterile Hanks Balanced Salt Solution (HBSS) with 50% standard concentration BD matrigel in the upper right flank area. Mice were monitored and staged on day 14 (post injection of tumor cells) when size reached approximately 400mg. Oral daily dosing with ARQ 531 at 100 mg/kg, vinblastine or vehicle began on stage day. Tumor measurements and body weights were collected three times a week with electronic calipers and balance. Tumor weight (mg) was calculated from the equation length x $(width)^2/2$. This formula can also be used to calculate tumor volume assuming unit density 1mg=1mm^3

***In vivo* PD analysis**
Tumor samples were pulverized in liquid nitrogen and lysed in cell lysis buffer with phosphatase and protease inhibitors. Lysates were collected and heated at 95°C for 5 minutes with Laemmli buffer. Proteins were resolved on Novex 4-12% Tris-Glycine gels and then transferred to nitrocellulose membranes using XCell SureLock® Mini-Cell at 30v for 1.5 hr. Membranes were blocked with 5% milk in TBS-Tween20 at RT for 1 hr and then blotted with primary antibodies with 1% BSA in TBS-Tween20 overnight at 4°C and secondary antibodies at RT for 1 hr. Membranes were incubated with GE ECL Prime reagent for 2 min and images were captured in FUJI LAS3000 Image System. Antibodies used in this study were anti-pBTK(Y223) and anti IgG antibodies. Ratio of pBTK(Y223)/Actin was determined using densitometry analysis. The percentage of vehicle group was designated as 100%

ADME and pharmacokinetics studies
CYP450 inhibition and P-gp substrate/inhibition potential for ARQ 531 as well as pharmacokinetics studies in monkeys and dogs were determined and analyzed by Covance Laboratories Inc

REFERENCES

[1]Woyach JA, Furman RR, Liu TM, et al. Resistance mechanisms for the Bruton's tyrosine kinase inhibitor ibrutinib. N Engl J Med. 2014 Jun 12;370(24):2286-94
[2]Woyach JA, Johnson AJ. Targeted therapies in CLL: mechanisms of resistance and strategies for management. Blood. 2015 Jul 23;126(4):471-7

RESULTS

Binding mode of ARQ 531 with BTK

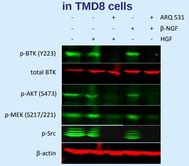
ARQ 531 is a reversible non-covalent ATP competitive inhibitor and does not require the C481 residue to bind to BTK

In vitro characterization of ARQ 531 on wild type and mutant BTK

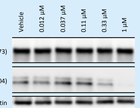
Inhibitor	Biochemical Assay		Transfected in HEK-293 Cells		Fold ratio between C481S and WT pBTK	TMD8 WT-pBTK IC_{50} (nM)
	WT-BTK IC_{50} (nM)	C481S-BTK IC_{50} (nM)	WT-BTK pBTK IC_{50} (nM)	C481S-BTK pBTK IC_{50} (nM)		
ARQ 531	0.85	0.39	490	790	**1.6**	11
Ibrutinib	0.037	9.03	6	1100	**183**	0.5

ARQ 531 is a potent BTK inhibitor in biochemical and in cell based assays and inhibits with similar potency both the wild type and the ibrutinib resistant C481S mutant. ARQ 531 shows potent anti-proliferative activity in BTK-dependent TMD8 cell line

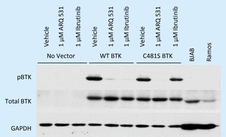
ARQ 531 effects on BTK, AKT, MEK, SRC, in TMD8 cells



ARQ 531 effects on AKT and ERK in AN3CA cells (BCR-, PIK3CAmut, PTEN-)



ARQ 531 Inhibition of WT and C481S mutated BTK in HEK- 293 cells



ARQ 531 inhibits the RAF/MEK/ERK and the PI3K/AKT/mTOR pathways with no direct activity on PI3K

In vitro kinase profiling

Kinase	IC_{50} (nM) ARQ 531	Kinase	IC_{50} (nM) ARQ 531	
BRK	2.45	ACK	146	
Lck	3.86	PAX2	190	
Yes	4.22	Fms	207	
BTK	4.23	FGFR2	218	Trk family
BMX	5.23	IRR	221	
TEC	5.80	INSR	221	
Blk	9.71	EGFR	290	
Trk3	11.7	FGFR1	313	
TrkA	13.1	KDR	316	
Hck	18.3	SIK	340	
LYNa	18.8	FLT1	374	
TrkC	19.1	FGFR3	403	
FGR	25.9	Fes	420	Src family
Tie2	29.4	MST1	509	
Fyn	32.2	MEK1	599	
RAF1	34.7	IGF1R	687	
TXK	36.4	PYK2	3000	Tec family
CSK	45.4	Arg	3229	
FRK	48.0	Aurora-B	10589	
Src	57.7	Abl	10684	
RET	110	ITK	>10000	
FLT4	113			

ARQ 531 potently inhibits Tec, Src and Trk family kinases

Reverse Phase Protein Array (RPPA) analysis in TMD8 cells

Pathways and targets activated (increase in phosphorylation)

Group	ARQ 531 tested at 0.3 μM	Normalized Value
BTK	p-EGF Receptor (Tyr992)	0.32
PAM	p-mTOR (Ser2448)	0.84
PAM	p-PDK1 (Ser241)	0.45
PAM	p-Tuberin/TSC2 (Ser939)	0.44
Angiogenesis	p-eNOS (Ser1177)	0.43
Cell cycle	p-Rb (Ser807/811)	0.36
Cytoskeleton	p-VASP (Ser239)	0.40
Cytoskeleton	p-Cofilin (Ser3)	0.34
DNA damage	p-CHK2 (Ser19/35)	0.51
DNA damage	p-CHK1 (Ser19)	0.37

Group	ARQ 531 tested at 3.0 μM	Normalized Value
BTK	p-EGF Receptor (Tyr1068)	0.11
RTK	p-PKCθ (Thr538)	0.09
Ras-Raf	p-CREB (Ser133)	0.11
PAM	p-mTOR (Ser2448)	0.75
PAM	p-PDK1 (Ser241)	0.27
Cytoskeleton	p-Cofilin (Ser3)	0.20
Energy homeostasis	p-Acetyl-CoA carboxylase (Ser79)	0.12
Forkhead	p-FoxO3a (Ser318/321)	0.11
TGF-β	p-TAK1 (Thr184/187)	0.10
DNA damage	p-ATM (Ser1981)	0.08

Pathways and targets inhibited (decrease in phosphorylation)

Group	ARQ 531 tested at 0.3 μM	Normalized Value
mRTK	p-Src (Tyr416)	0.18
Ras-Raf	p-p44/42 MAP kinase (Thr202/Tyr204)	0.39
Ras-Raf	p-Mek1 (Thr197/202)	0.06
Apoptosis	p-NDRG1 (Thr346)	0.16
Apoptosis	p-Bcl-2 (Ser70)	0.06
Cell cycle	p-Histone H3 (Thr11)	0.13
DNA damage	p-CHK2 (Thr68)	0.10
DNA damage	p-p53 (Ser6)	0.08
Mitosis marker	p-TACC3 (Ser558)	0.10
SAPK/JNK	p-SEK1/MKK4 (Thr261)	0.08

Group	ARQ 531 tested at 3.0 μM	Normalized Value
RTK	p-EGF Receptor (Tyr992)	0.07
RTK	p-SHP2 (Tyr1135)	0.40
RTK	p-VEGF Receptor 2 (Tyr1175)	0.32
RTK	p-HER2/ErbB2 (Tyr1221/1222)	0.37
Insulin receptor	p-IGF-1 Receptor (Tyr1131)/Insulin Receptor (Tyr1146)	0.34
mTOR	p-Src (Tyr416)	0.71
Ras-Raf	p-Mek1 (Thr197/202)	0.34
Ras-Raf	p-p44/42 MAP kinase (Thr202/Tyr204)	0.07
Apoptosis	p-NDRG1 (Thr346)	0.78
Cell cycle	p-Rb (Ser780)	0.38

Decrease in phosphorylation +1 0 -1 Increase in phosphorylation

ARQ 531 inhibits pEGFR at 3 μM concentration (but not at 0.3 μM), PI3K/AKT/mTOR (PAM) and Ras/Raf/MEK pathways

Cell cycle analysis in TMD8 cells

Inhibitor	Time (hr)	% Cells in cell cycle G1	S	G2/M	Blockage
Ctrl	24	69.6	25.2	5.2	
	48	75	20.4	4.7	
ARQ 531 (1μM)	24	91.1	9	0	G1
	48	95	5	0.08	G1
Ibrutinib (0.1μM)	24	90.9	8.5	0.5	G1
	48	95	4.7	0.35	G1
Staurosporine (1μM)	24	45.5	44.1	10.5	S, G2/M
	48	54.8	33.6	11.6	S, G2/M
Vinblastine (1μM)	24	29.2	45.7	25.2	S, G2/M
	48	32.9	36.1	31	S, G2/M

LDH cytotoxicity assay

Inhibitor	% LDH release at 33.33 μM			
	NIH-3T3	AsPC1	A2780	TMD8
ARQ 531	-2.3	6.5	2.8	3.9
Staurosporine	54.7	39.5	69.5	25.1

ARQ 531 does not induce significant LDH release after 18 hrs at the concentration range tested in the MTS assay

ARQ 531 causes arrest in G1 cell cycle phase, similar to ibrutinib, at both 24 and 48 hrs. This mechanism of cell growth inhibition is distinct from promiscuous kinase inhibitor staurosporine and cytotoxic agent vinblastine, as both caused S and G2/M arrest

Overall, cell cycle and LDH analyses suggest no cytotoxic effect by ARQ 531

Anti-proliferative activity of ARQ 531 in hematological malignant cell lines

Origin	Cell line	ARQ 531 GI_{50} (μM)	Ibrutinib GI_{50} (μM)	Known mutations/alterations
DLBCL	SU-DHL-6	0.079	0.78	EZH2 t(14;18), BCL2
follicular DLBCL	DOHH-2	0.1035	0.1865	CDKN2A, p14
DLBCL	TMD8	0.13	0.0017	ABC-DLBCL, HES1
AML	OCI-AML2	0.139	10.3	unknown
MCL	REC-1	0.18	0.00055	Notch1, INK4a, ATM, MHC2TA, PI3K
follicular DLBCL	SU-DHL-4	0.2	1.07	constitutive PI3K/AKT pathway
AML	MV-4-11	0.54	0.66	FLT3, TRKC
ALL	Molt-4	0.695	2.45	Notch1, NRAS, PIK3RA, PTEN, TP53
CML	K-562	1.435	4.705	HER3, FGFR4, RON, TP53, Notch1, TRKC
AML	KG-1	2.1	3.3	TP53, ALK, FGFR1, FGFR2, FLT3, RON, Notch2
AML	HL-60	2.8	21	NRAS, TP53
CLL	EHEB	2.9	4.9	unknown
MCL	Z-138	3	13.3	High expression of AKT
T-cell leukemia	HH	3.67	7.38	TP53
T-cell leukemia	Jurkat	7.5	9.1	ALK, HER2, VEGFR1, FAK, SYK, ITK, TEC, PTEN
MCL	MAVER-1	7.61	11.1	TP53, ATM, MYC
AML	HEL	8.8	14	TP53, JAK2
B-cell lymphoma	RL	10.6	2.54	EZH2, TP53 t(14;18)
AML	HEL-92-1-7	12.1		JAK2
Burkitt's lymphoma	Namalwa	14.1	21.2	TLR9, TNF, IL-6, and IL-10
Burkitt's lymphoma	EB-3	14.7		Myc
Burkitt's lymphoma	RAMOS-RA-1	15	12	unknown
myeoloma	NCI-H929	16	2.49	active RSK2-NTKD and AKT
DLBCL	DB	21		E2H2, TP53
Burkitt's lymphoma	Raji	26.9	22.6	c-Abl
Burkitt's lymphoma	P3-HR-1	33.4	14.7	constitutive PI3K/AKT pathway

ARQ 531 inhibits proliferation of diverse types of hematological cancer cells and potently inhibits cell lines that are addicted to BCR, PI3K/AKT, Notch signaling pathways

Effects on TNFα production

Inhibitor	IC_{50} (μM)
ARQ 531	0.156
Ibrutinib	3.99

ARQ 531 inhibits Fcγ-mediated TNFα production in peripheral blood mononuclear cells (PBMC) monocytes

Pharmacokinetics and ADME of ARQ 531

P-gp test in Caco-2 monolayers

Inhibitor	P-gp Substrate classification	P-gp Inhibition classification
ARQ 531	Negative	Positive

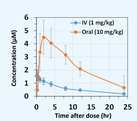
CYP 450 test in human liver microsomes

Cyp Isoform	IC_{50} (μM) 1A2	2C8	2C9	2C19	2D6	3A-M	3A-T
ARQ 531	>100	23.7	14.1	19.9	32.3	>100	>100

ARQ 531 is not a P-gp substrate and is not significantly degraded when incubated with human recombinant CYP enzymes

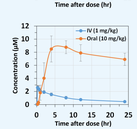
Mean pharmacokinetic parameters in plasma collected from male dogs after a single intravenous or oral dose of ARQ 531

Dose route and dosage	C_{max} (μM)	T_{max} (hours)	AUC_{0-t} (μM.hour)	$t_{1/2}$ (hours)	F (%)
IV (1mg/kg)	1.65	0.306	13.80	9.27	N/A
Oral (10mg/kg)	5	2.67	55	7.1	38

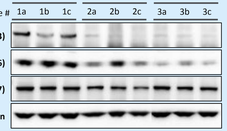


Mean pharmacokinetic parameters in plasma collected from male monkeys after a single intravenous or oral dose of ARQ 531

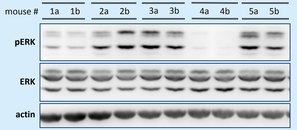
Dose route and dosage	C_{max} (μM)	T_{max} (hours)	AUC_{0-t} (μM.hour)	$t_{1/2}$ (hours)	F (%)
IV (1mg/kg)	2.71	0.167	23.80	13.6	N/A
Oral (10mg/kg)	9	6.67	173	N/A	72.4



In vivo target and pathway inhibition by ARQ 531 in TMD8 tumor xenograft model

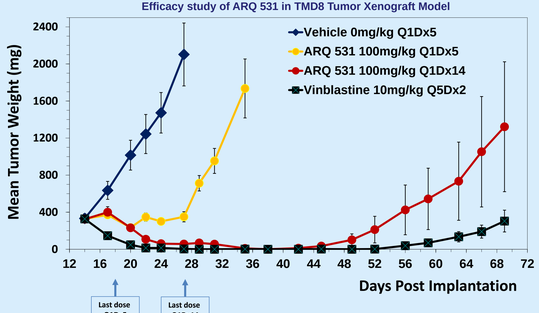


Tumors were removed 6 hours after a single oral dosing of ARQ 531 at 100mg/kg. Western blot analysis was performed to assess the phosphorylation levels of BTK, S6 and PLCγ2



Tumors were removed 2 or 24 hours after consecutive daily oral dosing of ARQ 531 at 100mg/kg. Western blot analysis was performed to assess the phosphorylation levels of ERK and pERK

Efficacy study of ARQ 531 in TMD8 Tumor Xenograft Model



ARQ 531 dosed for 14 days in mice bearing 400mg tumors caused long-lasting complete tumor regressions with tumors reaching 1000mg 38 days after dosing interruption and 46 days later than in the vehicle group

CONCLUSIONS

- **ARQ 531 is a potent reversible non-covalent inhibitor of BTK, inhibiting both the wild type and ibrutinib resistant BTK-C481S mutant with similar potency**
- **ARQ 531 has distinct kinase selectivity profile with strong inhibitory activity against several key oncogenic drivers from TEC, Trk and Src family kinases, inhibiting also the RAF/MEK/ERK and the PI3K/AKT/mTOR pathways, the latter related to ibrutinib resistance**
- **ARQ 531 potently suppresses cell proliferation of hematological malignancies *in vitro*, with B-Cell Receptor signaling inhibition**
- **ARQ 531 has high oral bioavailability, good ADME, pharmacokinetic and metabolic properties**
- **In the BTK driven TMD8 xenograft mouse model, ARQ 531 demonstrates strong *in vivo* target and pathway inhibition with sustained tumor growth inhibition when treatments are initiated at established tumor weights (400 mg)**
- **These results warrant further preclinical and clinical investigation of ARQ 531, particularly in the setting of ibrutinib-resistance**