Targeting Ibrutinib-Resistant BTK-C481S Mutation with ARQ 531, a Reversible Non-Covalent Inhibitor of BTK

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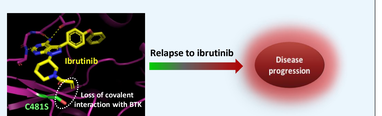
BACKGROUND

Targeting ibrutinib resistance mechanism

The B-cell receptor (BCR) signaling pathway is a central determinant of B-cell fate and function and Bruton tyrosine kinase (BTK) is a critical component of this signaling cascade. BTK, a nonreceptor tyrosine kinase, plays a significant role in B-cell development and is a unique therapeutic target in B-cell malignancies. Targeting BTK with the irreversible, inhibitor ibrutinib achieved an impressive objective response rate in patients with CLL. However, disease progression occurs more frequently in these patients with high-risk genomic features either shortly after the start of ibrutinib therapy or later with progressive CLL. Disease progression is often associated with resistance to ibrutinib with acquired BTK mutation on C481 residue which prevent covalent binding of ibrutinib to BTK. Here we present discovery and preclinical development of potent reversible BTK inhibitor, ARQ 531, capable of inhibiting activation of both wild-type and the C481S mutant of BTK and subsequent downstream oncogenic signaling in various hematological malignancies.



Relapse to ibrutinib → Disease progression

MATERIALS AND METHODS

BTK biochemical and kinase selectivity 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
In vitro PD assay
Cells were treated with increasing concentrations of inhibitors in SUDHL-4 for 2 hours, following stimulation with either anti-IgM or growth factors cells were lysed for Western blot analysis
BTK human PBMC functional assay
Fresh blood was collected from donors and PBMC was isolated using lymphoprep density gradient protocol. CD69 expression on CD20⁺ B-cells was assayed with increasing concentrations of ARQ 531 followed by IgM stimulation. The percentage of CD69 expression levels on CD20⁺ B-Cells was determined by flow cytometry analysis to estimate the potency of ARQ 531
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). Glo-values were determined using a four-parameter logistic curve fit
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)
Tumor model
Female SCID mice were implanted subcutaneously with 8x10⁶ 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 and the tumor volume was calculated by assuming unit density 1mg equals to 1mm³
In vivo PD analysis
In vivo Target and pathway inhibition was studied in mouse TMD8 xenograft model. Following oral dosing of BTK inhibitors tumor tissues and plasma were collected for Western blot analysis of phosphorylated proteins and ARQ 531 levels in plasma. Percent inhibition relative to the vehicle control was determined using densitometry analysis and the intensity of actin band was used as a loading control and the percentage of vehicle group was designated as 100%.
Collagen-induced arthritis model
DBA1/J mice were immunized with collagen to develop the arthritis, following the onset of arthritis, mice were randomized into treatment groups. Treatment was initiated by oral dosing of ARQ 531 at 25, 50 and 75 mg/kg and continued daily (QD at 24-hour intervals) through arthritis day 14. Clinical scores were assessed for each of the paws on study arthritis days 1–15. Dexamethasone at 3 mg/kg was used as a control to monitor inhibition of disease symptoms
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

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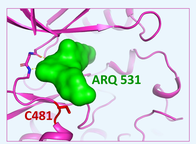
RESULTS

ARQ 531 is a reversible non-covalent inhibitor of BTK



Binding mode of ARQ 531 modeled based on the co-crystal structure of ARQ 531 analogue. Unlike ibrutinib ARQ 531 do not require C481 residue for binding to BTK

ARQ 531 potently inhibits both wild-type and the C481S mutant BTK

Inhibitor	Biochemical Assay		Transfected in HEK-293 Cells		Fold ratio between C481S and WT pBTK	WT-pBTK in TMD8 EC_{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	240	395	1.6	11
Ibrutinib	0.037	9.03	13	1700	126	0.5

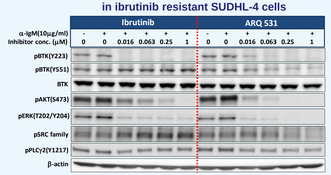
ARQ 531 is a potent inhibitor of BTK in biochemical and in cell based assays and inhibits with equipotency both the wild-type and the ibrutinib resistant C481S mutant. ARQ 531 shows strong target inhibition in TMD8 cell line

Kinase profiling of ARQ 531

Kinase	ARQ 531 IC_{50} (nM)
BRK	2.45
LCK	3.86
YES	4.22
BMX	5.23
TEC	5.80
BLK	9.71
TrkB	11.7
TrkA	13.1
HCK	**18.3**
LYNa	18.8
TrkC	19.1
FGR	25.9
Tie2	29.4
FYN	32.2
RAF1	34.7
TXK	36.4
CSK	45.4
FRK	48.0

Additional kinases that show strong inhibition (IC_{50} <50nM) are shown here. ARQ 531 demonstrates distinct kinase selectivity than ibrutinib. ITK is a TEC family kinase and showed very weak inhibition (IC_{50} >10 µM). Highlighted are TEC (green), SRC (yellow) and TRK (blue) family kinases.

ARQ 531 suppresses BTK signaling pathways in ibrutinib resistant SUDHL-4 cells



ARQ 531 is highly effective in inhibiting the upstream BTK activation step of Tyr551 residue phosphorylation by SRC family kinase (SFK) as well as the downstream signaling mediated by PI3K/AKT/mTOR and Raf/MEK/Erk pathways.

ARQ 531 inhibits activation of B-cells followed by CD69 expression on CD20⁺ B-cells

Inhibitor	IC_{50} (nM)
ARQ 531	42
Ibrutinib	<3

ARQ 531 inhibits Fcγ-mediated TNFα production in human PBMCs

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

RPPA analysis show suppression of BCR addict survival signaling by AQR 531 in TMD8 cells

Targets phosphorylation increased

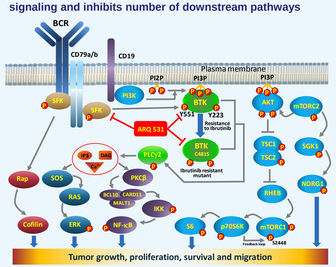
Group	ARQ 531 tested at 0.3 µM	Normalized Value	Group	ARQ 531 tested at 3.0 µM	Normalized Value
PI3K/AKT/mTOR	p-mTOR (Ser2448)	0.74	PI3K/AKT/mTOR	p-mTOR (Ser2448)	0.75
DNA damage	p-CHK2 (Ser19/33)	0.51	PI3K/AKT/mTOR	p-PDK1 (Ser241)	0.27
PI3K/AKT/mTOR	p-PDK1 (Ser241)	0.45	Cytoskeleton	p-Cofilin (Ser3)	0.20
PI3K/AKT/mTOR	p-Tuberin(T562) (Ser939)	0.44	Energy homeostasis	p-Acetyl-CoA carboxylase (Ser79)	0.12
Angiogenesis	p-eNOS (Ser1177)	0.43	Ras/Raf/MEK/ERK	p-CREB (Ser133)	0.11
Cytoskeleton	p-VASP (Ser239)	0.40	RTK	p-EGF Receptor (Tyr1068)	0.11
DNA damage	p-CHk2 (Ser19)	0.37	Forkhead	p-FoxO1a (Ser318/321)	0.11
Cell cycle	p-Rb (Ser807/811)	0.4	TGF-β	p-TAK1 (Thr184/187)	0.1
Cytoskeleton	p-Cofilin (Ser3)	0.3	RTK	p-PKCδ (Thr538)	0.1
RTK	p-EGF Receptor (Tyr992)	0.3	DNA damage	p-ATM (Ser1981)	0.1

Targets phosphorylation decreased

Group	ARQ 531 tested at 0.3 µM	Normalized Value	Group	ARQ 531 tested at 3.0 µM	Normalized Value
Ras/Raf/MEK/ERK	p-ERK1/2 (Thr202/Tyr204)	-0.39	Ras/Raf/MEK/ERK	p-ERK1/2 (Thr202/Tyr204)	-1.00
mTOR	p-Src (Tyr416)	-0.18	mTOR	p-Src (Tyr416)	-0.81
Apoptosis	p-NDRG1 (Thr346)	-0.16	Apoptosis	p-NDRG1 (Thr346)	-0.78
Cell cycle	p-Histone-H3 (Thr11)	-0.13	RTK	p-EGF Receptor (Tyr992)	-0.54
Mitosis marker	p-TACC3 (Ser558)	-0.10	Phosphatase	p-SHP2 (Tyr1105)	-0.40
DNA damage	p-CHk2 (Thr68)	-0.10	Cell cycle	p-Rb (Ser780)	-0.38
SAPK/JNK	p-SEK1/MAK4 (Thr261)	-0.08	RTK	p-HER2/ErbB2 (Tyr1221/1222)	-0.37
DNA damage	p-p53 (Ser6)	-0.1	RTK	p-IGF-I Receptor (Tyr1131)/Insulin Receptor (Tyr1146)	-0.3
Apoptosis	p-Bcl-2 (Ser70)	-0.1	Ras/Raf/MEK/ERK	p-Mnk1 (Thr197/202)	-0.3
Ras/Raf/MEK/ERK	p-Mnk1 (Thr197/202)	-0.1	RTK	p-VEGF Receptor 2 (Tyr1175)	-0.3

ARQ 531 selectively inhibits BCR signaling dependent PI3K/AKT/, Ras/Raf/MEK and Rap-GTPase-Cofilin pathways in TMD8 cells. Targets sensitive at both concentrations (0.3 µM and 3 µM) of ARQ 531 are highlighted in yellow and exhibit inhibitory effect on downstream signaling.

ARQ 531 targets oncogenic BCR addicted and ibrutinib resistant signaling and inhibits number of downstream pathways



SFK: Src Family Kinase

ARQ 531 inhibits proliferation of malignant cells both sensitive and resistant to ibrutinib

Origin	Cell line	ARQ 531 GI_{50} (µM)	Ibrutinib GI_{50} (µM)	BTK Gene or pBTK expression
GCB-DLBCL	SUDHL6	0.079	0.78	9.519
ABC-DLBCL	TMD8	0.13	0.0017	High*
AML	OCIAML2	0.139	10.3	9.963
GCB-DLBCL	DOHH2	0.16	0.4	10.248
MCL	REC1	0.18	0.00055	10.056
GCB-DLBCL	SUDHL4	0.2	1.07	10.349
AML	Eol1	0.395	1.05	9.800
AML	MV411	0.54	0.66	9.704
AML	BDCM	0.623	3.08	9.943
ALL	Molt4	0.695	2.02	5.008
AML	SKM1	0.978	30.9	9.697
CML	K562	1.435	4.705	8.370
AML	KG1	2.1	3.3	9.403
Myeloma	THP1	2.475	26.375	9.164
Myeloma	NCIH929	2.49	16	5.821
AML	HL60	2.8	21	9.861
CLL	EHEB	2.9	4.9	9.918
MCL	Z138	3	13.3	moderate*
Burkitt's lymph.	Daudi	3.05	3.32	10.209
Myeloma	U266B1	3.2	13	5.594
T-cell lymphoma	HH	3.67	7.38	5.282
T-cell lymphoma	Jurkat	7.5	9.1	4.724
MCL	MAVER1	7.6	11.1	moderate*
AML	HEL	8.8	14	10.772
AML	HEL9217	12.1		10.350
Burkitt's lymph.	NAMALWA	14.1	21.2	9.836
Burkitt's lymph.	EB3	14.7		8.566
Burkitt's lymph.	RAMOS	15	12	moderate*
AML	U937	17.45	13.05	10.301
GCB-DLBCL	DB	21		7.732
Burkitt's lymph.	Raji	26.9	22.6	10.077
Burkitt's lymph.	P3HR1	33.4	14.7	10.119

Gene expression scale: low — High

Cell lines sensitive to ibrutinib

* Expression by Western blot analysis of pBTK

ARQ 531 inhibits proliferation of diverse types of cell lines and shows potency in cell lines that are addict to BCR, Src-family kinase and PI3K/AKT pathways.
Gene expression data was extracted from Cancer Cell Line Encyclopedia (CCLE) database [Link to URL: http://www.broadinstitute.org/ccle
Reference: Barretina, Caponigro, Stransky et al. (2012) The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. Nature. 483:603-7]

P-gp substrate and inhibition potential of ARQ 531 using Caco-2 monolayers

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

ARQ 531 Inhibition of CYP 450 potential in human liver microsomes

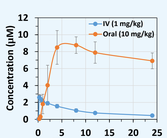
Cyp Isoform	IC_{50} (µM)						
	1A2	2C8	2C9	2C19	2D6	3A-M	3A-T
ARQ 531	>100	23.7	14.1	19.9	32.3	>100	>100

CYP phenotyping: No significant degradation of ARQ 531 was observed when incubated with human recombinant CYP enzymes

Pharmacokinetics of ARQ 531 in monkeys and dogs

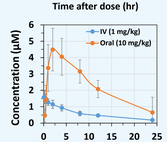
Mean pharmacokinetic parameters in plasma collected from male monkeys following a single intravenous or oral administration of ARQ 531

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

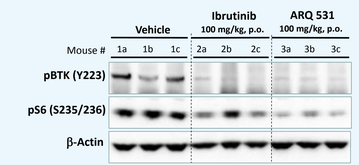


Mean pharmacokinetic parameters in plasma collected from male dogs following a single intravenous or oral administration of ARQ 531

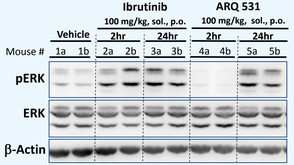
Dose route and dosage	C_{max} (µM)	T_{max} (hours)	AUC_{0-t} (µM.hour)	$t_{1/2}$ (hours)	F (%)
IV (1mg/kg)	1.65	0.31	13.80	9.27	N/A
Oral (10mg/kg)	5	2.67	55	7.1	38



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

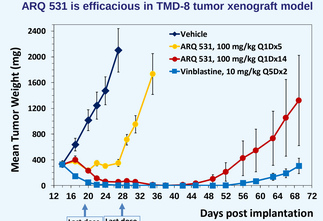


Tumors were removed at 6 hours after a single oral dose of ARQ 531 at 100mg/kg and western blot analysis was performed for assessing the phosphorylation levels of BTK and S6 targets.



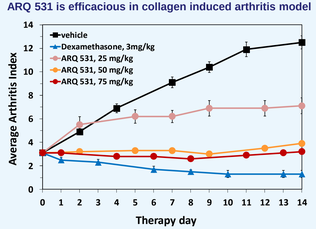
Tumors were removed at 2 or 24 hours after single oral dosing of ARQ 531 at 100mg/kg and Western blot analysis was performed to assess the phosphorylation levels of ERK and pERK.

ARQ 531 is efficacious in TMD-8 tumor xenograft model



Female SCID mice bearing TMD-8 tumors subcutaneously were administrated orally daily with ARQ 531 at 100 mg/kg or vehicle control. Tumor weight (mg) was determined three times a week, vehicle group was designated as 100% and percentage of tumor growth inhibition was calculated by measuring mean tumor weight. ARQ 531 caused complete tumor regression after 14 days of treatment.

ARQ 531 is efficacious in collagen induced arthritis model



BTK plays a crucial role in B cell differentiation and proliferation, making it an attractive target to treat inflammatory and autoimmune diseases. Inhibitory activity of ARQ 531 against BTK was tested in collagen induced model. Female DBA1/J female mice were administrated orally daily with ARQ 531, dexamethasone or vehicle control for 14 days. Arthritis score and body weights were measured three times weekly. ARQ 531 demonstrated potent efficacy against arthritis in mouse model.

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 and suppresses the key RAF/MEK/ERK, the PI3K/AKT/mTOR and Rap-GTPase-Cofilin pathways**
- **ARQ 531 potently inhibits proliferation of hematological malignant cell lines addicted to BCR signaling, both sensitive and resistant to ibrutinib**
- **ARQ 531 has high oral bioavailability, good ADME, pharmacokinetic and metabolic properties**
- **In the BTK driven TMD8 xenograft mouse model, ARQ 531 demonstrates excellent anti-tumor activity with durable response**
- **ARQ 531 demonstrates in vivo efficacy in a mouse collagen-induced arthritis (CIA) model**
- **These results warrant further preclinical and clinical investigation of ARQ 531, particularly in the setting of ibrutinib-resistance**