In Vitro and *in Vivo* Anti-tumor Activity of ARQ 751, a Potent and Selective AKT Inhibitor

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BACKGROUND

Dysregulation of the PI3K-AKT signaling pathway is associated with a number of cancers. AKT can be activated through aberrant receptor tyrosine kinases, gain-of-function mutations of PIK3CA, PTEN deficiency, and AKT amplification or activating mutations such as AKT1-E17K. We present here the preclinical characterization of ARQ 751, which has distinct physicochemical properties compared to our first generation inhibitor, ARQ 092.

MATERIALS AND METHODS

Biochemical IC_{50} determination and *in vitro* Kinase profile
Biochemical IC_{50} for ARQ 092 and ARQ 751 against AKT1, 2, and 3 was assessed in Reaction Biology (http://www. reactionbiology.com). *In vitro* Kinase profile was performed in either Carna Biosciences (http://www.carnabio. com).

Intrinsic tryptophan fluorescence quench assay
The binding of ARQ 092, ARQ 751, and MK-2206 to AKT1-WT and AKT1-E17K was followed by monitoring intrinsic tryptophan fluorescence quench. Dissociation constants (K_d) were calculated.

AKT1 plasma membrane translocation
NIH3T3 cells were transiently transfected with AKT1-GFP or AKT1-E17K plasmids. After 48 hours, cells were changed into serum free media for another 24 hours and treated with or without ARQ 751 at 1 μM for 2 hours and stimulated with PDGF at 50 ng/ml for 10 minutes. Cells were fixed and blocked & permeabolized. Images were captured using Olympus inverted epi-fluorescent microscope with 60x oil immersion objective. DAPI (was used to counter stain the nuclei.

Cell-based enzyme-linked immunosorbent assay (ELISA)
AN3CA cells were treated with ARQ 751 at concentrations of 2000, 500, 125, 31.3, 7.81, 1.95, 0.488, and 0.122 nM (n = 6) for 1 hr and pAKT (T308), pAKT (S473) and pPRAS40 (T246) in the cells were probed using ELISA. The chemiluminescent signal was detected using ELISA-Light Immunoassay System with CDP-Star and Sapphire-II Substrate/Enhancer Solution and PHERAstar. The relative phosphorylation rate was calculated in the MARS: Relative phosphorylation rate (%)=$(P/T)_{ARQ\ 751}/(P/T)_{Control}$x100. P: phospho proteins; T: total proteins.

Western blot analysis
293T cells were transiently transfected with pcDNAAKT1-E17K-GFP using Lipofectamine 2000. Proteins were extracted and resolved from extracts using SDS-PAGE followed by immunoblotting. pAKT(S473) and total AKT were assessed. Images were captured using FuJi LAS 3000. The intensity of bands were semi-quantitated using Multi Gauge software. pAKT level in untreated sample was designed as 100% after normalized with total AKT level.

OncoPanel analysis and Mutation analysis
Anti-proliferative effect of ARQ 751 on a panel of 240 cancer cell lines was evaluated by Eurofins Scientific(http://www.eurofins.com). Mutation analysis was performed based on information from COSMIC (http://cancer.sanger.ac.uk/ cancer genome/projects/cosmic/).

***In vivo* acute PK/PD and efficacy studies**
AN3CA cells were inoculated subcutaneously into female NCr *nu/nu* mice. For acute PD study, ARQ 751 was orally dosed at either 10 or 40 mg/kg. Tumor samples were assessed by western blot analysis. For PK study, BALB/c mice (N=3) were dosed orally with ARQ 751 at 25, 50, 75, and 150 mg/kg. Blood samples were collected at 1, 2, 4, 6, 8, 10, and 24 hrs. ARQ 751 plasma concentrations were determined using LC/MS/MS. For efficacy study, mice were inoculated with tumor cells or patient-derived tumor fragment and dosed with ARQ 751 at various dose regimen. The tumor length and width (mm) were measured using a digital caliper. Tumor volume (mm^3)=1/2 x (tumor length) x (tumor width)^2.

RESULTS

Biochemical potency and selectivity of ARQ 751

A

Biochemical Inhibition of AKT Kinases by ARQ 751

		Biochemical IC_{50} (nM)		
	Type	AKT1	AKT2	AKT3
ARQ 092	Allosteric	5.0	4.5	16
ARQ 751	Allosteric	0.55	0.81	1.3
MK-2206	Allosteric	40.5	29.5	36.4
GDC-0068	ATP-competitive	2.0	27.0	6.3

B

ARQ 751 Kinase selectivity

Kinase	% inh. at 5 μM
Blk(h)	40
Tie2	33
Haspin	30
Met	28
SGK3(119-end)	28
PASK	26
Other 238 kinases	<25

The biochemical IC_{50} values for ARQ 092, ARQ 751, MK-2206 and GDC-0068 were determined against full-length active forms of AKT1, 2, and 3 (A). The percent kinase inhibition of ARQ 751 was determined at a concentration of 5 μM against a panel of 245 kinases (B).

RESULTS

Binding affinity and AKT1 inhibition of ARQ 751

A

Binding Assay

Inhibitor	AKT1 Kd (nM)	AKT1-E17K Kd (nM)	Fold differenc between AKT1 wt- and E17K mutant
ARQ 092	7 .1	42	6
ARQ 751	1.2	8.6	7
MK-2206	11.6	1260	108

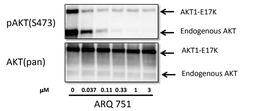
B

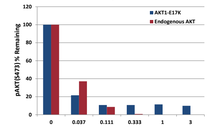
***In vitro* AN3CA PD**

		95% CI	
Target	IC_{50} (nM)	Lower	Upper
pAKT(T308)	5.1	4.8	5.49
pAKT(S473)	10.2	8.3	12.86
pPRAS40(T246)	48.55	42	56.91

C

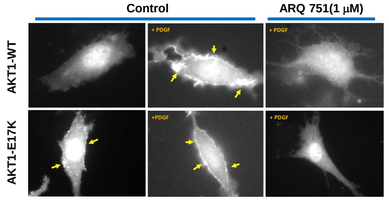
Inhibition of phosphorylation of AKT1-E17K





(A) The kd values of ARQ 092, ARQ 751 and MK-2206 were determined against AKT1-WT and AKT1-E17K using Intrinsic tryptophan fluorescence quench assay. (B) AN3CA cells were treated with ARQ 751 at various concentrations of ARQ 751 for 1 hr and pAKT (T308), pAKT (S473) and pPRAS40 (T246) in the cells were probed using Cell-based ELISA. The relative phosphorylation rate was calculated and IC_{50} values were determined. (C) 293T cells were transiently transfected with pcDNA-E17K-GFP and then treated with various concentrations of ARQ 751 for 2 hours. pAKT(S473) and total AKT were assessed. Densitometry analysis was performed and the relative pAKT(S473) level was determined as percent of pAKT/total AKT. The results indicate that the binding affinity predicts potency of ARQ 751.

Effect of ARQ 751 on AKT1 membrane translocation and AKT signaling

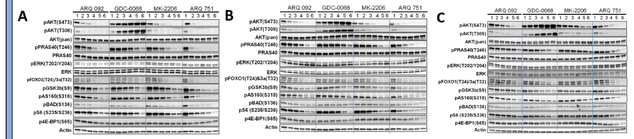


NIH3T3 cells were transiently with pcDNAAKT1-WT.GFP or pcDNAAKT1-E17K-GFP, starved for 24 hours and were treated with DMSO or ARQ 751 1 μM for 2 hrs. After cells were stimulated with PDGF at 50 ng/ml for 10 minutes, membrane translocation of AKT1-WT and AKT1-E17K was detected by a fluorescent microscope. Arrows show membrane accumulation of fluorescence of GFP

MDA-MB 453
(Breast, PIK3CAH1047R)

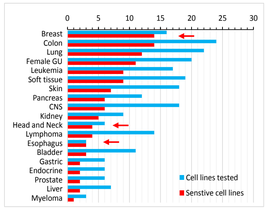
NCI-H1650
(NSLC, PTEN null)

KU-19-19
(Bladder, AKT1-E17K, NrasQ61R)

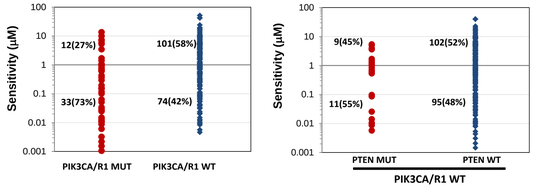


AKT singling pathway was analyzed in MDA-MB 453 (PIK3CA mut) (A), NCI-H1650 (PTEN null)(B), and KU-19-19 (AKT1 mut) (C) in response to treatment of ARQ 092, GDC-0068, MK-2206 and ARQ 751. Levels of pAKT, AKT downstream target pPRAS40 and pFOXO1(T24)/3a(T32) and pERK were assessed by western blot analysis. These data suggest that AKT inhibitors tested in this study displayed different potency in suppressing AKT pathway and ARQ 751 is the most potent inhibitor.

Potency of ARQ 751 in various cancer types



The sensitivity of ARQ 751 with different cancer types was determined. Assessment from a panel of 240 cancer cell lines showed the best anti-proliferative effects on esophageal (100%, 3 out of 3), breast (87.5%, 14 out of 16) and Head and Neck cancer (67%, 4 out of 6). Sensitive cells were defined as IC50<1 μM.



Somatic mutation analysis was performed for sensitive cell lines (IC_{50}<1 μM) and resistant cell lines (IC_{50} >1 μM). Scatter plot shows that 73% of cell lines with PIK3CA/R1 mutations (left) were sensitive to ARQ 751 but only 42% of cell lines wild type PIK3CA/R1 are sensitive(p=0.0002). However, there is no significant difference of ARQ 751 sensitivity between mutant and wild type PTEN status (right) (p=0.64). Those data suggest that PIK3CA/R1 but not PTEN is predictive for ARQ 751 sensitivity. MUT: mutant; WT: wild type

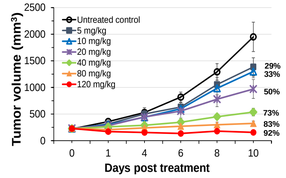
Breast Cancer Cell Lines	IC_{50}(μM)	PIK3CA	ER	PR	HER2
T47D	0.001	H1047R	+	+	-
EFM-19	0.002	H1047R	+	+	-
MCF-7	0.002	E545K	+	+	-
BT474	0.003	K111N	+	+	+
MDA-MB-453	0.006	H1047R	-	-	+
CAMA-1	0.009	WT	+	+	-
AU565	0.013	WT	+	+	+
KPL-1	0.029	E545K	+	+	-
MT-3	0.031	unknown	unknown	unknown	unknown
SK-BR-3	0.113	WT	-	-	+
Hs 578T	0.149	WT	-	-	-
BT-549	0.724	WT	-	-	-
BT-20	0.730	H1047R, P539R	-	-	-
MDA-MB-468	0.886	WT	-	-	-
MX1	1.976	unknown	unknown	unknown	unknown
MDA-MB-231	3.412	WT	-	-	-
MDA-MB-436	6.931	WT	-	-	-

The correlation of PIK3CA mutation with ARQ 751 sensitivity in breast cancer cell lines was determined. All breast cancer cell lines with PIK3CA mutations were sensitive to ARQ 751 (IC_{50}<1 μM). ER: estrogen receptor; PR: progesterone receptor; Her2: human epidermal growth factor receptor 2.

In vivo efficacy of ARQ 751

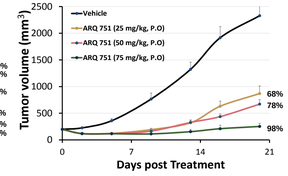
A

AN3CA
(Endometrial, PTENR130fs*, K561Q, FGFR2N549K&K310R)



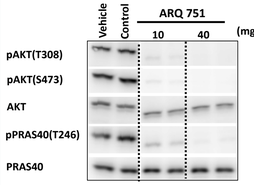
B

ST1062
(Endometrial PDX, AKT1-E17K)



C

***In vivo* AN3CA PD**



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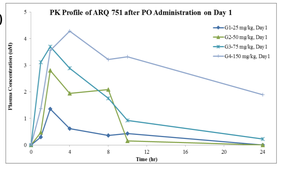
Mouse PK



(A) AN3CA cells were inoculated into female NCr *nu/nu* mice. ARQ 751 was orally dosed daily at 5, 10, 20, 40, 80, or 120 mg/kg.
(B) Athymic Nude mice (Crl:NU(NCr)-Foxn1^{nu}) were dosed with ARQ 751 at 25, 50, and 75 mg/kg at a schedule of 5 days on and 2 days off for 20 days. (C) AN3CA cells were inoculated subcutaneously into female NCr *nu/nu* mice. ARQ 751 was orally dosed at either 10 or 40 mg/kg. Tumor samples were assessed by western blot analysis after 6 hrs. (D) BALB/c mice were dosed treated orally with ARQ 751 at 25, 50, 75, and 150 mg/kg. Blood samples (N=3) were collected at 0, 1, 2, 4, 6, 8, 10, 24 hrs.

CONCLUSIONS

- **ARQ 751 is a potent and selective allosteric AKT inhibitor with different physicochemical properties and enhanced potency from ARQ 092, our first generation AKT inhibitor.**
- **ARQ 751 inhibits both inactive and active forms of AKT1 and AKT1-E17K mutant.**
- **Esophageal, breast, and head and neck cancer cells are the best responders in *in vitro* anti-proliferation activity. PIK3CA/PIK3R1 and AKT mutations are associated with an increased sensitivity of ARQ 751 in cancer cell lines.**
- **In *in vivo* studies, ARQ 751 exhibits potent anti-tumor activity in xenograft mouse models with activated PI3K/AKT pathway.**