AR-mTOR-26 – A Potent, Selective mTORC 1/2 Kinase Inhibitor for the Treatment of Malignancy

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Introduction

The PI3K/Akt pathway is constitutively activated in a large proportion of human cancers. mTOR kinase plays a vital role in this pathway as the key component of two independent signaling complexes (mTORC1 and mTORC2). Inhibition of mTOR kinase could therefore serve as an effective means of abrogating PI3K-dependent signaling. In addition, the activity of TORC1 is often aberrantly activated in a PI3K-independent manner enabling tumor cells to survive and proliferate despite the many negative influences of the tumor microenvironment. Therefore, targeting the activity of TORC1 and TORC2 would abrogate both PI3K signaling and the cancer cells' ability to survive in the harsh environment of the tumor. We report here the development of a potent and selective small molecule inhibitor of mTOR kinase designated AR-mTOR-26.

- On enzyme this compound exhibits low single digit nM activity against isolated mTOR kinase with >40-fold selectivity against the α, β and δ isoforms of PI3K as well as a panel of over 250 ser/thr and tyrosine kinases.
- On cells AR-mTOR-26 potently inhibits TORC1/2-dependent readouts pAKT(S473), p4E-BP1(S37/46) and pS6(S235/6) yet is significantly inactive against the PI3K-dependent readout pAKT(T308) confirming its selectivity against Class I PI3K-kinases.
- This cellular potency readily translates into broad anti-proliferative activity against a wide array of solid tumor and hematological cancer cell lines irrespective of mutational status (i.e. KRAS, PTEN, PIK3CA, etc.)
- AR-mTOR-26 possesses exceptional pharmacokinetic properties across multiple species, including mouse with exposures predicted to be biologically active.
- The potential for *in vivo* activity was confirmed in two tumor xenograft models both in terms of tumor growth inhibition as well as inhibition of mTOR-relevant targets in the tumors.

AR-mTOR-26 In Vitro Activity and Safety Profile

Enzyme	AR-mTOR-26 IC_{50} (nM)
mTOR	<0.5
p110α	21
p110β	240
p110γ	6
p110δ	176

Selectivity Data	AR-mTOR-26
261 Kinase Panel >50% inhibition at 1 uM (% control)	MKK7β (49)
58 Channel / Receptor Panel >50% inhibition at 10 uM (% Control in binding assay) [Functional assay IC_{50}]	Adenosine A3 (80) [>100 uM]
hERG channel (% inhibition)	14% at 1 uM 59% at 10 uM

•AR-mTOR-26 is potent inhibitor of mTOR enzyme that is >40-fold selective for the α, β and δ isoforms of the Class I PI3Ks and substantially selective vs. 261 other protein kinases.

•AR-mTOR-26 exhibited minimal effects on a panel of 58 receptors and was only a modest inhibitor of the hERG cardiac channel.

Preclinical Pharmacokinetics[1]

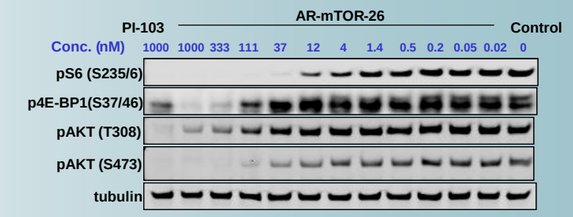
Species	Mouse		Rat		Dog	
Route	IV	PO	IV	PO	IV	PO
Dose (mg/kg)	2	10	2	10	2	10
CL (ml/min/kg)	8.8		4.7		14	
Vss (l/kg)	3.2		4.0		8.0	
$t_{1/2}$ (h)	3.9		N/A		9.8	
AUC (mg/h/ml)	3.7	6.8	7.2	14.2	2.5	9.1
Cmax (mg/ml)		0.9		0.5		0.4
%F		37		39		75

•Based upon its pharmacokinetic properties, both AR-mTOR-26 is predicted to produce biologically active exposures via PO dosing in mouse as well as other species.

Cellular pathway effects of AR-mTOR-26

In-cell western analysis of pathway inhibition [IC_{50} (nM)]

Compound	MOA	pS6	p4E-BP1	pAkt-S473	pAkt-T308
AR-mTOR-26	mTOR kinase	11	89	15	427
AR472303	mTOR kinase	37	215	44	124
AR454415	mTOR/PI3K	7	83	20	69
AR453517	mTOR/PI3K	142	485	65	172
AR466979	PI3Kα/δ	560	2666	186	251

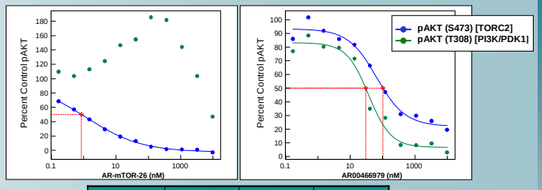


•In cells, AR-mTOR-26 is a potent inhibitor of TORC1 and TORC2-dependent readouts (pS6, p4E-BP1 and pAKT-S473)

•The compound also effects a substantial but incomplete loss of pAKT(T308) phosphorylation in marked contrast to PI3K inhibitor AR00466979 which is equipotent on both readouts

Methods. LNCaP cells were used for in-cell western analysis employing phospho-specific antibodies to the indicated TORC1, TORC2 or PI3K-dependent substrates. PC3 cells were used for the immunoblotting analysis employing the same antibodies.

mTOR vs. PI3K selectivity in cells



	Construct:	AKT [T308E]	AKT [S473E]
Compound	MOA	pAKT-S473	pAKT-T308
AR-mTOR-26	mTOR kinase	4	>2500
AR472303	mTOR kinase	2	>2500
AR454415	mTOR kinase	2	>2500
AR453517	mTOR/PI3K	170	178
AR466979	PI3Kα/δ	63	37

•AR-mTOR-26 potently inhibits the TORC2 readout pAKT-S473 but is substantially inactive against the PI3K-dependent readout pAKT-T308.

•Over the dose range analyzed, data indicate that AR-mTOR-26 actually activates the PI3K pathway likely due to abrogation of a downstream negative feedback loop.

•This profile is in marked contrast to PI3K inhibitor AR00466979 which potently inhibits both readouts.

Methods. To measure the cellular activities of PI3K and TORC2 on AKT independently, DNA constructs expressing mutant forms of AKT substituted with glutamate at one of the regulatory phosphorylation sites T308 or S473 were generated. Expression constructs were then transfected into HEK-293T cells, treated with compound and processed for in-cell western analysis probing for either pAKT(S473) or pAKT(T308). Upper panel depicts typical dose/response curves for AR-mTOR-26 and AR00466979. Lower panel is a table of representative IC_{50} values obtained for the indicated compounds in the pAKT T308E and pAKT S473E cell assays.

Cell proliferation

Solid Tumor Lines **GI_{50} (nM)**

Cell Line:	BxPC -3	DU145	Malme -3M	OVCAR -8	PC3	LNCaP	DLD -1	BT -474	22RV1	H460	HCT116
Mutation:	WT	LKB1	BRAF V600E	ERBB2 G776V	PTEN -	PTEN -	KRAS G13D	PI3KCA K111N	PI3KCA E545K	PI3KCA E545K KRAS Q61H	PI3KCA H1047R KRAS G12D
AR-mTOR-26	34	46	34	76	21	17	38	100	37	34	40
AR454415	54	81	60	51	36	21	17	85	34	44	102
AR453517	1942	233	109	305	115	295	134	596	402	366	324
AR466979	316	710	354	1685	360	415	458	312	761	604	767

Hematologic Tumor Lines **GI_{50} (nM)**

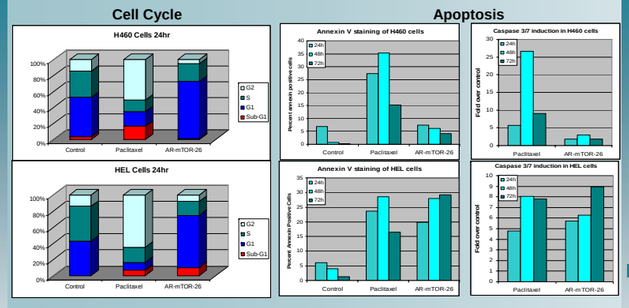
Cell Line:	K562	GDM -1	THP1	HEL	MOLT4	MO7e	MV4 -11	MOLM13	MOLT3	HL -60
Mutation:	BCR -Abl	NRAS	BRAF V600E	ERBB2 G776V	PTEN -	PTEN -	KRAS G12D	PI3KCA K111N	PI3KCA E545K	PI3KCA E545K KRAS Q61H
AR-mTOR- 26	41	76	69	41	17	31	13	14	20	90
AR454415	51	231	154	76	57	59	52	24	55	248
AR453517	1068	620	492	1319	151	728	295	143	158	164
AR466979	6789	1030	778	5647	207	3820	349	344	271	169

•Across both solid tumor and leukemic cell lines, the mTOR-selective inhibitors are more broadly active than the PI3K inhibitor AR00466979

•Relative potency of these compounds is largely independent of mutational status of the PI3K or RAS/RAF/Mek/Erk pathways

Methods. Indicated cell lines were plated out at a density of 5-10K cells/well of a 96-well plate, treated with compound for 3-5 days and then assessed for viability/proliferation using Celltiterblue (Promega).

Understanding the mechanism of anti-proliferation



•AR-mTOR-26 treatment results in a profound G1 arrest in both cell lines examined, as expected for this mechanism [left panels].

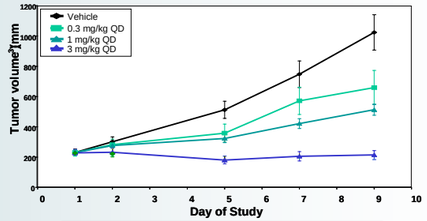
•Appearance of sub-G1 population in the HEL cells is indicative of apoptosis which was confirmed by detection of enhanced Annexin V binding and Caspase 3/7 induction [right panels, top].

•NCI-H460 cells, while sensitive to AR-mTOR-26 treatment in terms of growth arrest did not exhibit any of the hallmarks of apoptosis.

•Methods. Cell cycle analysis: NCI-H460 (NSCLC) and HEL 92.1.7 (Leukemia) cells were treated for 24h with either 170 nM (H460) or 200 nM (HEL) AR-mTOR-26 for 24h and assessed for cell cycle distribution by propidium iodide staining and subsequent FACS analysis [*left panel*]. Apoptosis assays: HEL and H460 cells were treated with compound (same concentrations as for cell cycle) for 24-72h and stained using Annexin V or monitored for caspase 3/7 activation (CaspaseGlo 3/7, Promega) *[right panel]*.

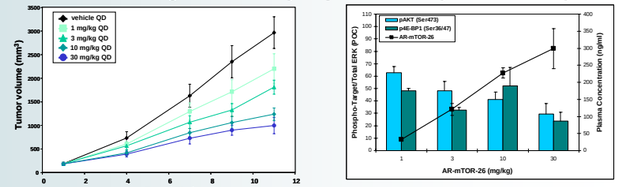
In vivo efficacy[1]

PC3-NCI tumor xenograft efficacy study



Treatment	Dose (mg/kg)	Route/ Schedule	% TGI	Mean % Regression
Vehicle	---	PO/QD	N/A	0
AR-mTOR-26	0.3	PO/QD	35.6	0
AR-mTOR-26	1.0	PO/QD	49.9	0
AR-mTOR-26	3.0	PO/QD	79	20.7

H460 tumor xenograft efficacy & pharmacodynamic study



Treatment	Dose (mg/kg)	Route/ Schedule	% TGI	TORC1 p4E-BP1 Thr37/46 (POC)	TORC2 pAKT Ser473 (POC)
AR-mTOR-26	1	PO/QD	26	48.2	62.9
AR-mTOR-26	3	PO/QD	39	32.8	48.1
AR-mTOR-26	10	PO/QD	58	52.3	41.1
AR-mTOR-26	30	PO/QD	66	23.7	29.4

•AR-mTOR-26 dose-dependently inhibits growth of both PC3-NCI and NCI-H460 tumor xenografts with a corresponding inhibition of pAKT(S473) and p4E-BP1 in tumors (H460)

Methods. All studies were performed using female nude mice bearing growth staged PC3-NCI or H460 tumors. Tumor size was measured on the indicated days over the course of each study. Percent TGI was calculated as follows: 100 x (Treated/Vehicle control). Test compound administration and tumor growth metrics are displayed for each study as indicated. Phosphorylation of 4E-BP1 and pAkt (S473) were assessed by immunoblot and normalized to total ERK expression. Results are expressed as percent of control (POC).

Summary

AR-mTOR-26 is a potent and selective mTOR kinase inhibitor that exhibits:

- **High selectivity for mTOR vs. PI3K *in vitro* and in cells**
- **Broad anti-proliferative activity against a panel of solid and hematologic cancer cell lines harboring many important oncogenic mutations**
- **Pre-clinical pharmacokinetics that support efficacy following once-daily oral dosing**
- ***In vivo* efficacy in multiple models including the rapalog-resistant cell line (NCI-H460)**
- **Inhibition of TORC1 and TORC2 signaling *in vivo***

[1]All *in vivo* studies were performed in accordance with IACUC guidelines and in harmony with the Guide for Laboratory Animal Care and Use