Poster #16
Session #2

Discovery of a first-in-class dual microtubule- and pan-RAF–targeting inhibitor

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Introduction

- Microtubule-targeting agents (MTAs) are effective first-line cancer therapies, but novel agents are needed to overcome resistance mechanisms, minimize toxicities, improve delivery, and enhance outcomes with new combination strategies[1,2]
- Several kinase inhibitors have demonstrated microtubule-targeting activity, with some dual inhibitors demonstrating lower toxicities; however, no reported compounds have demonstrated dual RAF kinase– and microtubule-targeting activities[2]
- Additionally, treatment with microtubule inhibitors cause an increase in phosphorylated and activated ERK, which may drive resistance to microtubule inhibitors[3]
- Mutations in the RAS/MAPK pathway are among the most common tumor drivers, with *RAS* genetic alterations driving 30% of all cancers;[4] thus, we sought to develop a compound with dual microtubule- and pan-RAF–targeting inhibitory activity

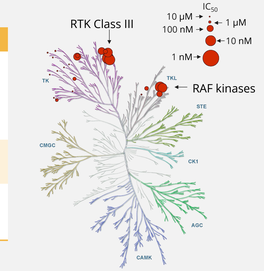
Methods

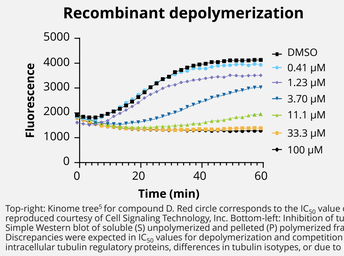
- Kinase inhibition and off-rate analysis were measured using recombinant enzymes
- Tubulin polymerization inhibition and binding-site competition were measured with recombinant tubulin
- X-ray crystallography enabled structure-based design
- Cellular microtubule modulation was determined by a microtubule sedimentation assay, and cellular inhibition of pERK and pRSK was measured by AlphaLISA, ELISA, or HTRF assays
- Cellular proliferation was monitored via resazurin
- Spindle formation, cell cycle, and apoptosis were measured by immunofluorescence, flow cytometry, and Western blotting
- PK in plasma, brain, and cerebrospinal fluid compartments were measured following oral dosing in rats
- In vivo efficacy was assessed in *RAF*- and *RAS*-mutant mouse xenograft models

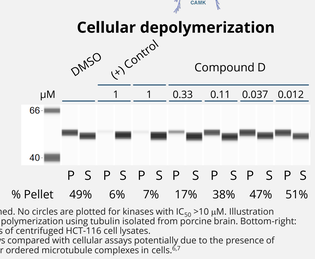
Results

Figure 1. Compound D is a potent and selective inhibitor of RAF kinases and a microtubule destabilizing agent

Assay		Compound D
RAF kinases IC_{50} (nM)	ARAF	320
	BRAF	55
	CRAF	23
	BRAF V600E	23
Residence time (hours)	BRAF	34
	CRAF	8.6
Tubulin depolymerization IC_{50} (nM)	recombinant	6200
	cellular	130

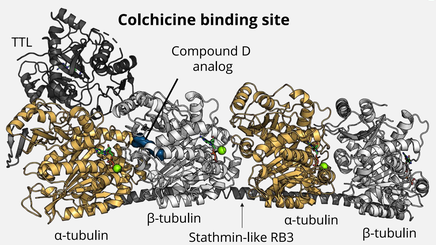


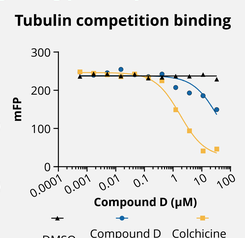


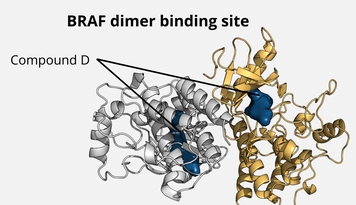


Top-right: Kinome tree[5] for compound D. Red circle corresponds to the IC_{50} value obtained. No circles are plotted for kinases with IC_{50} >10 µM. Illustration reproduced courtesy of Cell Signaling Technology, Inc. Bottom-left: Inhibition of tubulin polymerization using tubulin isolated from porcine brain. Bottom-right: Simple Western blot of soluble (S) unpolymerized and pelleted (P) polymerized fractions of centrifuged HCT-116 cell lysates.
Discrepancies were expected in IC_{50} values for depolymerization and competition assays compared with cellular assays potentially due to the presence of intracellular tubulin regulatory proteins, differences in tubulin isotypes, or due to higher ordered microtubule complexes in cells.[6,7]

Figure 2. Compound D binds at the colchicine binding site of α-/β-tubulin and binds to BRAF dimers to inhibit the MAPK signaling pathway







MAPK signaling inhibition

Cell line	pERK IC_{50} (nM)	pRSK IC_{50} (nM)
A375	160	260
BXPC-3	110	560
HCT-116	64	18

Top-left: 2.57 Å x-ray crystal structure of a compound D analog bound to α-/β-tubulin dimers in complex with TTL and stathmin. Top-right: Compound D competes with a BODIPY-colchicine probe as measured by fluorescence polarization. Bottom-left: 2.75 Å x-ray crystal structure of compound D bound to a BRAF dimer.

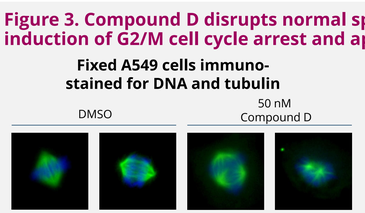
Table 1. Compound D is a potent inhibitor of proliferation in a variety of tumor cell lines

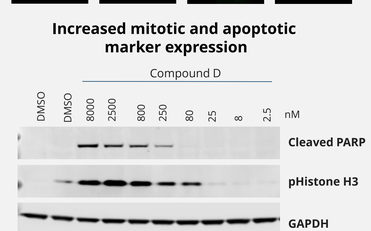
Mutated gene	Mutation class	Cell line	Compound D IC_{50} (nM)	Naporafenib IC_{50} (nM)
BRAF	*BRAF* Class I	A375	39	460
	BRAF Class II	H2405	120	470
	BRAF Fusion	WM3928	58	90
	BRAF Class III	WM3629	8.0	3.0
KRAS	*KRAS G12C*	MiaPaca-2	140	1300
		H358	64	390
	KRAS G12D	HPAF-II	140	2600
		Pa16c	120	6400
	KRAS other mutations	A549	190	2100
		PSN-1	88	1900
		HCT-116	190	1800
		Calu-6	52	800
		Pa02c	590	3700

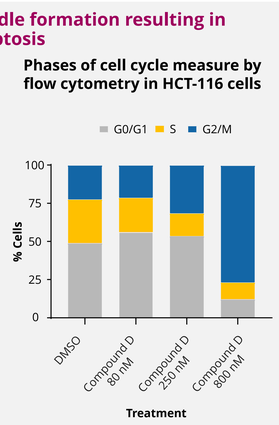
Table 2. Compound D can overcome resistance mechanisms including MDR1 overexpression in cell lines

Cell line	Docetaxel IC_{50} (nM)	Colchicine IC_{50} (nM)	Compound D IC_{50} (nM)
HEK293	0.5	11	85
HEK293 + MDR1 overexpression	130	210	64
A2780	0.5	6.0	68
A2780 + MDR1 overexpression	2.0	160	88

Figure 3. Compound D disrupts normal spindle formation resulting in induction of G2/M cell cycle arrest and apoptosis







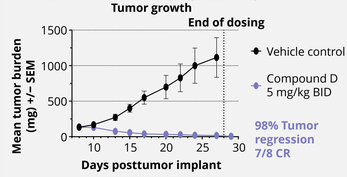
Top-left: Images of fixed A549 cells immuno-stained for DNA (DAPI; blue) and tubulin (green). Right: Bar graph distribution of cells in G0/G1, S, and G2/M phases of the cell cycle measured by flow cytometry in HCT-116 cells treated with increasing concentrations of compound D for 16 hours. Bottom-left: Western blot of cleaved PARP and pS10 of histone H3 following treatment with compound D.

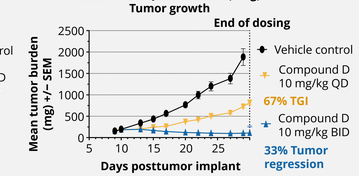
Table 3. Compound D shows favorable ADME and PK properties and is brain penetrable

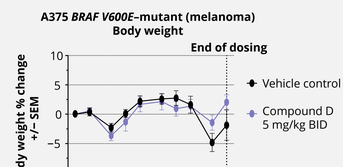
Property	Measurement	Compound D
Thermodynamic solubility	pH 1.6 (µM)	<1.5
Caco2	P_{app} A-B (10^{-6} cm/s)	12.5
	P_{app} B-A /P_{app} A-B	0.6
MDR1	P_{app} A-B (10^{-6} cm/s)	11.2
	P_{app} B-A /P_{app} A-B	0.8
BCRP	P_{app} A-B (10^{-6} cm/s)	19.6
	P_{app} B-A /P_{app} A-B	1.2
Rat PK[a]	CL (mL/min/kg)	6.8 (low)
	V_{dss} (L/kg)	2.7 (moderate)
	F %	35.4
Monkey PK[b]	CL (mL/min/kg)	10.8
	V_{dss} (L/kg)	1.92
	F %	49.6
BBB[c,d]	AUC [brain]/AUC [plasma]	0.88
	K_{puu}	1.17
	Classification[8]	High

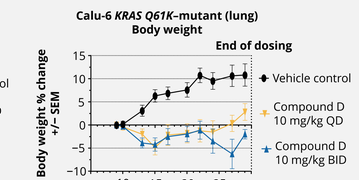
[a]PK values were measured at 1 mg/kg IV and 10 mg/kg PO doses. [b]PK values measured at 1 mg/kg IV and 3 mg/kg PD doses. [c]Free fraction was determined based on percent rat brain and plasma binding. [d]Brain and plasma concentrations measured in Wistar rats after a single oral 5 mg/kg dose.

Figure 4. Oral treatment of compound D resulted in tumor regression or tumor growth inhibition in *BRAF*- and *KRAS*-mutant mouse xenograft models









CONCLUSIONS

- Compound D is the first reported tubulin destabilizer that also targets RAF kinases
- Compound D is orally available and can penetrate the blood-brain barrier
- In *BRAF*- and *KRAS*-mutant mouse xenograft models, compound D inhibits tumor growth as a single agent
- Our preclinical data supports the exploration of therapeutic opportunities for developing agents that target both microtubules and RAF kinases

Abbreviations: Å, angstrom; ADME, absorption, distribution, metabolism, and excretion; AGC, protein kinases A, G, and C; AlphaLISA, Amplified Luminescent Proximity Homogeneous Assay; ARAF, A-Raf serine/threonine kinase; AUC, area under the curve; BBB, blood-brain barrier; BCRP; breast cancer resistance protein; BID, twice daily; BODIPY, boron–dipyrromethene; BRAF, B-Raf proto-oncogene, serine/threonine kinase; CAMK, calcium/calmodulin-dependent protein kinases; CK1, casein kinase 1; CL, clearance; CMGC, includes cyclin-dependent kinases, MAPKs, glycogen synthase kinases, and CDC-like kinases; CR, complete response; CRAF, C-Raf serine/threonine kinase; DAPI, 4′, 6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; ELISA, Enzyme-Linked Immunosorbent Assay; F, absolute bioavailability; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HTRF, Homogeneous Time-Resolved Fluorescence; IC_{50}, half maximal inhibitory concentration; IV, intravenously; K_{puu}, unbound tissue-to-plasma partition coefficient; *KRAS*, Kirsten RAS virus oncogene homolog; MAPK, mitogen-activated protein kinases; MDR1, multidrug resistance 1; mFP, milli-fluorescence polarization units; MTA, microtubule-targeting agent; P_{app} A-B, apparent permeability from apical to basolateral direction; P_{app} B-A, apparent permeability coefficient from basolateral to apical direction; PARP, poly-ADP ribose polymerase; PD, pharmacodynamic; pERK, phosphorylated extracellular-signal-regulated kinase; PK, pharmacokinetics; PO, by mouth; pRSK, phosphorylated 90 kilodalton ribosomal S6 kinase; QD, once daily; RAF, rapidly accelerated fibrosarcoma; RAS, rat sarcoma; RTK, receptor tyrosine kinase; SEM, standard error of the mean; STE, sterile; comprises homologs of yeast Sterile 7, 11, and 20 kinases; TGI, tumor growth inhibition; TKL, tyrosine kinase-like kinase; TTL, tubulin tyrosine ligase; V_{dss}, volume of distribution at steady state.

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DICSLOSURES

All authors are or were fulltime employees of Deciphera Pharmaceuticals, LLC.

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