NST-628 is a novel molecular glue that inhibits signaling and pathway reactivation in oncogenic RAS-MAPK cancers

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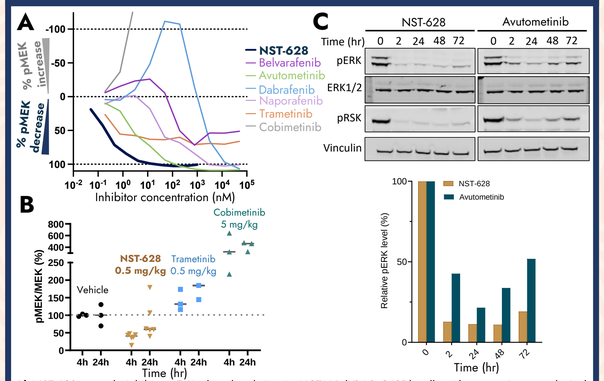
Abstract

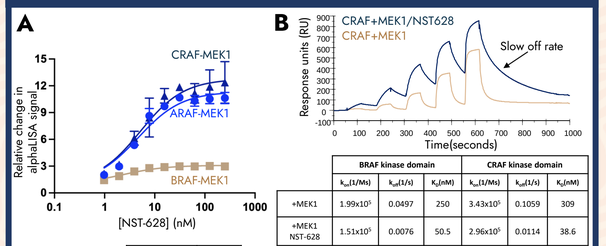
Alterations in the RAS-MAPK pathway are identified in nearly 30% of cancers and are associated with poor patient prognosis. Although available therapies target various nodes of the RAS-MAPK pathway, clinical anti-tumor activity is limited by paradoxical bypass mechanisms, feedback signaling and pathway reactivation, and genetic resistance mechanisms. NST-628 aims to overcome the limitations of traditional RAS-MAPK pathway inhibitors by stabilizing all RAF-MEK complexes in a conformation that blocks MEK phosphorylation, to sequester these nodes from dynamic signaling processes that promote RAF-mediated bypass signaling. In comparison with clinically approved RAS-MAPK pathway inhibitors, only NST-628 robustly and durably decreases pathway reactivation, as measured by MEK phosphorylation, in KRAS-G13D tumor models in vitro and in vivo. Immunoprecipitation of endogenous ternary complexes demonstrates that NST-628 promotes potent stabilization of CRAF-MEK, BRAF-MEK, and ARAF-MEK complexes with complete shutdown of MEK, ERK, and RSK phosphorylation. Moreover, NST-628 treatment completely prevents RAF paralog heterodimerization in RAS-driven cells. Dose-dependent panRAF-MEK complex stabilization was also confirmed using recombinant pre-formed CRAF-MEK, BRAF-MEK, and ARAF-MEK complexes, and MEK and RAF paralog affinity increases in the presence of NST-628 were quantified via surface plasmon analysis (SPR). In this assay, NST-628 significantly increased the affinity of MEK with both inactive monomeric and active dimeric RAF complexes. In parallel, we structurally characterized NST-628 bound to CRAF-MEK, BRAF-MEK, ARAF-MEK complexes using x-ray crystallography and cryogenic electron microscopy (cryo-EM). NST-628 binds the interfacial allosteric pocket along the MEK activation loop, thereby preventing RAF-mediated activation of MEK. We also demonstrate RAF paralog-specific pocket features that NST-628 can engage, helping us rationalize the large increase in affinity observed for MEK and CRAF in the presence of NST-628. The increased stabilization of CRAF-MEK complex by NST-628 may also contribute to the decreased pathway reactivation in specific biomarker-driven tumors. Through our robust biophysical and cellular characterization coupled with novel structural insights, we have defined the unique mechanism of action for NST-628 and provide insight into its best-in-class potential in RAS-MAPK activated cancers.

NST-628 prevents inhibitor-induced RAS-MAPK pathway reactivation *in vitro* and *in vivo*



A) NST-628 potently inhibits MEK1 phosphorylation in HCT116 (KRAS G13D) cells without causing paradoxical activation. **B)** The potency and overall magnitude of inhibition is superior to all other RAF and MEK inhibitors tested. MEK phosphorylation was also monitored in ***in vivo*** KRAS G13D tumor models 4 hours and 24 hours after a single inhibitor dose, and of the inhibitors tested, only NST-628 blocks MEK hyperphosphorylation at both time points. **C)** In addition to preventing MEK phosphorylation, NST-628 durably impairs downstream pathway signaling through ERK and RSK.

NST-628 enhances interactions between recombinant MEK and RAF in active or autoinhibited conformations



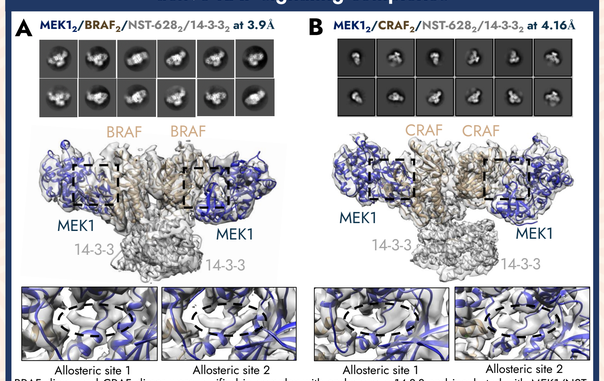
	EC50 (nM)	Top
ARAF-MEK1	4.659	11.35
BRAF-MEK1	2.319	3.093
CRAF-MEK1	5.155	12.78

BRAF kinase domain	k_{on}(1/Ms)	k_{off}(1/s)	K_d(nM)	CRAF kinase domain	k_{on}(1/Ms)	k_{off}(1/s)	K_d(nM)
+MEK1	$1.99x10^5$	0.0497	250		$3.43x10^5$	0.1059	309
+MEK1 NST-628	$1.51x10^5$	0.0076	50.5		$2.96x10^5$	0.0114	38.6

BRAF WT FL (autoinhibited)	k_{on}(1/Ms)	k_{off}(1/s)	K_d(nM)	BRAF S365A FL (activated)	k_{on}(1/Ms)	k_{off}(1/s)	K_d(nM)
+MEK1	$1.60x10^5$	0.0527	328		$1.35x10^5$	0.0502	382
+MEK1 NST-628	$1.77x10^5$	0.0086	48.5		$1.55x10^5$	0.0097	62.4

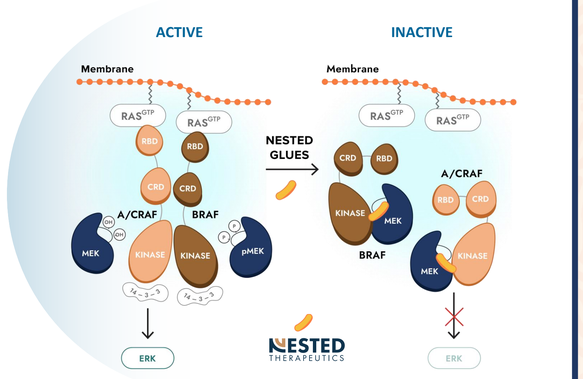
A) Changes in alphaLISA signal were measured with increasing NST-628 dose for recombinant, preformed MEK-RAF complexes and single digit nanomolar EC50s were observed for all. **B)** SPR-based ternary complex assays quantified changes in affinity between RAF and MEK upon NST-628 treatment and demonstrate that NST-628 increases the affinity ~5 times for BRAF-MEK1 and ~9 times for CRAF-MEK1. **C)** NST-628 also enhances the affinity of MEK1 to recombinant full-length BRAF-14-3-3 complex in the autoinhibited conformation (WT BRAF) and activated dimer conformation (S365A BRAF).

NST-628 engages MEK bound to RAF homodimers to inhibit active RAF signaling complexes



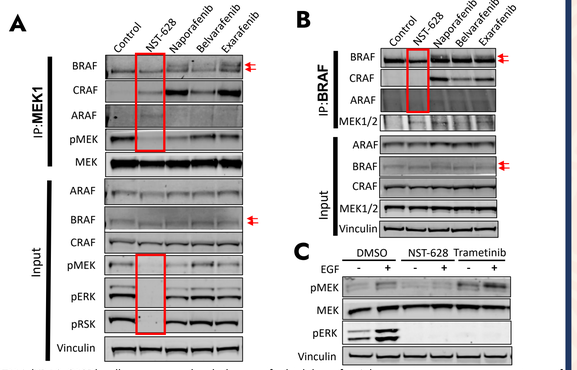
BRAF dimer and CRAF dimer were purified in complex with endogenous 14-3-3 and incubated with MEK1/NST-628. CryoEM datasets were collected for each complex and structures were determined of BRAF$_2$-MEK1$_2$-14-3-3$_2$ at 3.9Å resolution **(A)** and CRAF$_2$-MEK1$_2$-14-3-3$_2$ at 4.2Å resolution **(B)**. Both complexes adopt a similar architecture with a RAF homodimer bound to MEK1. Although the resolutions of the structures are modest, NST-628 density is visible in the allosteric site of MEK1 and this demonstrates that NST-628 engages active RAF signaling complexes.

Designing a RAS-MAPK pathway inhibitor that acts on multiple nodes and blocks MEK phosphorylation



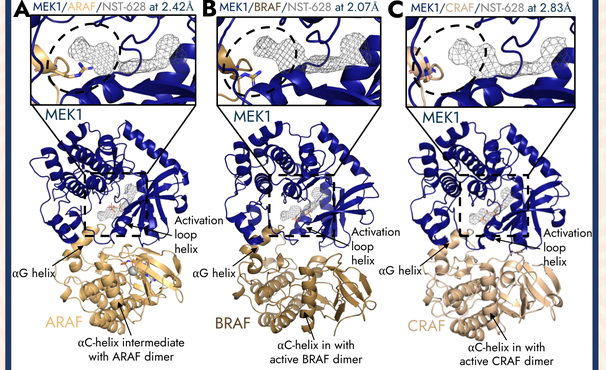
RAS-MAPK pathway alterations are highly oncogenic and found in nearly 30% of all cancers. Current therapies targeting the RAS-MAPK pathway have limited anti-tumor efficacy due to various forms of biological and genetic feedback. We aim to develop an inhibitor that blocks RAS-MAPK pathway activation by stabilizing pan-RAF-MEK complexes and preventing feedback-induced MEK1 hyperphosphorylation.

NST-628 stabilizes endogenous pan-RAF-MEK complexes and prevents RAF heterodimerization



HCT116 (KRAS G13D) cells were treated with the specified inhibitor for 2 hours prior to immunoprecipitation of endogenous MEK1 **(A)** or endogenous BRAF **(B).** In MEK1 IPs, NST-628 is the only inhibitor that stabilizes CRAF-MEK, BRAF-MEK, and ARAF-MEK complexes while dramatically decreasing MEK, ERK, and RSK phosphorylation. In BRAF IPs, NST-628 is the only inhibitor that completely blocks RAF heterodimerization. **C)** NST-628 also blocks pathway signaling under EGF stimulation, suggesting that it can prevent receptor tyrosine kinase-mediated pathway reactivation.

NST-628 binds the MEK allosteric site to engage pan-RAF-MEK complexes



Recombinant RAF-MEK1 complexes were co-crystallized with NST-628 and structures were determined of ARAF-MEK1 (2.42Å resolution; **(A)**), BRAF-MEK1 (2.07Å resolution; **(B)**), and CRAF-MEK1 (2.83Å resolution; **(C)**). In all structures, unambiguous density corresponding to NST-628 (grey mesh) is observed in the allosteric site of MEK1 adjacent to the activation loop helix. This binding mode blocks phosphorylation of the activation loop of MEK1 and enables engagement of unique αG-helix features of each RAF.

Conclusions and Acknowledgements

- NST-628 durably inhibits MAPK pathway signaling and blocks MEK hyperphosphorylation.
- NST-628 engages endogenous and recombinant ARAF-MEK, BRAF-MEK, and CRAF-MEK complexes and blocks RAF heterodimerization
- NST-628 engages MEK in active, dimeric and autoinhibited RAF complexes
- NST-628 has best-in-class potential due to its unique mechanism of action, balanced metabolic profile, strong ***in vivo*** efficacy (see poster A088), and central nervous system penetrance (see poster A089)

This work was done in collaboration with many talented contract research organizations including, but not limited to, Viva Biotech, Beactica, HD Bio, Pharmaron, and Wuxi Chemistry

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