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The paradox-breaker BRAF inhibitor plixorafenib (PLX8394; FORE8394) synergizes with MEK inhibitors (MEKi) in BRAF V600 and non-V600 alterations, with higher potency compared to early generation BRAFi and pan-RAFi

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Background

- Approved BRAF inhibitors (BRAFi) for BRAF V600-mutated tumors lead to paradoxical MAPK pathway activation associated with toxicities, acquired resistance, and secondary malignancies
- BRAFi combined with a MEK inhibitor (MEKi) is more effective than monotherapy in some settings and overcomes MAPK pathway paradoxical activation, but results in severe side effects and a high discontinuation rate
- ~35% of BRAF mutations occur outside the V600 codon and are not targeted by approved BRAFi

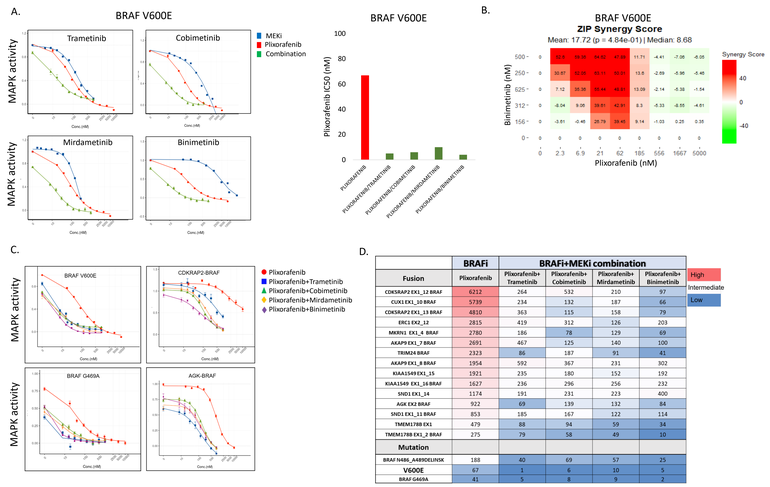
Plixorafenib (FORE8394; PLX8394)

- A selective, potent paradox-breaker BRAFi that targets mutated BRAF monomers and homodimers and BRAF-CRAF heterodimers without inducing RAF dimer formation
- Demonstrated robust anti-tumor activity as a single agent against BRAF-altered tumors including CNS tumors, with durable long-term tolerability, no dose limiting toxicities, infrequent symptomatic G3 AEs, infrequent fever, and no skin toxicities observed with approved BRAFi in clinical settings
- This work evaluates the combination of plixorafenib and MEKi in nonclinical models and explored the feasibility of the combination for clinical use

Methods

- High-throughput cell-based functional assay quantifies MAPK signaling pathway activation using fluorescent imaging coupled with image analysis of cells expressing the mutated protein together with a fluorescently labeled ERK2 as a signaling pathway reporter (Zimmerman, L. *et al. Sci. Rep.* 63, 4192 (2020))
- High-throughput assay results are validated using standard western blot and cell viability assay

The combination of plixorafenib with MEKi is synergistic



Fusion	BRAFi	BRAFi+MEKi combination			
	Plixorafenib	Plixorafenib+ Trametinib	Plixorafenib+ Cobimetinib	Plixorafenib+ Mirdametinib	Plixorafenib+ Binimetinib
CDK5RAP2 EX1_12 BRAF	6212	264	532	210	97
CUX1 EX1_10 BRAF	5739	234	132	187	66
CDK5RAP2 EX1_13 BRAF	4810	363	115	158	79
ERC1 EX2_12	2815	419	312	126	203
MKRN1 EX1_4 BRAF	2780	186	78	129	69
AKAP9 EX1_7 BRAF	2691	467	125	140	100
TRIM24 BRAF	2323	66	187	91	41
AKAP9 EX1_8 BRAF	1954	592	367	231	302
KIAA1549 EX1_15	1921	235	180	152	192
KIAA1549 EX1_16 BRAF	1627	236	296	256	232
SND1 EX1_14	1174	191	231	223	400
AGK EX2 BRAF	922	69	139	132	84
SND1 EX1_11 BRAF	853	185	167	122	114
TMEM178B EX1	479	88	94	59	34
TMEM178B EX1_2 BRAF	275	79	58	49	10
Mutation					
BRAF N486_A489DELINSK	188	40	69	57	25
V600E	67	1	6	10	5
BRAF G469A	41	5	8	9	2

High
Intermediate
Low

Figure 1. (A) Left: MAPK pathway activity in BRAF V600E+ cells treated with plixorafenib and the MEKis trametinib, cobimetinib, mirdametinib, and binimetinib as single agents and combination. Combinations show the response of the cells to plixorafenib at different concentrations in combination with an IC:25 concentration of each of the MEKi. **Right:** IC:50 summary of plixorafenib monotherapy and the 4 combinations. **(B)** SynergyFinder analysis between plixorafenib and the MEKi binimetinib. **(C)** MAPK pathway activity in BRAF V600E, BRAF G469A, CDK5RAP2-BRAF and AGK-BRAF cells treated with plixorafenib single agent and in combination with the 4 MEKis at the IC:25 concentration of each of the MEKis. **(D)** IC:50 (nM) summary of BRAF alterations for plixorafenib single agent and in combination with the 4 MEKis at IC:25 concentration of each of the MEKis.

Plixorafenib with low dose binimetinib is more potent than vemurafenib, tovorafenib, and lifirafenib with binimetinib in BRAF V600E mutated cells

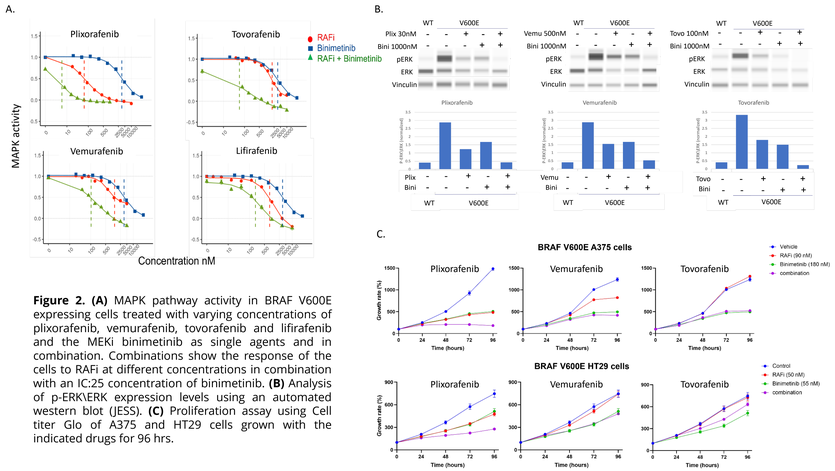


Figure 2. (A) MAPK pathway activity in BRAF V600E expressing cells treated with varying concentrations of plixorafenib, vemurafenib, tovorafenib and lifirafenib and the MEKi binimetinib as single agents and in combination. Combinations show the response of the cells to RAFi at different concentrations in combination with an IC:25 concentration of binimetinib. **(B)** Analysis of p-ERK\ERK expression levels using an automated western blot (JESS). **(C)** Proliferation assay using Cell titer Glo of A375 and HT29 cells grown with the indicated drugs for 96 hrs.

Plixorafenib with low dose binimetinib is more potent than vemurafenib, tovorafenib, and lifirafenib with binimetinib in BRAF non-V600 mutated cells

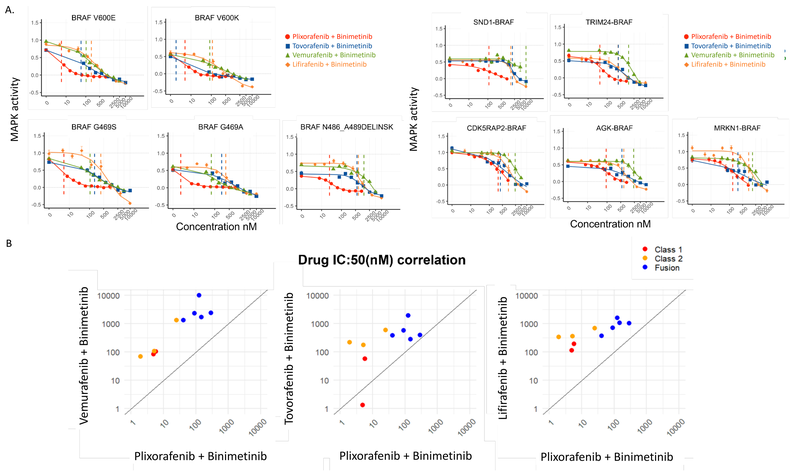


Figure 3. (A) MAPK pathway activity in BRAF V600 and non V600 mutated cells treated with the combinations of binimetinib with plixorafenib, tovorafenib, vemurafenib and lifirafenib. Binimetinib was used at the IC:25 concentration for each BRAF alteration. **(B)** IC:50 correlation between plixorafenib and binimetinib combination to 3 other combinations presented in (A).

Plixorafenib acquired resistant cells induced by long-term exposure in stepwise increment of concentration of plixorafenib demonstrated upregulation of MAPK pathway, which are sensitized by the addition of the MEKi binimetinib

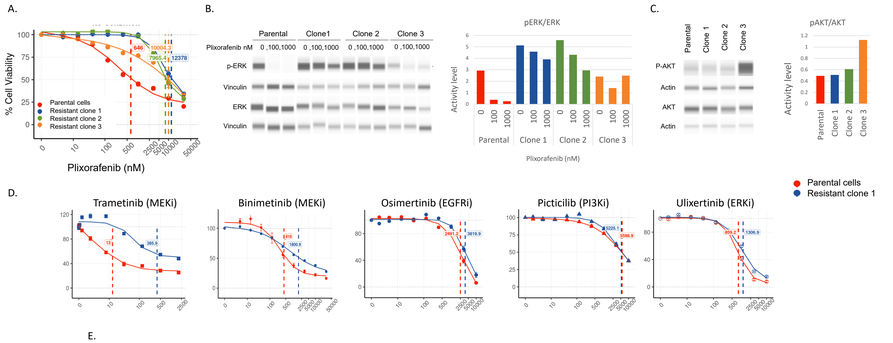


Figure 4. Plixorafenib acquired resistant cell clones were developed from established human cancer cell line ES-2 (Ovarian Ca., BRAF V600E, MEK1 p.D67N) and A375 (Melanoma, BRAF V600E,mutation w/o other driver mutations) by long term continuous exposure of cells to plixorafenib in stepwise increments of concentration. **(A)** Cell viability assay showing the acquired resistance of 3 clones derived from the BRAF V600E mutated ES-2 ovarian cancer cells. The assay was performed for 72 hrs and analyzed using CellTiter Glo. **(B)** Western blot analysis (JESS™) showing the p-ERK activation and response to plixorafenib at the indicated concentrations of ES-2 parental cells and 3 resistant clones. **(C)** Western blot analysis (JESS™) showing the p-AKT activation of ES-2 parental cells and 3 resistant clones. **(D)** Cell viability assays showing the response of ES-2 parental and resistant clone 1 cells to the indicated drugs. The assay was performed for 72 hrs and analyzed using CellTiter Glo. **(E)** Analysis of synergy between plixorafenib and binimetinib in 2 plixorafenib acquired resistant cell line clones derived from ES-2 and A375 cells. The assay was performed for 72 hrs and analyzed using CellTiter Glo, in the drug concentrations indicated. The "SynergyFinder" server was used for the analysis.

Conclusions

- Plixorafenib in combination with a MEKi (trametinib, cobimetinib, mirdametinib, or binimetinib) showed synergistic inhibition of MAPK pathway activity in BRAF-altered tumor models compared with plixorafenib or any of the MEK inhibitors as single agents
- Plixorafenib in combination with binimetinib showed greater potency against BRAF V600 and non-V600 alterations compared with binimetinib combinations with vemurafenib (a first-generation BRAFi) or tovorafenib or lifirafenib (pan-RAF inhibitors)
- Plixorafenib + binimetinib is active and synergistic at clinically relevant nanomolar concentrations; the combination also exhibited synergistic activity in plixorafenib-resistant cells
- The robust anti-cancer activity of plixorafenib, a novel BRAFi, combined with a MEKi support the potential of dual MAPK pathway suppression for the treatment of:
  - Cancers harboring BRAF V600 or non-V600 alterations
  - BRAF V600 mutated cancers with acquired resistance

Fore