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Titration of signalling output: insights into clinical combinations of MEK and AKT inhibitors

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Background: We aimed to understand the relative contributions of inhibiting MEK and AKT on cell growth to guide combinations of these agents.

Materials and methods: A panel of 20 cell lines was exposed to either the MEK inhibitor, PD0325901, or AKT inhibitor, AKT 1/2 inhibitor. p-ERK and p-S6 ELISAs were used to define degrees of MEK and AKT inhibition, respectively. Growth inhibition to different degrees of MEK and AKT inhibition, either singly or in combination using 96-h sulphorhoda-mine assays was then studied.

Results: A significantly greater growth inhibition was seen in $BRAF^M$ and $PIK3CA^M$ cells upon maximal MEK ($P = 0.004$) and AKT inhibition ($P = 0.038$), respectively. $KRAS^M$ and $BRAF/PIK3CA/KRAS^{WT}$ cells were not significantly more likely to be sensitive to MEK or AKT inhibition. Significant incremental growth inhibition of the combination of MEK + AKT over either MEK or AKT inhibition alone was seen when MEK + AKT was inhibited maximally and not when sub-maximal

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inhibition of both MEK + AKT was used (11/20 cell lines versus 1/20 cell lines; $P = 0.0012$).

Conclusions: $KRAS^M$ cells are likely to benefit from combinations of MEK and AKT inhibitors. Sub-maximally inhibiting both MEK and AKT within a combination, in a majority of instances, does not significantly increase growth inhibition compared with maximally inhibiting MEK or AKT alone and alternative phase I trial designs are needed to clinically evaluate such combinations.

Key words: combination, MEK inhibitor, AKT inhibitor

introduction

MEK and AKT are important nodes in signal transduction pathways critical to growth and function of cancer cells and normal tissue (supplementary Figure S1, available at *Annals of Oncology* online). Early clinical studies of MEK and AKT inhibitors have shown that it is possible to achieve therapeutic drug levels and modulate the respective targets [1–6]. Combining MEK and AKT inhibitors for the treatment of cancer is of interest for multiple reasons. First, inhibition of either MEK and AKT would only partially inhibit signalling output, resulting in sub-optimal growth inhibition of cancer cells [7]. Secondly, in defined models, pharmacological inhibition of MEK causes an increase in signalling through AKT [8, 9]. Thirdly, there is growing evidence of intra-tumoral heterogeneity within cancers [10] and this could lead to areas of a tumour that are differentially sensitive to an MEK or AKT inhibitor alone. There is pre-clinical evidence of the activity of MEK inhibitor + AKT inhibitor combinations [11] and, more generally, of drugs inhibiting the RAS-RAF-MEK and PI3K-AKT-m-TOR axis [12–14]. Early clinical activity of these combinations has been demonstrated [15, 16] and combinations of MEK and AKT inhibitors have now entered biomarker integrated targeted therapy studies such as BATTLE-2 (NCT-01248247), which uses an adaptive randomization design to use the combination in the setting of non-small-cell lung cancer.

The individual toxicities of MEK and AKT inhibitors are now fairly well defined, with ocular toxicities being limited to MEK inhibitors [1–3] and hyperglycaemia limited to AKT inhibitors [4, 6, 17, 18]. Overlapping toxicities include rash and diarrhoea [1–4, 6, 17, 18]. Thus, there are considerable challenges in combining these agents [16]. It is possible to circumvent such toxicities by altering the scheduling of both agents [19].

Over-arching questions that govern combinations of MEK and AKT inhibitors are: (i) which tumours are susceptible to combinations of MEK and AKT inhibitors; (ii) is combined maximal inhibition of MEK or AKT better than maximal MEK or AKT inhibition alone; (iii) does sub-optimally inhibiting signalling due to overlapping toxicity compromise the chances of success of combinations of MEK and AKT inhibitors? We aimed to answer these three questions in pre-clinical models.

materials and methods

cell lines

Source, authentication, mutations and tissue of origin of cell lines are documented in the supplementary Data and Table S1, available at *Annals of Oncology* online.

Details of the drugs used and ELISAs carried out to quantify inhibition of signalling are in the supplementary Data, available at *Annals of Oncology* online.

definition of inhibition of MEK and AKT

ELISA readings were normalized to the DMSO control being assessed as 0% and the maximal inhibition of signalling output achieved as 100%. The drug concentration required to cause 25%, 50%, 75% and 100% of maximal reduction of p-S6 or p-ERK levels was then calculated. GraphPad Prism (v6.0, GraphPad Software, Inc., La Jolla, CA) was used for the analysis. 100%, 75%, 50%, 25% inhibition of MEK was defined as maximal reduction in levels of p-ERK or 75%, 50%, 25% of maximal reduction of levels of p-ERK. 100%, 75%, 50%, 25% inhibition of AKT was defined as maximal reduction in levels of p-S6 or 75%, 50%, 25% of maximal reduction of levels of p-S6.

growth inhibition assays

Each of the 20 cell lines was exposed for 96 h to concentrations of PD0325901 and AKT 1/2 kinase inhibitor to inhibit signalling of p-S6 and p-ERK by different degrees, as specified in each experiment. Growth inhibition was calculated using sulphorhodamine assays, as described previously [20]. A similar experiment was done in one randomly chosen cell line from each group ($BRAF^M$, $PIK3CA^M$, $KRAS^M$ and $BRAF/PIK3CA/KRAS^{WT}$) using a clinically used MEK inhibitor (AZD6244) and AKT inhibitor (AZD5363).

statistical analysis

Differences between growth inhibition upon being exposed to concentrations that maximally inhibited MEK and AKT in a given cell line were analysed using a *t*-test. A one-way ANOVA was carried out to detect differences between maximal growth inhibition caused by inhibiting MEK by 100% and combinations such as inhibition of MEK 100% + AKT 25%, MEK 100% + AKT 50%, MEK 100% + AKT 25% and MEK 100% + AKT 100%. *Post hoc* Dunnett's tests were carried out only if the ANOVA showed a significant difference. Similar analysis was carried out while testing differences between growth inhibition caused by 100% AKT inhibition and combinations of 100% AKT inhibition and increasing concentrations of MEK inhibition. A one-way ANOVA was also conducted to detect differences between 100% MEK or AKT inhibition and sub-optimal combinations of MEK and AKT, *post hoc* Dunnett's tests were carried out only if the ANOVA showed a significant difference. Differences between the number of cell lines categorized by mutation or degrees of inhibition were carried out using Fisher's exact test. GraphPad Prism (v6.0, GraphPad Software, Inc.) was used for the analysis.

results

A panel of 20 cell lines (5 $BRAF^M$, 5 $PIK3CA^M$, 5 $KRAS^M$, 5 $BRAF/PIK3CA/KRAS^{WT}$), was exposed to a concentration of PD0325901 and AKT1/2 kinase inhibitor that caused maximal MEK and AKT inhibition, respectively, for 96 h and growth inhibition compared with a DMSO-treated control was analysed (Figure 1A) . The ED_{50} concentrations of PD0325901 and AKT 1/2 kinase inhibitors to inhibit MEK and AKT are documented in supplementary Table S2, available at *Annals of Oncology* online. Six of 17 cell lines showed more significantly greater growth inhibition upon maximal MEK inhibition compared
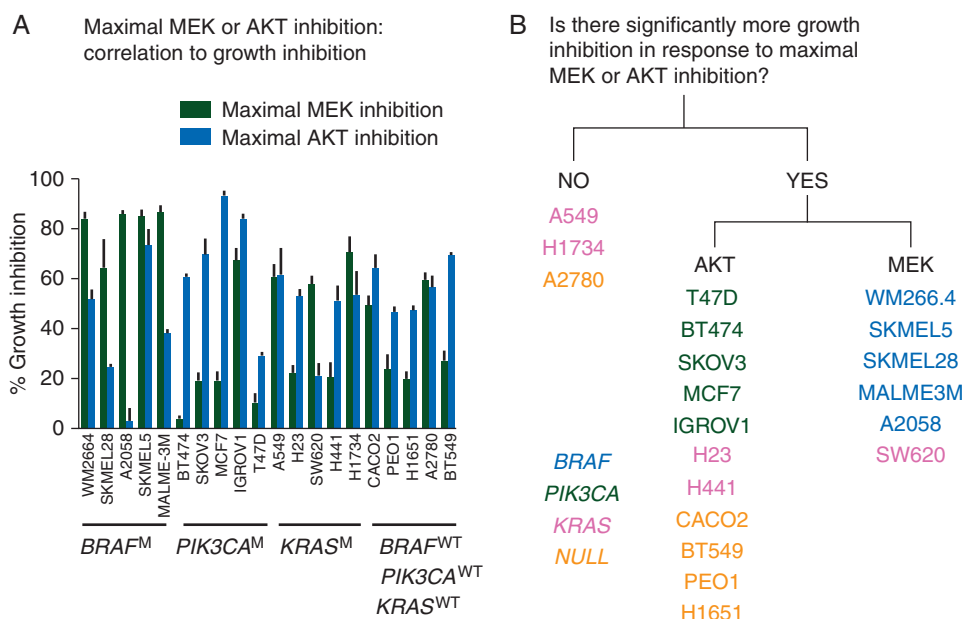
Figure 1. Dependence of growth inhibition on maximal inhibition of signalling output. A panel of 20 cell lines was exposed to concentrations of PD0325901 and AKT1/2 kinase inhibitor which caused maximal MEK and AKT inhibition, respectively, for 96 h. (A) The percentage growth inhibition in cell lines caused by maximal MEK and AKT inhibition. The black (green online) columns represent growth inhibition caused by maximal MEK inhibition and the grey (blue online) columns represent growth inhibition caused by maximal AKT inhibition; (B) the cell lines were classified according to the presence or absence of a statistical difference between maximal MEK and AKT inhibition.

with maximal AKT inhibition and 11/17 showed significantly greater inhibition upon maximal AKT inhibition compared with maximal MEK inhibition. Three cell lines were equally sensitive to maximal MEK and AKT inhibition (Figure 1B). $BRAF^M$ and $PIK3CA^M$ cells were more sensitive to growth inhibition by maximal MEK inhibition; $P = 0.0004$ and maximal AKT inhibition; $P = 0.038$, respectively. $KRAS^M$ and $BRAF/PIK3CA/KRAS^{WT}$ cells were not significantly more likely to be sensitive to MEK or AKT inhibition.

Four cell lines, i.e. SKMEL-28 ($BRAF^M$), T47D ($PIK3CA^M$), A549 ($KRAS^M$) and A2780 ($BRAF/PIK3CA/KRAS^{WT}$), were exposed to a clinically used MEK inhibitor (AZD6244) and AKT inhibitor (AZD5363) that caused maximal MEK and AKT inhibition, respectively, and this caused growth inhibitory patterns similar to that caused by PD0325901 and AKT 1/2 kinase inhibitor (supplementary Data, available at *Annals of Oncology* online). This suggests that the growth inhibitory patterns seen of PD0325901 and AKT 1/2 kinase inhibitor unlikely to be due to off target effects.

The panel of cell lines was then exposed to maximal inhibition of MEK, in addition to increasing degrees of AKT inhibition (i.e. 25%, 50%, 75% and 100%) (Figure 2A). Only 1/5 $BRAF^M$ cells had incremental growth inhibition by the addition of AKT inhibition to the maximal MEK inhibition. The panel of cells was also exposed to maximal inhibition of AKT in addition to increasing degrees of MEK inhibition (i.e. 25%, 50%, 75% and 100%) (Figure 2B). Of the $PIK3CA^M$ lines, 3/5 cell lines showed significant incremental benefit of the addition of MEK inhibition to maximal AKT inhibition. The findings are summarized in Figure 2C. In the 20 cell line panel studied, maximal MEK + AKT inhibition

caused greater growth inhibition than maximal inhibition than either maximal MEK or maximal AKT in 11/20 cell lines.

Next, the panel was exposed to different combinations of sub-maximal inhibition of MEK + AKT (25% + 25%, 50% + 50% and 75% + 75%) (Figure 3A). Only 1/20 cell lines showed significantly greater growth inhibition upon sub-maximal inhibition of MEK + AKT compared with either maximal MEK or maximal AKT inhibition.

Thus, in the cell line panel studied, in 11/20 cell lines, maximal MEK + AKT inhibition caused significantly greater growth inhibition compared with maximal inhibition cause by singly inhibiting MEK or AKT; however, a sub-maximal inhibition of MEK + AKT caused superior growth inhibition caused by singly maximally inhibiting MEK or AKT in only 1/20 cell lines; $P = 0.0012$ (Figure 3B).

Interestingly, in both HUVEC and Ker-CT cells (representing non-cancer 'normal' cells) sub-optimal inhibition of MEK or AKT did not cause significantly more growth inhibition compared with maximal AKT or MEK inhibition alone (whichever the cell line was sensitive to, or both, if the cell line was sensitive to both if the cell line was equally sensitive to MEK and AKT inhibition) (details in supplementary Data and Figure S2, available at *Annals of Oncology* online).

discussion

Currently, there are eight ongoing phase I studies of MEK and AKT inhibitors in combination or which have recently completed recruitment (http://clinicaltrials.gov/ct2/results?term=mek+akt). There is an urgent and unmet need to understand the relative contributions of inhibition of both these targets on cell

A

	MEK 100 AKT 0	MEK 100 AKT 25	MEK 100 AKT 50	MEK 100 AKT 75	MEK 100 AKT 100	Significant?
WM266.4	83.77	86.66	87.83	89.52	86.94	N
SKMEL-28	64.03	70.28	73.31	74.24	74.03	N
A2058	85.38	87.55	87.16	87.09	86.83	N
SKMEL-5	84.62	85.02	86.26	88.83	92.33	Y
MALME-3	86.32	86.13	86.37	89.16	89.04	N
BT474	3.98	24.17	24.01	37.8	63.21	Y
SKOV-3	18.75	38.65	48.14	58.16	79.59	Y
MCF-7	17.29	30.09	47.7	67.45	94.61	Y
IGROV1	66.92	77.44	80.81	87.24	91.38	Y
T47D	9.75	12.99	16.14	23.16	35.02	Y
A549	60.32	64.29	63.19	73.59	75.25	N
H23	21.9	44.79	50.19	64.58	72.6	Y
SW620	57.58	58.78	58.04	60.35	67.47	N
H441	20.08	50.45	57.22	64.11	69.3	Y
H1734	70.28	81.45	83	84.83	85.94	Y
CACO2	48.94	64.69	69.36	75.35	78.05	Y
PEO1	23.34	29.14	27.23	32.05	57.19	Y
H1651	19.65	30.15	29.45	42.02	56.21	Y
A2780	59.46	71.36	72.52	76.01	84.24	Y
BT549	26.82	50.71	55.74	65.31	76.24	Y

$BRAF^M$ (WM266.4–MALME-3); $PIK3CA^M$ (BT474–T47D); $KRAS^M$ (A549–H1734); $BRAF^{WT}/PIK3CA^{WT}/KRAS^{WT}$ (CACO2–BT549)

B

	MEK 0 AKT 100	MEK 25 AKT 100	MEK 50 AKT 100	MEK 75 AKT 100	MEK 100 AKT 100	Significant?
WM266.4	51.51	65.01	69.66	79.77	86.94	Y
SKMEL-28	24.68	48.08	62.73	69.54	74.03	Y
A2058	2.86	24.02	52.96	85.86	86.83	Y
SKMEL-5	73.41	84.08	91.54	92.01	92.33	Y
MALME-3	37.86	51.57	77.15	86.49	89.04	Y
BT474	60.53	61.15	63.18	62.52	63.21	N
SKOV-3	69.4	73.24	75.05	79.19	79.59	Y
MCF-7	93.04	94.01	94.59	95.38	94.61	N
IGROV1	83.85	89.19	90.25	90.94	91.38	Y
T47D	29.21	29.88	30.79	31.3	35.02	Y
A549	61.22	58.96	70.16	73.52	75.25	N
H23	53.01	58.41	63.78	64.39	72.6	Y
SW620	20.94	42.58	56.13	61.33	67.47	Y
H441	51.07	58.04	62.58	65.63	69.3	Y
H1734	53.66	60.57	63.01	72.37	85.94	Y
CACO2	64.13	67.72	68.88	73.26	78.05	Y
PEO1	46.44	50.3	54.42	52.74	57.19	Y
H1651	47.03	50.66	51.77	54.33	56.21	N
A2780	56.16	63.93	71.19	72.82	84.24	Y
BT549	69.08	72.18	74.36	75.4	76.24	Y

C

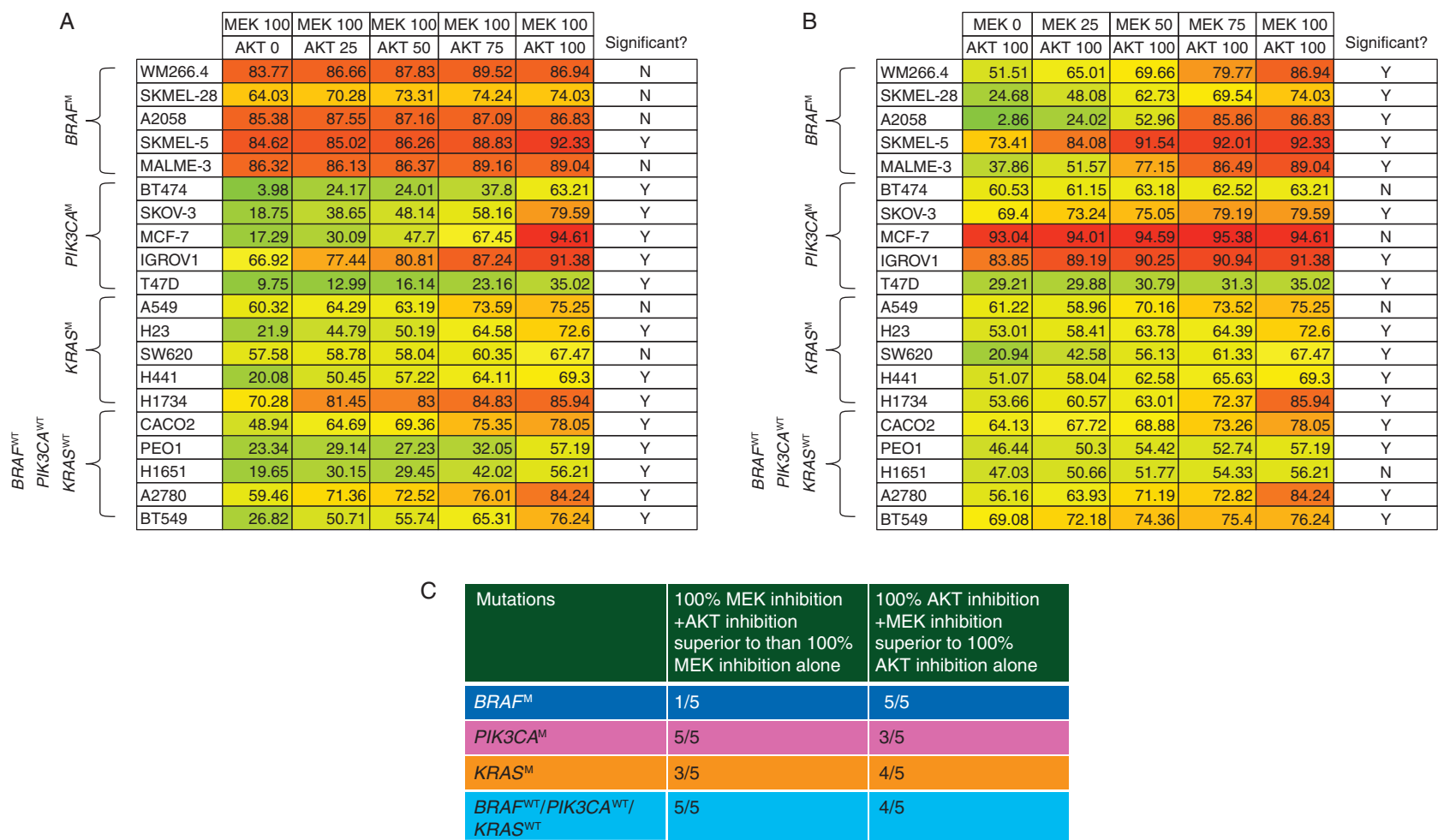| Mutations	100% MEK inhibition +AKT inhibition superior to than 100% MEK inhibition alone	100% AKT inhibition +MEK inhibition superior to 100% AKT inhibition alone
$BRAF^M$	1/5	5/5
$PIK3CA^M$	5/5	3/5
$KRAS^M$	3/5	4/5
$BRAF^{WT}/PIK3CA^{WT}/KRAS^{WT}$	5/5	4/5

Figure 2. Percentage growth inhibition caused by combinations of MEK and AKT inhibitors. Cell lines were exposed to combinations of concentrations of PD0325901 and AKT1/2 kinase inhibitor at which different degrees of MEK and AKT inhibition occurred for 96 h. A one-way ANOVA was carried out to detect differences between growth inhibition caused by maximal MEK or AKT inhibition alone compared with combinations. *Post hoc* Dunnett's test was only carried out if the ANOVA showed a significant difference. (A) Cell lines exposed to 100% MEK inhibition and increasing levels of AKT inhibition; (B) cell lines exposed to 100% AKT inhibition and increasing levels of MEK inhibition. (C) A summary of numbers of cell lines where there was significantly greater growth inhibition caused by the combinations of MEK + AKT over that caused by maximal inhibition of MEK or AKT alone.
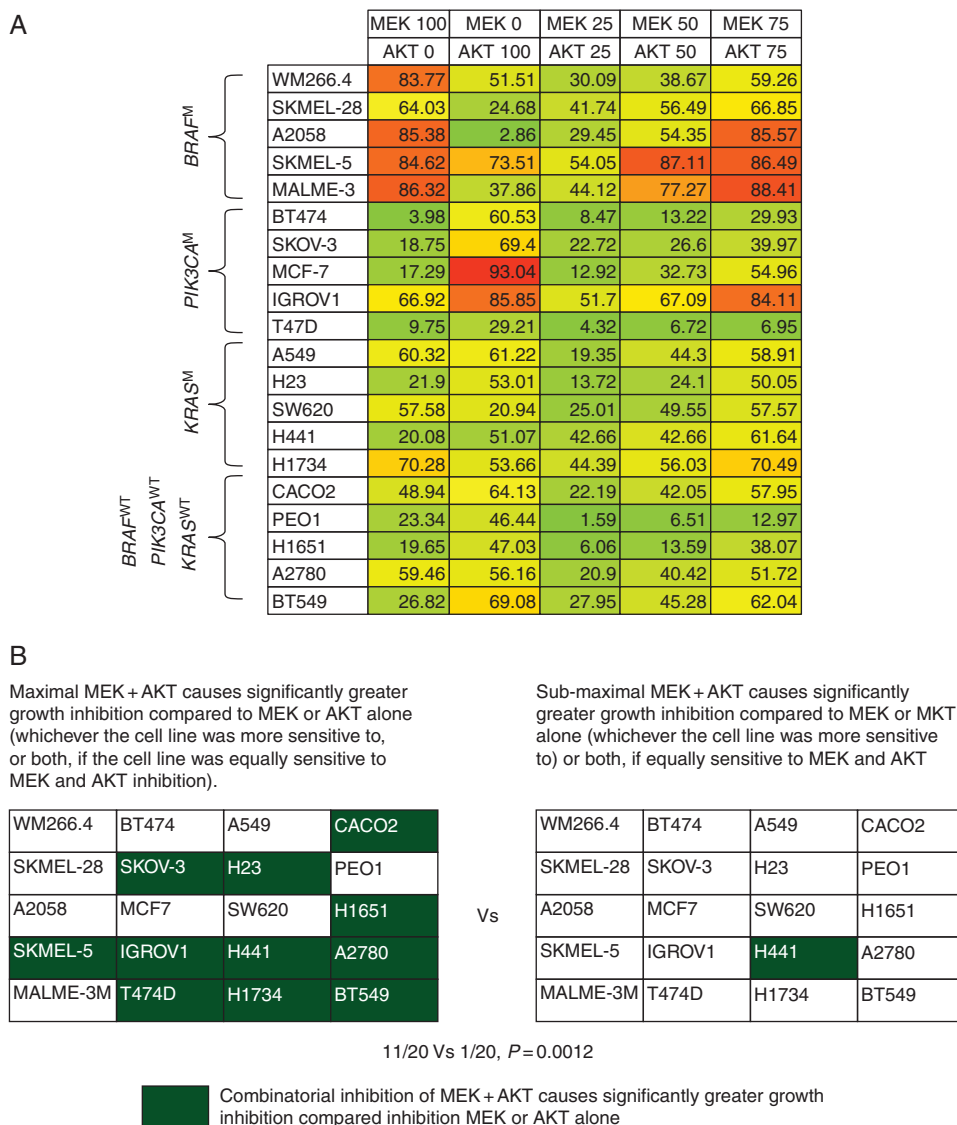
A

		MEK 100	MEK 0	MEK 25	MEK 50	MEK 75
		AKT 0	AKT 100	AKT 25	AKT 50	AKT 75
$BRAF^M$	WM266.4	83.77	51.51	30.09	38.67	59.26
	SKMEL-28	64.03	24.68	41.74	56.49	66.85
	A2058	85.38	2.86	29.45	54.35	85.57
	SKMEL-5	84.62	73.51	54.05	87.11	86.49
	MALME-3	86.32	37.86	44.12	77.27	88.41
$PIK3CA^M$	BT474	3.98	60.53	8.47	13.22	29.93
	SKOV-3	18.75	69.4	22.72	26.6	39.97
	MCF-7	17.29	93.04	12.92	32.73	54.96
	IGROV1	66.92	85.85	51.7	67.09	84.11
	T47D	9.75	29.21	4.32	6.72	6.95
$KRAS^M$	A549	60.32	61.22	19.35	44.3	58.91
	H23	21.9	53.01	13.72	24.1	50.05
	SW620	57.58	20.94	25.01	49.55	57.57
	H441	20.08	51.07	42.66	42.66	61.64
	H1734	70.28	53.66	44.39	56.03	70.49
$BRAF^{WT}$ $PIK3CA^{WT}$ $KRAS^{WT}$	CACO2	48.94	64.13	22.19	42.05	57.95
	PEO1	23.34	46.44	1.59	6.51	12.97
	H1651	19.65	47.03	6.06	13.59	38.07
	A2780	59.46	56.16	20.9	40.42	51.72
	BT549	26.82	69.08	27.95	45.28	62.04

B

Maximal MEK + AKT causes significantly greater growth inhibition compared to MEK or AKT alone (whichever the cell line was more sensitive to, or both, if the cell line was equally sensitive to MEK and AKT inhibition).

WM266.4	BT474	A549	CACO2
SKMEL-28	SKOV-3	H23	PEO1
A2058	MCF7	SW620	H1651
SKMEL-5	IGROV1	H441	A2780
MALME-3M	T474D	H1734	BT549

Vs

Sub-maximal MEK + AKT causes significantly greater growth inhibition compared to MEK or MKT alone (whichever the cell line was more sensitive to) or both, if equally sensitive to MEK and AKT

WM266.4	BT474	A549	CACO2
SKMEL-28	SKOV-3	H23	PEO1
A2058	MCF7	SW620	H1651
SKMEL-5	IGROV1	H441	A2780
MALME-3M	T474D	H1734	BT549

11/20 Vs 1/20, $P = 0.0012$

Combinatorial inhibition of MEK + AKT causes significantly greater growth inhibition compared inhibition MEK or AKT alone

Figure 3. Percentage growth inhibition caused by combinations of MEK and AKT inhibitors: Effects of sub-maximal inhibition. Cell lines were exposed to combinations of concentrations of PD0325901 and AKT1/2 kinase inhibitor at which different degrees of MEK inhibition and AKT inhibition occurred for 96 h. (A) Cell lines were exposed to 100% MEK inhibition, 100% AKT inhibition or different combinations of sub-maximal MEK + AKT inhibition. (B) A summary of the number of cell lines where the maximal and sub-maximal combinations of MEK + AKT caused significantly greater growth inhibition compared with MEK or AKT inhibition alone (whichever the cell line was more sensitive to, or both, if the cell line was equally sensitive to MEK and AKT inhibition).

growth in order to optimize these combinations because overlapping toxicities make them difficult to deliver [16, 19].

The cell lines with different mutations have been selected from various tumour types. It is now emerging that context specificity is important to feedback loops in signalling pathways [21], and future work will be better done in specific disease contexts. We considered a variety of methods that could potentially be used to understand individual contributions of MEK and AKT signalling. We chose to study p-ERK as a downstream readout of MEK inhibition as it downstream of MEK. We did not choose p-AKT Ser473 to study AKT inhibition as allosteric and ATP competitive drugs used to inhibit AKT caused diametrically opposite effects on phosphorylation at this site [4, 22]. We were not able to get consistent results showing reduction of p-m-TOR following AKT inhibition thus chose p-S6. This study

could be potentially strengthened by *in vivo* experiments but would require an extremely large cohort of animals so was deemed as not justified. Our study did not to study feedback loops activating receptor tyrosine that can be caused by MEK or AKT inhibition as this has been done before [23].

When MEK and AKT inhibitors were used as single agents, the findings that $BRAF^M$ and $PIK3CA^M$ cell lines in the panel were significantly more sensitive to MEK and AKT inhibitors, respectively. However, $KRAS^M$ cell lines in the panel were either significantly more sensitive to AKT (H23, H441) or MEK (SW620) inhibition in some instances or equally sensitive to MEK and AKT inhibition (A549, H1734), suggesting that it is likely that cells with KRAS mutations are not predominantly dependant on MEK or AKT signalling alone for cell growth. Our findings that $KRAS^M$ cancers could respond to combinations of
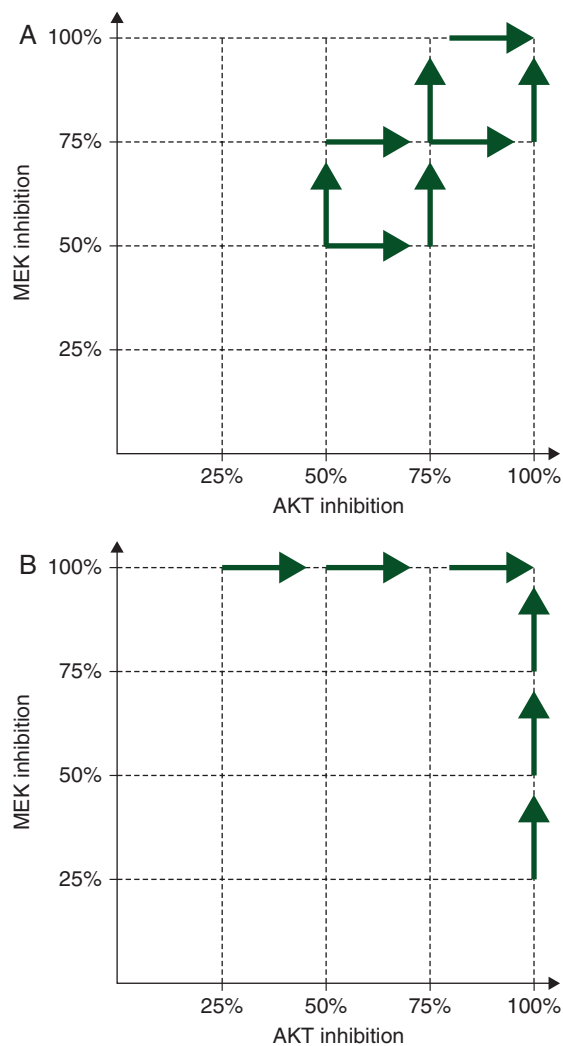
Figure 4. Dose escalation paradigms in phase I studies of combinations of MEK and AKT inhibitors. (A) Current dose escalation strategies of MEK and AKT inhibitors start at doses of MEK and AKT inhibitors lower than the doses that cause maximal pharmaco-dynamic effects for both drugs defined in phase I studies. (B) The proposed dose escalation schedule at two different cohorts, the first starts with the dose of the MEK inhibitor that causes maximal pharmaco-dynamic effects and additional cohorts of increasing doses of AKT inhibitor are added. The second starting cohort will start with a dose of AKT inhibitor that causes maximal pharmaco-dynamic effects and additional cohorts of patients with increasing does of MEK inhibitor will be added. This model always has a dose of MEK or AKT inhibitor that maximally inhibits its intended target.

MEK + AKT inhibitors is interesting and other groups have shown MEK + AKT inhibitors could be effective in cells with mutations both in *KRAS* and *PIK3CA* [24].

All five *BRAF*^{M} cell lines that were significantly more sensitive to MEK inhibition and only 1/5 *BRAF*^{M} cell lines showed incremental growth inhibition by addition of maximal AKT inhibition to maximal MEK inhibition. The cell line SKMEL-5 was shown to be sensitive to MEK inhibition but additive growth inhibition was observed upon AKT inhibition. SKMEL-5 cells are known to have a *BRAF* mutation but no PTEN loss [25] and previous studies have suggested signalling through IGFR-1 is a potential mechanism of resistance to MEK inhibitors in this cell line [9].

Our findings are interesting and show that, for the first time, combinations of MEK + AKT inhibitors are unlikely to be of added benefit to MEK inhibitors alone in cell line models studied which have only *BRAF* mutations. It is possible that cell lines with *BRAF* and *PIK3CA* mutations may benefit from the combination. The findings contradict pre-clinical studies that suggest melanomas with BRAF mutation may benefit from inhibition of dual MEK and PI3K pathway inhibition [13, 26]. All the *PIK3CA*^{M} cell lines studied were more sensitive to AKT inhibition and *PIK3CA*^{M} sensitizing cells to AKT mutation has been shown before [27]. However, 3/5 PIK3CA^M cell lines in our study showed significantly more growth inhibition with the combination of maximal AKT + MEK inhibition suggesting that the combination of MEK + AKT inhibition may be of benefit to *PIK3CA*^{M} cancers.

Crucially, our studies show for the first time that in only 1/20 cell lines did a combination of sub-maximal inhibition of MEK + AKT cause a significantly greater growth inhibition compared with growth inhibition caused by maximal MEK or AKT inhibition alone (whichever the cell line was sensitive to, or both, if the cell line was equally sensitive to MEK and AKT inhibition). This is particularly relevant as it is often difficult to deliver full does of combinations of MEK and PI3K pathway inhibitors in the clinic [16]. However, the effect of sub-optimal inhibition of signal transduction on evolution of resistant clones has not been explored in the present study and such experiments may provide further insights into the use of combinations of MEK and AKT inhibitors. The dependence on the degree of inhibition of p-ERK to clinical response in *BRAF*^{M} cancers has been previously described and supports our findings [28]. Currently, clinical trials exploring these combinations often started with sub-optimal inhibitory doses of both MEK and AKT inhibitors. The present pre-clinical data suggest that it is preferable to start with the dose of the MEK or AKT inhibitor which causes maximal pharmaco-dynamic inhibition and then add progressively higher doses of the second drug, with the aim of reaching the maximal doses of both drugs (Figure 4). If it is not possible to clinically achieve maximal inhibition of one or both pathways as continuous dosing schedules, intermittent schedules could be considered [19].

Experiments in this manuscript also attempted to answer the effects of inhibition of MEK and AKT on normal tissue and thus potential toxicity experienced by patients. Importantly, sub-optimal MEK or AKT inhibition in combination when compared with maximal MEK and AKT alone did not cause more growth inhibition in both HUVEC and Ker-CT cell lines, suggesting no additional toxicity once MEK or AKT is completely inhibited if a second inhibitor is added. In clinical practice that is not true as we struggle to deliver full doses of combinations of both drugs. It is possible that this is due to the selective susceptibility of different cell types that make up normal tissue such as skin and the gut.

This study provides insights into ways of clinically combining MEK and AKT inhibitors.

disclosure

AS, PT, JSB and UB are employees of The Institute of Cancer Research, which has commercial interests in the discovery and development of AKT and AGC kinase inhibitors.

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