MAPK pathway inhibition enhances the efficacy of an anti-endothelin B receptor drug conjugate by inducing target expression in melanoma.

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Abstract

Therapies targeting the MAP kinase pathway in melanoma have produced significant clinical responses; however, duration of response is limited by acquisition of drug resistance. Rationale drug combinations may improve outcomes in this setting. We assessed the therapeutic combination of an antibody drug conjugate (ADC) targeting the Endothelin B receptor (EDNRB) with small molecule inhibitors of the MAP kinase signaling pathway in melanoma. Cell lines and tumor models containing either mutant BRAF or NRAS, or wildtype for both, were exposed to small molecule inhibitors or BRAF and MEK. Expression of EDNRB was analyzed and the therapeutic impact of combining the anti-EDNRB ADC with the BRAF and MEK inhibitors was assessed. Increased expression of EDNRB in response to inhibition of BRAF and/or MEK was observed and augmented the antitumor activity of the ADC. Enhanced target expression and ADC anti-tumor activity were realized irrespective of the response of the tumor model to the BRAF or MEK inhibitors alone and could be achieved in melanoma with mutant NRAS, BRAF or neither mutation. Cells that acquired resistance to BRAF inhibition through long-term culture retained drug-induced elevated levels of EDNRB expression. Expression of EDNRB was not enhanced in normal human melanocytes by inhibition of BRAF and the combination of the ADC with MAPK inhibitors was well tolerated in mice. The anti-EDNRB ADC combines well with BRAF and MEK inhibitors and could have therapeutic utility in the majority of human melanoma cases.
Introduction

Antibody drug conjugates (ADC) have emerged as a promising new class of cancer therapeutics. High-throughput screening technologies have enabled the identification of highly specific targets that serve as selective entry points for antibodies appended with extremely potent cytotoxic agents. Numerous antibody drug conjugates are now in various stages of clinical development and one was recently granted accelerated approval based on profound responses achieved in CD30-positive lymphomas (1-3). To some extent, the criteria for applying an ADC approach in cancer is simplified by the use of drugs that kill cells by generalized mechanisms rather than relying upon specific genetic attributes of the target. Therefore, there is an opportunity for ADC therapy to serve as a more tolerable, more effective alternative to standard of care chemotherapy, which is frequently used in combination with targeted treatments.

Expression of EDNRB is highly restricted in normal adult tissues and is overexpressed in the majority of metastatic melanomas (4, 5). We have recently described an anti-endothelin B receptor (EDNRB) ADC comprised of a humanized monoclonal antibody conjugated to monomethylauristain E (MMAE) that is efficacious in mouse xenograft models of human melanoma (5). Upon binding to cells, the antibody is rapidly internalized and the released drug disrupts cell proliferation by interfering with microtubule dynamics. The ADC has demonstrated efficacy in human melanoma xenograft models expressing amounts of cell-surface EDNRB comparable to that in the patient population. In both rodent and primate models the anti-EDNRB ADC exhibits
favorable pharmacokinetic properties and is well tolerated at exposures predicted to be efficacious based on tumor xenograft studies (unpublished observation, JA, PP).

Metastatic melanoma is a highly aggressive cancer with a 5-year survival rate of less than 10% and median survival of less than 8 months (6-8). The incidence of melanoma has increased over the past few decades and the annual mortality rate has surpassed 9000 in North America. Standard of care treatment for metastatic melanoma includes administration of the alkylating agent dacarbazine and the cytokine Interleukin-2 (9). However, the response rate to these therapies is very low and durable responses are rare. More recently, a number of innovative therapies targeting the MAPK pathway have produced significant responses in advanced metastatic disease (10, 11). In particular, vemurafenib, an FDA approved small molecule inhibitor of BRAF, has demonstrated increased overall survival in the treatment of metastatic melanoma containing activating BRAF mutations (12, 13). Additional compounds targeting BRAF, as well as MEK, which resides immediately downstream of BRAF, are also under development (14-17). The incidence of BRAF mutations in metastatic melanoma is approximately 50% with an additional 15% harboring NRAS mutations (18). Thus, the majority of melanoma patients will qualify diagnostically for treatment with these agents.

Because of the importance of MAPK pathway inhibition in the treatment of melanoma, combination therapy has the potential to provide improved outcomes, and is likely to become standard of care. However, it is beneficial to establish a biological or pharmacological rationale for combination strategies in advance. Considering the emergence of MAPK pathway inhibitors and their anticipated widespread adoption in the treatment of melanoma, we assessed their impact on
the expression levels of EDNRB and the efficacy of the corresponding ADC in the preclinical setting. Using melanoma cell lines and tumor xenografts, we examined several scenarios under which the drugs might influence the activity of the anti-EDNRB ADC: mutant BRAF, responsive to BRAF inhibition; mutant BRAF, intrinsically resistant to BRAF inhibition; wildtype BRAF and NRAS; and wildtype BRAF and mutant NRAS. Finally, we examined mutant BRAF cells adapted in long-term culture to resist BRAF inhibition. Under all scenarios described, MAPK pathway inhibitors elevate the levels of EDNRB on melanoma cells and thereby improve the efficacy of the ADC.
Materials and Methods:

MAPK pathway inhibitors- All MAPK pathway inhibitor compounds were synthesized at Genentech. The chemical structures of PLX4032 (19), GDC-0973 (20) and G590945 (21) have been described previously. The chemical structure of GDC-0623 is presented in Suppl. Fig. S1A.

Immunological procedures- Flow Cytometry or fluorescence-activated cell sorting (FACS), western blotting and production of in-house antibodies against EDNRB were conducted as described previously (5). In-house generated anti-EDNRB antibodies (humanized 5E9 and murine 1H1.8.5) were used for EDNRB cell surface and total protein detection. Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) and p44/42 MAPK (ERK1/2) antibodies (9101 and 9102; Cell Signaling Technology) were used to evaluate MAPK activities.

Cell culture:

The cell lines A2058, COLO 829, IPC-298, SK23-mel, SK-MEL-5, UACC-257 and WM-266-4 were obtained from the American Type Culture Collection or NCI-60 [National Cancer Institute (NCI)]. The cell lines 928mel and 537mel were a generous gift from Paul Robbins (Center for Cancer Research, Tumor Immunology Section, NCI). The Genentech in-house cell bank acquired cell lines over a period of more than 10 years. Cell lines were tested for mycoplasma using the MycoAlert™ Mycoplasma Detection Kit from Lonza and MycoSensor PCR Assay Kit from Stratagene and authenticated by STR and SNP profiling before
distribution to end users. Cells were used for less than 6 months after distribution. Cells were cultured in appropriate media at 37°C and 5% CO₂. Normal adult normal human epidermal melanocytes were obtained from Cascade Biologics, Invitrogen by end user and not authenticated further.

The UACC-257X2.2 cell line is a derivative of the parental UACC-257 cell line (NCI-Frederick Cancer DCT Tumor Repository) optimized for growth \textit{in vivo}. Parental UACC-257 cells were injected subcutaneously in the right flank of female NCr nude mice, one tumor was harvested, dissociated and grown \textit{in vitro} resulting in the UACC-257X1.2 cell line. The UACC-257X1.2 line was injected again subcutaneously in the right flank of female NCr nude mice in an effort to improve the \textit{in vivo} growth characteristics of the cell line. A tumor from this study was collected and again adapted for \textit{in vitro} growth to generate the UACC-257X2.2 cell line. This cell line retains high-level expression of EDNRB as determined by Flow Cytometry.

To develop cell lines resistant to BRAF inhibitor PLX4032, V600E BRAF cell lines 928 mel, UACC-257X2.2 and V600D BRAF cell line WM-266-4 were grown in culture media containing step-wise increasing concentrations of PLX4032 over a period of 10-12 weeks to obtain a sub-population of these cells that were viable in relatively high concentrations of the inhibitor. PLX4032 resistant lines, 928 mel and UACC-257X2.2 could be maintained in culture media containing 2 μM PLX4032 whereas PLX4032-resistant WM-266-4 cell line could be maintained in culture media containing 1 μM PLX4032.

To study the effects of signaling pathway inhibitors on melanoma cells, $2 \times 10^6$ cells were plated on
4-well dishes and treated overnight with various inhibitors at the indicated concentrations. To study the MAPK phosphorylation status of cell lines rendered step-wise resistant to BRAF inhibitor PLX4032, parental and PLX4032 resistant lines were serum starved overnight on 60 mm dishes, in the presence of 10 μM BRAF inhibitor PLX4032 as indicated. Cells from the experiments described above, were harvested, either live for Flow Cytometry or into a 1% n-Dodecyl β-D-maltoside (DDM) containing lysis buffer to prepare lysates for further analysis by immunoblotting.

To assess the effects of PLX4032 on cell viability, cells were plated at 1,500 per well in 50 μL of normal growth medium in 96-well clear-bottom plates. Twenty-four hours later, an additional 50 μL of culture medium with serial dilutions of PLX4032 or controls was added to triplicate wells. Five days later, cell survival was determined using CellTiter-Glo Luminescent Cell Viability Reagent (G7572; Promega Corporation) and with an EnVision 2101 Multilabel Reader (Perkin-Elmer).

Transcript analysis- mRNA transcript analysis were performed in triplicate on a 7500 Real Time PCR thermal cycler, Applied Biosystems (ABI) using ABI reagents. Either FAM-TAMRA or FAM-BHQ fluorophore labeled probes were used with flanking primers for the detection of various genes. Primer and probe sets were designed as follows:

Human EDNRB; Forward primer-5’-TCACCGATTTCCCTGATTAACC, Reverse primer-5’-GCATAAGCATGACTTAAAGCAGTT, probe-5’-AATTGCTCTGTATTTGGTGAGCAAAAGATTCAA; Murine EDNRB: Forward primer-5’-GAAGCAGTCCTGCCTGAAG, Reverse primer-5’-ATTGCTGGACCGGAAGTT, probe, 5’- TCAAAGCCAACGATCACGGATATGAC; GAPDH (Glyceraldehyde
3-phosphate dehydrogenase): Forward primer-5’- GAGTCCCTGCCACACTCA, Reverse primer-5’-GGGGTCTACATGGCAACTG, probe-5’-CCCCACCACACTGAATCTCCCC; Hprt1 (Hypoxanthine phosphoribosyl transferase 1): Forward primer-5’-CACATCAAAGACAGCATCTAAGAA, Reverse primer-5’-CAAGTTGGAAAATACAGTCAACATT, probe-5’-TTTTGTTCTGTCTGGAATTATTTTAGTGTGTTC; RPL19 (Ribosomal protein L19), Human RPL19: Forward primer-5’-AGCGGATTCTCATGGAACA, Reverse primer-5’-CTGGTCAGCCAGGAGCTT, probe-5’-TCCACAAGCTGAAGG CAGACAAGG; Murine RPL19: Forward primer-5’-AGCGCATCCTCATGGAGC, Reverse primer-5’-CTGGTCAGCCAGGAGCTT, probe-5’-TCCACAAGCTGAAGGCAGACAAGG. Fold changes in EDNRB transcript were normalized against house keeping gene transcripts in corresponding samples.

Xenograft models:

All studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (Ref: Institute of Laboratory Animal Resources (NIH publication no. 85-23), Washington, DC: National Academies Press; 1996). Antibodies were conjugated with monomethyl auristatin E (MMAE) as described previously (22). The drug to antibody ratio for 5E9-vc-MMAE was 3.5 and the drug to antibody ratio for control IgG-vc-MMAE was 3.3. Six to eight-week-old female mice (unless indicated otherwise) were inoculated subcutaneously in the dorsal right flank. Mice strains were selected as indicated, see details below:

10 X 10^6 COLO 829 or 5 X 10^6 A2058 cells in HBSS with Matrigel were inoculated into CRL Nu/Nu mice from Charles River Laboratories.

10 X 10^6 A2058 cells in HBSS with Matrigel were inoculated into 8- to 12- week-old Athymic nude mice from Harlan laboratories (Fig. 2F).
5 X 10^6 SK23-mel cells or UACC-257X2.2 cells in HBSS with Matrigel were inoculated into NCR Nude mice from Taconic Laboratories. When tumor volumes reached between 100 to 300 mm^3 in size, the mice were grouped out for treatment.

Efficacy of anti-EDNRB ADC in combination with signaling pathway inhibitors was analyzed using melanoma xenograft models where the drugs were administered either as single agents or in combination as indicated. For efficacy studies, average tumor volumes with standard deviations were determined from 9-10 animals per group. When tumor volumes reached approximately 200 mm^3 (day 0), animals were randomized into groups of 9-10 each and orally administered with PLX4032 twice a day or G590945 once a day or MEK inhibitors GDC-0973 once a day, for a total of 21 days. On day 1, animals were administered with a single intravenous (IV) injection of either humanized anti-EDNRB ADC or anti-gD ADC (human isotype) conjugated to MMAE through the valine-citrulline linker (22). Animal weights and tumor volumes were measured twice per week until study end.

Effects of signaling pathway inhibitors on the phosphorylation of ERK protein and on EDNRB transcript in xenografts were evaluated in pharmacodynamic studies. For these studies, flash frozen tumor material was collected from 5-6 animals per group. When tumor volumes reached approximately 200 mm^3 (day 0) animals were randomized into groups of 5-6 each and administered orally with either PLX4032 twice a day up to day 3 or GDC-0973 once a day up to day 21 starting on
day 0. Animals were harvested 2 hours after the last dose, except in the case of the 28-day time point on the SK23-mel xenograft time course study (Suppl. Fig. S5A), in which case, animals were harvested 7 days after the last dose to allow wash out of the drug. Flash frozen tumor material collected from the animals was processed to evaluate EDNRB transcript and MAPK phosphorylation.

The effect of MAPK inhibitors dosing regimen on EDNRB in normal tissues was evaluated by monitoring murine EDNRB transcript levels (normalized to RPL19). Non-tumor bearing CRL Nu/Nu mice from Charles River Laboratories were dosed twice daily with either vehicle control or 30 mg/kg PLX4032 and SK-23 mel tumor bearing NCR Nude mice from Taconic Laboratories were dosed once daily with either vehicle control or 7.5 mg/kg GDC-0973. Normal tissues were harvested from mice 2h post the 7th dose of PLX-4032 or the 4th dose of GDC-0973 and processed to evaluate murine EDNRB and RPL19 transcripts.
Results

Several studies have demonstrated that the expression of melanocyte lineage markers, such as tyrosinase, MITF, PMEL17 and EDNRB, are suppressed by MAPK signaling (23-26). Since the expression level of a cell-surface target can affect the efficacy of an ADC, we evaluated the suitability of combining the anti-EDNRB ADC with the BRAF inhibitors PLX4032 (27) and G590945; and two chemically distinct MEK inhibitors, GDC-0973 and GDC-0623. We started with the COLO 829 human melanoma model, which contains a V600E BRAF mutation and is responsive to MAPK pathway inhibition. Treatment of the COLO 829 melanoma cell line with PLX4032, as well as with the MEK inhibitors GDC-0623 and GDC-0973, resulted in a dramatic increase in levels of total and cell surface EDNRB protein (Fig. 1A and B). The MAPK pathway inhibitors also produced an increase in EDNRB mRNA transcript, consistent with gene activation as the basis for increased EDNRB protein (Fig. 1C). Additional melanoma cell lines SK-MEL-5, 928mel, UACC-257 and WM-266-4, all with V600 BRAF mutations, and 537mel, which is wildtype for BRAF, all underwent increased EDNRB protein and mRNA in response to MEK inhibitors GDC-0973 and GDC-0623 and BRAF inhibitor G590945 (Suppl. Fig S1 A-F). Data analysis for all in vivo efficacy studies is detailed in Suppl. Tables S1 and S2 and methods used for determining tumor growth inhibition is described in Supplementary Materials and Methods.

To assess the impact of BRAF inhibition on EDNRB expression in vivo, we established COLO 829 tumor xenografts in mice. PLX4032 was administered at a dose of 10 or 30 mg/kg twice per day for 3 days (starting on day 0) and the tumors were harvested 2 hours after the final dose. Analysis of
mRNA by RT-PCR revealed a comparable increase in EDNRB mRNA levels in animals treated with either dose level of PLX4032 relative to vehicle (Fig. 1D). Inhibition of the MAPK pathway was confirmed by immunoblotting for phosphorylated ERK in tumor lysates (Fig. 1E). The impact of PLX4032 on the levels of EDNRB in the COLO 829 tumor xenografts prompted us to evaluate this combination in an efficacy study. To specifically examine an interaction of the two drugs, we examined dose responses with either agent alone and then intentionally selected doses that would not produce complete responses as single agents. We administered a single sub-optimal dose of the anti-EDNRB ADC alone, which produced a modest retardation in tumor growth, whereas PLX4032 alone, dosed twice a day at 30 mg/kg for 21 days, resulted in an approximate 50% inhibition (Fig. 1F). However, combining the two agents at these same doses resulted in complete tumor stasis for the treatment period. To rule out a possible impact of the PLX4032 on clearance of the ADC, we measured ADC blood levels and observed comparable exposure in the presence or absence of PLX4032 (Suppl. Fig. S2). We also tested the combination of a BRAF inhibitor distinct from PLX4032, G590945 (21), and the ADC at varying doses in UACC-257X2.2, a second model of melanoma harboring mutant BRAF. At all dose levels examined, the combination of drugs produced better responses than either alone (Suppl. Fig. S3).

While the anti-EDNRB ADC and PLX4032 combine favorably in the COLO 829 and UACC-257X2.2 tumor efficacy models, this could result from additive independent effects not attributable to any specific facilitation of the ADC by the BRAF kinase inhibitor. To explore this further we tested the A2058 melanoma model, which harbors a V600E BRAF mutation but is intrinsically refractory to MAPK pathway inhibitors (Suppl. Fig. S4.) Treatment of cultured A2058 cells with PLX4032 resulted in a modest increase in total EDNRB protein and a corresponding decrease in ERK phosphorylation.
(Fig. 2A and B). As expected, administration of 10 mg/kg of PLX4032 twice daily for 21 days did not significantly inhibit tumor xenograft growth (Fig. 2C). Nevertheless, this dose of PLX4032 further enhanced the efficacy observed with a single 6 mg/kg dose of anti-ENDRB ADC (Fig. 2C). Increasing the dose of PLX4032 to 30 mg/kg still did not yield significant tumor growth inhibition, nor did it facilitate the activity of the ADC beyond that observed in combination with 10 mg/kg PLX4032.

We next tested the MEK inhibitor GDC-0973 with the A2058 model, which elicited a stronger induction of EDNRB and a more pronounced inhibition of phosphorylated ERK relative to PLX4032 (Fig. 2D and E). Administration of 5 or 10 mg/kg of GDC-0973 for 3 days to animals bearing A2058 tumors resulted in an induction of EDNRB transcript (Fig. 2F). However, no anti-tumor activity was observed with the A2058 model when animals were administered the maximum tolerated dose of 7.5 mg/kg GDC-0973 over a 21-day period (Fig. 2G). Although ineffective as a single agent, GDC-0973 strongly enhanced the activity of the anti-ENDRB ADC (Fig. 2G). Administration of a negative control ADC that does not recognize A2058 cells, exhibited no activity either alone or in combination with GDC-0973 (Fig. 2G, inset). Thus in this particular example the effect of combining the MAPK pathway inhibitor with the ADC cannot simply be additive, and argues for a mechanistic interaction between the two agents. Moreover, these data imply that MAPK pathway inhibitors could have utility in melanoma patients that exhibit intrinsic resistance to these drugs as single agents.

The clinical application of PLX4032 is reserved for melanoma patients with tumors containing detectable BRAF V600 mutations (27, 28). This accounts for approximately 50% of melanomas, while another 15% are mutant for NRAS, with the remainder wildtype for both BRAF and NRAS.
To represent the latter category, we implemented the SK23-mel model, which contains both wildtype BRAF and NRAS. As anticipated, PLX4032 produced limited inhibition of ERK phosphorylation and a modest increase in EDNRB protein as detected by immunoblotting and no increase in cell-surface EDNRB was detected by flow cytometry (Fig. 3A and B). By contrast, the MEK inhibitor GDC-0973 elicited dramatic effects on both ERK phosphorylation and EDNRB expression (Fig. 3A and B).

Dosing of mice bearing SK23-mel tumor xenografts either once with 6 mg/kg of the anti-EDNRB ADC or daily with 7.5 mg/kg of GDC-0973 significantly inhibited tumor growth (Fig. 3C). Nevertheless, combining the agents at these doses was more efficacious than either agent alone. Anti-tumor activity was no longer apparent when the ADC was dosed singly at 3 mg/kg, and activity with GDC-0973 alone was reduced when the dose was lowered to 3 mg/kg. However, when the drugs were combined at these doses, marked tumor inhibition was restored. These data suggest that MEK inhibition would augment the efficacy of the anti-EDNRB ADC in melanoma with wildtype BRAF and NRAS.

Finally, we examined the melanoma cell line IPC-298 that harbors mutant NRAS and wildtype BRAF. It was of interest to test this cell line because recent studies have demonstrated a paradoxical activation of the MAPK pathway by BRAF inhibitors in melanoma cells containing NRAS mutations (29-31). Moreover, NRAS mutations were detected in de novo squamous tumors that arise in melanoma patients treated with PLX4032 (32). Thus, if the EDNRB gene is a target of suppression by the MAPK pathway, then PLX4032 should reduce its expression in mutant NRAS melanoma cells. Indeed, addition of PLX4032 to IPC-298 melanoma cells, harboring NRAS Q61L, increased the
phosphorylation of ERK and decreased the expression of EDNRB (Fig. 4A and B). By contrast, MEK inhibition effectively inhibited the MAPK pathway in the IPC-298 cell line, resulting in a concomitant increase in the expression of EDNRB. These results are consistent with the repression of EDNRB by MAPK signaling.

Although it is apparent that the MEK inhibitor augments the efficacy of the ADC, this affect might require a specific dosing schedule in order to maximally exploit the benefit of EDNRB induction by the inhibitor. To examine this, EDNRB transcript and the inhibition of MAPK pathway was assessed in a time course experiment. Mice bearing SK23-mel tumor xenografts were dosed once a day with either 7.5 mg/kg of the MEK inhibitor GDC-0973 or the corresponding vehicle control for a period up to 21 days, initiated on day 0. Tumors were harvested from each group on day 1, 3, 7, 14, 21 and 28, where day 28 represents a 7-day wash out period for the drug. Maximal EDNRB induction was maintained between days 3-7, decreasing gradually thereafter with recovery to near basal levels following the washout at day 28 (Suppl. Fig. 5A). These results point to a broad scheduling window of opportunity for administrating the ADC with respect to that of the MEK inhibitor. Moreover, the induction of EDNRB is already apparent at Day 1, in both the SK23-mel and COLO 829 tumor models, suggesting that the drugs could be initiated simultaneously (Suppl. Fig 5A and B).

Melanomas can acquire resistance to BRAF inhibition through a variety of reported mechanisms (29, 33-36). To determine whether EDNRB remains elevated following acquired resistance to PLX4032, resistant melanoma cell lines containing V600E BRAF were derived with prolonged
exposure to PLX4032 over a period of 10-12 weeks. The IC50’s for the parental cell lines, 928mel, UACC-257X2.2 and WM-266-4 are approximately 0.15, 0.15 and 0.26 μM of PLX4032, respectively. Following chronic exposure to increasing concentrations of PLX4032, the 928mel and UACC-257X2.2 derivatives were no longer inhibited by concentrations as high as 2 μM PLX4032 and WM-266-4 was resistant up to 1μM. Analysis of cell surface levels of EDNRB by flow cytometry revealed that all three PLX4032-resistant derivatives retained elevated expression of EDNRB relative to their corresponding parental lines (Fig. 5A). The WM-266-4 and 928 mel drug-resistant derivatives both exhibited lower basal levels of phosphorylated ERK, consistent with a resistance mechanism involving reduced reliance upon MAPK signaling (Fig. 5B). By contrast, the UACC-257X2.2 resistant derivative maintained phosphorylated ERK levels comparable to the parental cell line. However, addition of PLX4032 still resulted in diminution of MAPK signaling as determined by phosphorylated ERK. The UACC-257X2.2 derivative also underwent a further increase in EDNRB upon addition of the MEK inhibitor GDC-0973 (Fig. 5C).

Our data indicate that the level of EDNRB is elevated in response to MAPK inhibition and thereby enhances the efficacy of the ADC directed against it. However, increased on-target toxicity might be expected should a comparable enhancement occur in normal tissues. The ADC used in our studies cross reacts with mouse EDNRB, yet no overt signs of enhanced toxicity were observed when combined with MAPK inhibitors (unpublished observation). Nevertheless, we compared the expression of the EDNRB mRNA transcript in the normal tissue of animals administered vehicle control and MAPK inhibitors. Low levels of EDNRB mRNA transcript were detected by RT-PCR in a variety of normal mouse tissues overall, and no significant relative increases were noted in response to inhibitors of either MEK or BRAF (Fig. 6 A and B, Table S3). The normal tissue
measurements for MEKi administration were performed in SKmel-23 tumor bearing animals where the increase in EDNRB mRNA is readily apparent in the tumor material from these animals (Fig. 6B). We also examined cultured adult normal human epidermal melanocytes, which express detectable cell surface levels of EDNRB. Incubation with the BRAF inhibitor did not increase EDNRB cell-surface expression, consistent with the specificity of this drug for cells containing mutant BRAF (Fig. 6C). However, an increased level of cell surface EDNRB was observed in normal melanocytes in response to the MEK inhibitor. Based on the relative insensitivity of normal melanocytes to ADCs containing MMAE, likely attributable to their slow doubling time, the potential for toxicity in normal skin would be expected to be limited (37).

To determine the mechanism of cell death, we performed caspase activation assays on melanoma cell lines exposed to MAPK pathway inhibitors, the ADC or the combination. As expected, the raf inhibitor PLX4032 had no effect on caspase 3/7 activation in the BRAF wildtype SK23mel cell line nor did its presence add to the caspase 3/7 activation observed with the ADC alone (suppl. Fig 6 B, C). By contrast, addition of the MEK inhibitor, which was ineffective alone, resulted in an approximate 7-fold increase in caspase activation relative to that observed with ADC. In the A2058 and colo829 cells, Caspase 3/7 activation was detected upon inhibition of MEK or BRAF and, when combined with the ADC, the signal appeared additive to that observed with the ADC alone. An assessment of early and late apoptosis, using Annexin V and propidium iodide staining, yielded results consistent with Caspase 3/7 (suppl. Fig. 6 B,C).
Discussion

It has been appreciated for many decades that the use of drugs in combination can confer enhanced benefit to cancer patients (38). Combining drugs of various modalities, such as anti-metabolites, DNA damaging agents and microtubule disrupting agents, combats the diversity inherent to cancer and diminishes the likelihood of drug resistance. Even modern therapies targeting defined genetic attributes of a cancer are foiled by second site mutations or mechanistic redundancies that result in drug resistance and relapse (39-41). Thus, combining rational therapies in a mechanistically oriented manner will be instrumental in overcoming intrinsic and acquired drug resistance in cancer therapy. In particular, understanding and exploiting the mechanisms by which cancer cells compensate for or respond to targeted therapies will facilitate development of new drug combinations. Here we have taken advantage of a basic biological mechanism resident to melanoma by targeting a response to drugs that interfere with MAPK signaling.

Melanoma cells appear to repress cellular markers of the melanocytic lineage via activation of the MAPK pathway (23-26). To some extent, this might reflect a normal developmental process employed by melanocytic precursors for lineage determination. For example, in vivo ectopic activation of MEK in pigmented retinal cells drives their trans-differentiation into neural-like epithelium (42). The transcription factor MITF, a master regulator of melanocytic differentiation, is the likely effector in this signaling outcome as a similar transdifferentiation is observed in mice harboring germ-line MITF mutations (43). Moreover, it is known that the receptor tyrosine kinase c-kit, which is critical for differentiation of neural crest cells to melanocytes, regulates MITF via the MAPK pathway (44, 45). Thus, the near universal adoption of hyperactive MAPK signaling, through
the oncogenic activation of NRAS, BRAF or c-kit, (46, 47), could represent the cooption of a progenitor cell mechanism by melanoma cells. This hyperactivation, in combination with additional oncogenic events, some of which suppress senescence, appears to maintain melanoma cells in a de-differentiated state accompanied by the repression of melanocytic lineage markers. Acute inhibition of MAPK signaling produces a pronounced rebound in the expression of these markers, thereby creating new potential targets for therapies.

The sub-classification of melanoma is typically based on clinical observations and histology. However, distinctions based on gene copy number, mutations and gene expression signatures are also apparent and are particularly relevant in the application of rational drugs. Accordingly, BRAF inhibitors are beneficial only in V600BRAF-mutated melanoma (27) and the utility of the anti-EDNRB ADC will depend, in part, on the expression level of the endothelin B receptor (5).

Nevertheless, the presence of a V600 mutation does not guarantee a response to either BRAF or MEK inhibition, as some of these melanomas appear intrinsically resistant to the drugs. However, in some resistant cells, such as the A2058 in our study, inhibition of phosphorylated ERK and up-regulation of EDNRB still occurs in response to MAPK pathway inhibition, resulting in a more favorable response to the anti-EDNRB ADC. This is consistent with a previous study in which a number of melanoma cell lines resistant to PLX4032, up-regulated the expression of melanocyte-specific markers in response to the drug (23). This same study used gene expression signatures to define a category of melanoma cell lines that maintained high levels of melanocytic antigens, relative to those with low levels. However, cell lines with low levels underwent increased expression of the antigens in response to PLX4032. This was also evident for EDNRB in our study, and suggests that the level of the receptor on naïve melanoma cells might not be a suitable
diagnostic for the ADC when used in combination with MAPK inhibitors. Taken together, the data indicate that the combination of a MAPK pathway inhibitor with the anti-EDNRB ADC could benefit patients in which either drug alone is inert.

In melanomas lacking BRAF mutations, inhibition of the kinase is ineffective and can even exacerbate MAPK signaling and tumor growth, particularly when NRAS is mutated (48). This so-called BRAF paradox is consistent with the down-regulation of EDNRB that we observed in response to PLX4032 in NRAS mutant IPC-298 melanoma cells. Thus, melanoma diagnostically positive for NRAS mutations is excluded from treatment with BRAF inhibitors and would not benefit from combination with the ADC. However, MEK inhibitors retain their ability to inhibit MAPK signaling in these cells and consequently promote the up-regulation of EDNRB. Moreover, inhibition of MAPK signaling in cells wildtype for both BRAF and NRAS up-regulates EDNRB and augments the anti-tumor activity of the ADC. Thus hyperactive MAPK signaling, independent of any specific mutational status, appears to be the key diagnostic marker for combining the ADC with MAPK inhibitors. That the vast majority of melanomas are addicted to MAPK signaling, suggests that a broad melanoma patient population might benefit from the combination.

Acquired resistance to BRAF inhibition has been observed in melanoma patients who relapse following prolonged administration of PLX4032 (19, 49). Acquired resistance can also be recapitulated in vitro and a variety of mechanisms that account for it have been described (29, 33-36). Most of these resistance mechanisms involve the maintenance of MAPK signaling through the circumvention of BRAF, as evidenced by ERK phosphorylation. In other cases, phosphorylated ERK is still reduced by BRAF inhibition, yet the cells remain viable, indicating a diminished reliance upon
MAPK signaling (34). We generated clones resistant to PLX4032 from three independent melanoma cell lines, one of which appeared to maintain elevated phospho-ERK, whereas the other two exhibited lowered levels. Nevertheless, all three resistant lines retained elevated levels of EDNRB relative to the corresponding parental cells. Thus despite potential differences in the manner by which the melanoma cells might resist MAPK pathway inhibition, the elevated expression of EDNRB remained intact.

Scheduling the dosing of a therapeutic regimen that relies upon an interaction between two drugs might require specific timing to ensure an efficient interaction. The anti-EDNRB ADC attains maximum blood exposures levels shortly after administration but clears slowly with a half-life on the order of 9 days in rodents and non-human primates (unpublished data). Our results show the EDNRB remains elevated throughout the 21-day course of daily dosing with a MEK inhibitor. Thus the two drugs should have ample opportunity to interact, precluding a need for precise dose scheduling. Nevertheless, our data show that maximal elevation of EDNRB levels by the MEK inhibitor was reached within 1-3 days of administration and could therefore have implications for achieving an optimal combinatorial effect.

One potential outcome related to safety is the increased expression of EDNRB on normal melanocytes in response MAPK inhibition. Although this did not occur in response to BRAFi, we did observe upregulation of cell-surface EDNRB by MEK inhibitors. Thus, the potential loss of normal melanocytes targeted by the ADC could be exacerbated in the presence of MEK inhibition. This could affect pigmentation in hair or skin, possibly manifested as vitiligo-like symptoms. Although clearly undesirable, such outcomes ought to be readily monitorable and manageable in the clinic.
Despite the increase of EDNRB on normal melanocytes, we believe the affect of the ADC will be milder than that on melanoma cells due to the anti-mitotic nature of MMAE. In a separate study targeting PMEL17 in melanoma we have reported that the impact of anti-PMEL17vcMMAE on the viability of normal melanocytes is substantially tempered relative to that on melanoma cells (36).

Overall, our study shows that inhibition of MAPK signaling potentiates the efficacy of the anti-EDNRB ADC irrespective of the mutational background or response of a melanoma to MAPK pathway inhibition alone. The data further supports the retention of elevated EDNRB expression on melanoma cells, despite prolonged exposure and acquired resistance to a BRAF inhibitor. Combining the anti-EDNRB ADC with MAPK pathway inhibitors could provide enhanced benefit to wide range of melanoma patients.

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References


Figure legends

Figure 1: MAPK pathway inhibitors increase EDNRB expression and ADC activity in a V600E mutant BRAF melanoma model. A. EDNRB, phosphorylated ERK (Perk) and total ERK (erk) proteins were evaluated in lysates from COLO 829 cells treated overnight with the indicated concentrations of PLX4032 (BRAFi) and GDC-0973 and GDC-0623 (MEKi-973 and MEKi-623). B. Cell-surface levels of EDNRB were monitored by flow cytometry on untreated COLO 829 cells (NA, green tracing) or cells treated overnight with 1 μM PLX4032 (BRAFi, red tracing) or 0.1 μM GDC-0973 or GDC-0623 (MEKi-973, MEKi-623, red tracings). Gray tracing indicates cells treated with secondary detection reagent only. C. EDNRB mRNA transcript levels, relative to reference transcript RPL19, were evaluated in COLO 829 cells treated overnight with 0, 0.1, 1 and 10 μM of PLX4032 (BRAFi). Error bars were derived from measurements performed in triplicate for each sample. D. EDNRB transcript levels, normalized to GAPDH, measured in COLO 829 tumor xenografts harvested 2 hours post the 7th dose of vehicle, 10 or 30 mg/kg (MPK) of PLX4032. Each bar represents an individual tumor and CL is untreated COLO 829 cells included as a reference. Error bars were derived from measurements performed in triplicate. E. Phosphorylated (Perk) and total ERK (erk) in COLO 829 xenografts treated with 30 mg/kg PLX4032 or vehicle control were analyzed by immunoblotting. F. Mice bearing COLO 829 tumor xenografts were administered 30 mg/kg PLX4032 (BRAFi) twice a day for 21 days, a single IV injection of 3 mg/kg anti-EDNRB ADC on day 1, or the combination (ADC+BRAFi). Average tumor volumes with standard deviations were determined from 9 animals per group.

Figure 2. A V600E mutant BRAF tumor model, resistant to MAPK pathway inhibition responds to the combination of anti-EDNRB ADC plus BRAF or MEK inhibitor. A. EDNRB, phosphorylated erk (Perk) and total ERK (erk) proteins are evaluated in lysates from A2058 cells treated overnight with 0, 0.1, 1 and 10 μM PLX4032 (BRAFi). B. Cell-surface levels of EDNRB monitored by flow cytometry on untreated A2058 cells (NA, green tracing) or cells treated overnight with 1μM PLX4032 (BRAFi, red tracing). Gray tracing indicates cells treated with secondary detection reagent only. C. Mice bearing A2058 tumor xenografts were administered vehicle, 10 or 30 mg/kg PLX4032 (BRAFi) twice a day for 21 days, a single IV injection of 6 mg/kg anti-EDNRB ADC on day 1, or the combinations
(ADC+BRAFi). Average tumor volumes with standard deviations were determined from 10 animals per group. D and E. Analysis of A2058 cells treated with GDC-0973 (MEKi-973) as described for BRAF inhibition in A and B. F. EDNRB transcript levels, normalized to HPRT1, measured in A2058 tumor xenografts 2 hours after the third dose of vehicle or 5 or 10 mg/kg (MPK) of GDC-0973. Each bar represents an individual tumor and error bars were derived from measurements performed in triplicate for each sample. G. Mice bearing A2058 tumor xenografts were administered 7.5 mg/kg of GDC-0973 (MEKi) once a day for 21 days, a single IV injection of 6 mg/kg of anti-EDNRB ADC on day 1, or the combination (ADC+MEKi). Inset shows control groups in which an irrelevant IgGvcMMAE ADC (ADC-con) was administered at 6 mg/kg alone or with 7.5 mg/kg GDC-0973 daily (MEKi+ADC-con). Average tumor volumes with standard deviations were determined from 9 animals per group.

Figure 3. MEK inhibition enhances anti-EDNRB ADC activity in a melanoma model with wildtype NRAS and BRAF. A. EDNRB, phosphorylated ERK (Perk) and total ERK proteins (erk) evaluated in lysates from SK23-mel cells treated overnight with the indicated concentrations of PLX4032 (BRAFi) or GDC-0973 (MEKi). B. Cell-surface levels of EDNRB monitored by flow cytometry on untreated SK23-mel cells (NA, green tracing) or cells treated overnight with 1 μM PLX4032 or GCD-0973 (BRAFi, MEKi, red tracings). Gray tracing indicates cells treated with secondary detection reagent only. C. Mice bearing SK23-mel tumor xenografts were administered the indicated mg/kg dose of GDC-0973 (MEKi) once a day for 21 days, a single IV injection of 3 or 6 mg/kg of anti-EDNRB ADC on day 1, or the combinations (ADC+MEKi). Average tumor volumes with standard deviations were determined from 10 animals per group.

Figure 4. Opposing effects of BRAF and MEK inhibitors on EDNRB expression in cells expressing mutant NRAS. A. EDNRB, phosphorylated ERK (Perk) and total ERK proteins (erk) were evaluated in lysates from IPC-298 cells treated overnight with the indicated concentrations of PLX4032 (BRAFi), GDC-0973 and GDC-0623 (MEKi-973 and MEKi-623). B. Cell-surface levels of EDNRB monitored by flow cytometry on untreated IPC-298 cells (NA, green tracing) or cells treated overnight with 1 μM PLX4032, GDC-0973 and GDC-0623 (RAFi, MEKi-973 and MEKi-623, red tracings). Gray tracing indicates cells treated with secondary detection reagent only.
Figure 5. Melanoma cells adapted to resist BRAF inhibition retain elevated levels of cell-surface EDNRB. A. Cell-Surface EDNRB expression monitored by flow cytometry on live parental (green tracing) and BRAF inhibitor-resistant (red tracing) melanoma cell lines 928 mel, WM-266-4 and UACC-257X2.2. Gray and light blue tracings indicate parental and resistant cells, respectively, treated with secondary detection reagent only. B. Phosphorylated ERK (Perk) and total ERK (erk) proteins evaluated in lysates from the indicated parental (P) and resistant (R) cell lines untreated or treated overnight with 10 μM PLX4032 (BRAFi). C. Cell-surface EDNRB expression on live parental and BRAF inhibitor resistant UACC-257X2.2 cells untreated (green tracing) or treated (red tracing) overnight with 0.1 μM GDC-0973 (MEKi).

Figure 6. Effect of MAPK pathway inhibitors on EDNRB mRNA transcript levels in normal mouse tissues and on surface EDNRB in human melanocytes. A. Murine EDNRB mRNA levels (normalized to murine RPL19) were compared in the indicated normal tissue panels harvested from naive CRL nude mice 2h post 7th dose of either vehicle control (-BRAFi) or 30 mg/kg BRAF inhibitor PLX4032 (+BRAFi). B. Murine EDNRB mRNA levels (normalized to murine GAPDH) were compared in the indicated normal tissue panels harvested from SK23-mel tumor bearing NCR Nude mice 2h post 4th dose of either vehicle control (-MEKi) or 7.5 mg/kg GDC-0973 (+MEKi). Error bars were derived from measurements performed on 5 mice per group in triplicate for each sample. Unpaired t-test analyses showed no significant increases in EDNRB expression in normal tissues of mice treated with either BRAFi or MEKi as compared to control. C. Adult normal human epidermal melanocytes were treated overnight with 1μM of BRAF inhibitor PLX4032 (left panel) or MEK inhibitor GDC-0973 (right panel) and cell-surface EDNRB expression was monitored by flow cytometry (red tracing). Gray tracing indicates cells treated with secondary detection reagent only and green tracing indicates surface EDNRB expression on untreated cells.
Fig 2

A. BRAFi (μM)

B. Perk

C. Tumor volume (mm³)

D. BRAFi 10

E. MEKi-973 (μM)

F. EDNRB

G. EDNRB ADC
Fig 3

A

BRAFi (μM)

EDNRB

Perk

erk

MEKi-973 (μM)

EDNRB

Perk

erk

B

NA +RAFi

% of Max

NA +MEKi

% of Max

C

Tumor volume (mm³)

ADC 3

vehicle

MEKi

ADC 6

MEKi 7.5

ADC 3+MEKi 3

ADC 6+MEKI 7.5

MEKi (QD)

ADC

day

0 5 10 15 20 25 30 35

0 500 1000 1500

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Fig 6

A

Heart

Lung

Kidney

Skin

Liver

Small Intestine

B

Heart

Lung

Large Intestine

Small Intestine

Skin

Sk23-mel

tumors

C

NHEM-Adult

+BRAFi

+MEKi

% of Max

FL1-H

% of Max

FL1-H
# Molecular Cancer Therapeutics

**MAPK pathway inhibition enhances the efficacy of an anti-endothelin B receptor drug conjugate by inducing target expression in melanoma.**

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