Pharmacologically enhanced expression of GPNMB increases the sensitivity of melanoma cells to the CR011-vcMMAE antibody-drug conjugate

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ABSTRACT

GPNMB is a melanoma-associated glycoprotein that is targeted by the CR011-vcMMAE antibody-drug conjugate (ADC). Previous studies have shown that CR011-vcMMAE induces the apoptosis of GPNMB-expressing tumor cells in vitro and tumor regression in xenograft models. This ADC is currently in clinical trials for melanoma. In the present investigation, a variety of compounds were examined for their ability to increase the expression of GPNMB by cancer cells. These experiments lead to the identification of three distinct groups of compounds that increased GPNMB, some of which were shown to enhance the sensitivity of melanoma cells to CR011-vcMMAE. These data indicate that it may be possible to increase the anticancer activity of CR011-vcMMAE through pharmacological enhancement of GPNMB expression for potential therapeutic benefit.

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1. Introduction

GPNMB is a cell-surface proteoglycan expressed by melanoma and glioblastoma cells (Kuan et al., 2006; Tse et al., 2006). We previously generated a fully human monoclonal antibody to the extracellular domain of GPNMB, conjugated the antibody to the cytotoxic agent monomethylauristatin E (MMAE) via a protease-sensitive linker to facilitate the intracellular release of the MMAE cytotoxin within tumor cells, and found that this antibody-drug conjugate (ADC) effectively targeted GPNMB-expressing tumor cells in vitro and in xenograft models (Pollack et al., 2007; Tse et al., 2006). This ADC, denoted CR011-vcMMAE, is currently in Phase I clinical testing in patients with metastatic malignant melanoma.

A variety of parameters can potentially influence the activity of a given ADC (Carter, 2001). For example, cancers often exhibit intra/inter-tumoral heterogeneity of target expression which may affect ADC activity since a minimum threshold density of target molecules at the cell-surface may be required for efficient tumor-targeting. The rates of internalization and processing of the target-ADC complex, steps which are required for the intracellular delivery of active cytotoxic drug within tumor cells, are also important. Another parameter that can influence ADC activity is that of target shedding. Shedding of plasma membrane proteins is a common phenomena which can potentially reduce ADC activity by decreasing the expression of the relevant target on the surface of tumor cells and by generating soluble target protein that may compete with surface-associated protein for ADC binding (Dello Sbarba and Rovida, 2002; Eichenauer et al., 2007).

With the goal of maximizing the anticancer activity of CR011-vcMMAE, a screen was conducted in the present investigation to identify compounds that could increase the surface expression and/or decrease the shedding of GPNMB by tumor cells. We
focused our analysis on the two cancer types that are associated with GPNMB expression: melanomas and glioblastomas.

Melanomas can be categorized based on mutational status, with approximately 15–30% and 50–70% of these tumors harboring activating mutations in either NRAS or BRAF, respectively, and a small percentage possessing wild-type NRAS/BRAF (Gray-Schopfer et al., 2007). Cell lines representing each of these genetically distinct melanoma subtypes were included in the present investigation. In contrast to melanomas, glioblastomas rarely harbor activating NRAS or BRAF mutations (Knobbe et al., 2004).

Tumor cells harboring mutations NRAS or BRAF mutations exhibit constitutive activation of the ERK signaling pathway which is comprised of the RAS GTPase and three kinases: RAF, MEK and ERK (Dhillon et al., 2007). In normal quiescent cells, the components of this pathway are enzymatically inactive. Following an appropriate stimulus such as a growth-factor receptor-ligand interaction, the components of the ERK pathway are sequentially activated, culminating in the regulation of numerous cytoplasmic and nuclear proteins via either direct or indirect ERK-mediated phosphorylation. In contrast to the situation that exists in normal cells, tumors harboring mutations NRAS or BRAF exhibit constitutive activation of the ERK pathway in the absence of external stimuli.

In addition to screening for compounds that increased the expression of GPNMB, proof-of-principle experiments were performed in the present investigation to determine whether such compounds could be used to enhance the anticancer activity of CR011-vcMMAE, as hypothesized. Finally, both basal and inducible GPNMB expression was compared with that of other melanoma-associated tumor targets so that our findings regarding GPNMB could be viewed in a broader context. The results presented herein extend our understanding of GPNMB regarding GPNMB could be viewed in a broader context. The activity of CR011-vcMMAE, as hypothesized. Finally, both basal such compounds could be used to enhance the anticancer activity of CR011-vcMMAE. The A375 melanoma cell line was initially employed since this cell line harbors a mutant form of BRAF and thus represents a common molecularly-defined subclass of melanoma. Moreover, this cell line expresses a very low basal level of GPNMB which we believed might facilitate the detection of potentially weak GPNMB inductions. A variety of compounds were surveyed for their ability to increase GPNMB expression as detected by immunoblotting, and a representative experiment is presented in Figure 2A. In this particular experiment, a number of compounds were found to increase GPNMB expression, the majority of which were inhibitors of the ERK signaling pathway. This included inhibitors of RAF, MEK and ERK, in addition to the HSP90 inhibitor geldanamycin, which is known to inhibit the ERK pathway due to the dependency of BRAF on HSP90 function (Roberts and Der, 2007). (Note that although the induction of GPNMB by geldanamycin was relatively weak in the experiment presented in Figure 2A in which cells were exposed to compound for just 24 h, more robust GPNMB induction was observed in additional experiments in which A375 and other melanoma cells (i.e. WM2664) were exposed to geldanamycin for 48 h; data not shown).

In the experiment presented in Figure 2A, potential inhibition of the ERK pathway was investigated by immunoblotting for phospho-ERK (pp-ERK), which represents the active form of this signaling molecule. The results of this analysis demonstrated that, as expected, ERK phosphorylation/activation was increased GPNMB expression, the majority of which were inhibitors of the ERK signaling pathway. This included inhibitors of RAF, MEK and ERK, in addition to the HSP90 inhibitor geldanamycin, which is known to inhibit the ERK pathway due to the dependency of BRAF on HSP90 function (Roberts and Der, 2007). (Note that although the induction of GPNMB by geldanamycin was relatively weak in the experiment presented in Figure 2A in which cells were exposed to compound for just 24 h, more robust GPNMB induction was observed in additional experiments in which A375 and other melanoma cells (i.e. WM2664) were exposed to geldanamycin for 48 h; data not shown).

Not all compounds that were examined in this screen increased the expression of GPNMB. For example, inhibitors of JNK and aurora kinases were ineffective via immunoblotting (Figure 2A). In addition, numerous other compounds such as dacarbazine, IFNα, IFNγ, carmustine, cisplatin, paclitaxel and inhibitors of mTOR (rapamycin), PI3K (LY94002), IκB (BMS345541), methyltransferase (5-AZA, decitabine), histone deacetylase (belinostat) and the proteasome (bortezomib) were ineffective as determined by immunoblotting and/or flow cytometry analysis (data not shown).

2. Results

2.1. GPNMB is expressed in melanoma and glioblastoma cell lines and exhibits a unique expression profile compared to other melanoma-associated targets

A microarray analysis of the transcriptional profile of GPNMB on the NCI-60 cancer cell line panel revealed that GPNMB was most consistently expressed in cell lines derived from melanoma (9/10) and CNS (4/6) tumors (Figure 1A). The selective expression of GPNMB in melanoma and CNS (particularly glioblastoma) cell lines was confirmed at the protein level by immunoblotting, where GPNMB migrated as a doublet of ~130 and 110 kDa (Figure 1B). In addition to GPNMB, the expression of 8 other melanoma-associated molecules that are under consideration as potential targets for melanoma therapy was examined (Figures 1A,B). In comparison to the other melanoma targets examined, GPNMB exhibited a unique expression profile that was consistent with its potential utility for the targeted therapy of melanoma and glioblastoma.

2.2. Inhibitors of the ERK signaling pathway induce GPNMB expression

GPNMB expression has been shown to be both necessary and sufficient for the activity of the CR011-vcMMAE (Tse et al., 2006), and thus it is likely that the activity of this ADC is influenced by the level and consistency of GPNMB expression on tumor cells. Since tumors often exhibit heterogeneous expression of potential therapeutic targets, a screen was performed to attempt to identify compounds which increased the expression of GPNMB with the idea that such compounds might be used therapeutically to increase the anticancer activity of CR011-vcMMAE. The A375 melanoma cell line was initially employed since this cell line harbors a mutant form of BRAF and thus represents a common molecularly-defined subclass of melanoma. Moreover, this cell line expresses a very low basal level of GPNMB which we believed might facilitate the detection of potentially weak GPNMB inductions. A variety of compounds were surveyed for their ability to increase GPNMB expression as detected by immunoblotting, and a representative experiment is presented in Figure 2A. In this particular experiment, a number of compounds were found to increase GPNMB expression, the majority of which were inhibitors of the ERK signaling pathway. This included inhibitors of RAF, MEK and ERK, in addition to the HSP90 inhibitor geldanamycin, which is known to inhibit the ERK pathway due to the dependency of BRAF on HSP90 function (Roberts and Der, 2007). (Note that although the induction of GPNMB by geldanamycin was relatively weak in the experiment presented in Figure 2A in which cells were exposed to compound for just 24 h, more robust GPNMB induction was observed in additional experiments in which A375 and other melanoma cells (i.e. WM2664) were exposed to geldanamycin for 48 h; data not shown).

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Figure 1 – Expression of GPNMB and other melanoma-associated targets in cell lines representative of various cancer types. (A) Microarray transcript analysis of melanoma-associated targets on the NCI-60 panel of cancer cell lines. Data is available at http://cgap.nci.nih.gov/Genes/GeneFinder. The cell lines marked with an asterisk (MDA-MB-435 and MDA-N) were originally classified as breast carcinomas, but are actually melanomas (http://dtp.nci.nih.gov/docs/misc/common_files/MDA-MB-435-update.html; note that MDA-N is a subline of MDA-MB-435). (B) Immunoblotting of melanoma-associated targets on whole-cell lysates harvested from the indicated cancer cell lines. Information on the cell lines categorized as "miscellaneous" is as follows: NCI-H23 (lung); MDAMB231 (breast); OVCAR3 (ovarian); Caki2 (renal); HT29 (colon). Additional nomenclature on the targets is as follows: GPNMB (Glycoprotein NMB); MART1 (Melanoma Antigen Recognized by T Cells 1; MLANA; MELAN A); TYRP1 (Tyrosine-Related Protein 1); TYRP2 (Tyrosine-Related Protein 2); TRP2; DCT; Dopachrome Tautomerase); TRP1 (Tyrosine-Related Protein 1); TRP1; GP75); PMEL17 (Melanocyte Protein 17; SILV; GP100); MAGEA1 (Melanoma Antigen Family A1); MCSP (CSPG4; MEL-CSPG; Melanoma-Associated Chondroitin Sulfate Proteoglycan 4); MTf (MF12; Melanotransferrin; Melanoma-Associated Antigen P97); MCAM (Melanoma Adhesion Molecule; CD146; MUC18). See Table 1 for additional information on the cell lines. An antibody to ERK was used as a loading control.
2.3. Induction of GPNMB by inhibitors of the ERK signaling pathway is influenced by NRAS/BRAF mutational status and tumor type

To examine whether GPNMB induction by ERK-pathway inhibitors was influenced by tumor type and/or NRAS/BRAF mutational status, a variety of cell lines representing melanomas and non-melanomas with or without mutations in NRAS or BRAF (Table 1; Abi-Habib et al., 2005; Gupta et al., 2000; Ikediobi et al., 2006; Lev et al., 2004) were exposed to ERK-pathway inhibitors and examined for GPNMB expression via immunoblotting (Figure 3A). This analysis showed that melanomas harboring mutations in either NRAS (SKMEL2) or BRAF (A375, WM2664, G361, SKMEL28, UACC62) exhibited an induction of GPNMB following exposure to inhibitors of the ERK pathway, while non-melanomas harboring mutations in NRAS (HT1080) or BRAF (HT29) did not. In addition, neither melanoma (MEWO) nor non-melanoma (SF539 glioblastoma) cell lines possessing wild-type NRAS/BRAF exhibited GPNMB induction following exposure to ERK pathway inhibitors. This data indicates that the induction of GPNMB in a particular cell line by inhibitors of the ERK pathway is dependent upon the presence of a mutation in NRAS or BRAF in addition to the proper cellular context/tumor type.

Next, the effect of kinetics and drug concentration on the induction of GPNMB by inhibitors of MEK, RAF and ERK was examined (Figure 3B). This experiment confirmed that these compounds induced GPNMB expression in melanoma cell lines harboring mutations in NRAS (SKMEL2) or BRAF (A375), but not in a non-melanoma cell line possessing wild-type NRAS/BRAF (SF539 glioblastoma). Results obtained with a MEK inhibitor (Figure 3B, top) showed that the level of GPNMB induction increased with exposure time and in some instances exhibited

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Table 1 – Cell line characterization

<table>
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<tr>
<th>Cell line</th>
<th>Cancer type</th>
<th>BRAF/NRAS mutations</th>
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<td>A375</td>
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Figure 3 – The effect of ERK-pathway inhibitors on the expression of GPNMB in cell lines representative of various cancer types. (A) Immunoblotting for GPNMB on whole-cell lysates harvested from various cancer cell lines following exposure to the indicated compounds for 48 h. Compounds and concentrations were as follows: MEKi: U0126 (20 μM); RAFKi: 553013 (10 μM); ERKi: FR180204 (50 μM). The top panel consists of melanoma cell lines harboring mutations in NRAS (SKMEL2) or BRAF (A375, WM2664, G361, SKMEL28, UACC62). The bottom panel consists of a melanoma (MEWO) and a glioblastoma (SF539) cell line known to be wild-type for NRAS and BRAF, two glioblastoma cell lines (U118MG, XF498) for which the NRAS/BRAF mutational status is unknown, a colon cancer cell line (HT29) which harbors a BRAF mutation and a fibrosarcoma cell line (HT1080) which harbors an NRAS mutation. (B) Immunoblotting for GPNMB on whole-cell lysates harvested from various cancer cell lines following exposure to the indicated compounds for 12, 24 or 48 h. Compounds were as follows: MEKi (top panel): U0126; RAFKi (middle panel): 553013; ERKi (bottom panel): FR180204. Drug concentrations shown are in μM. The cell lines utilized (A375, SKMEL2, SF539) are described above. See Table 1 for additional details on the cell lines used in this figure. In both (A) and (B), an antibody to phospho-ERK was used to determine the level of ERK activation and an antibody to ERK was used as a loading control.
dose-responsiveness. As indicated by phospho-ERK levels, the MEK inhibitor exhibited robust and sustained inhibition of the ERK pathway in A375 and SKMEL2 cell lines, and transient inhibition in SF539 cells. Results using a RAF inhibitor (Figure 3B, middle) were similar to those found with the MEK inhibitor, although it is interesting to note that the RAF inhibitor showed no evidence of phospho-ERK inhibition in SF539 cells and thus this compound appears to exhibit selectivity for mutant BRAF (also see Figure 3A). Finally, exposure of cells to an ERK inhibitor showed strong induction of GPNMB in A375 cells, but only following an exposure time of 48 h (Figure 3B, bottom). Little or no GPNMB induction was seen in SKMEL2 or SF539 cells. This compound produced a relatively weak inhibition of the ERK pathway as indicated by an examination of phospho-ERK levels. Consistent with the immunoblotting results, analysis by flow cytometry demonstrated that the MEK, RAF and ERK inhibitors induced GPNMB surface expression in a melanoma (A375), but not in a glioblastoma (SF539) cell line (Table 2, top).

2.4. Inhibitors of the ERK signaling pathway sensitize melanoma cells to the growth-inhibitory activity of CR011-vcMMAE

Having established that inhibitors of the ERK pathway enhance overall and cell-surface GPNMB expression in melanoma cell lines harboring mutant NRAS or BRAF, we next wanted to determine whether this translated into increased sensitivity to the growth-inhibitory effects of the GPNMB-targeting ADC, CR011-vcMMAE. To this end, the effect of exposing melanoma cells to a MEK inhibitor prior to the addition of CR011-vcMMAE was examined. The UACC62 melanoma cell line was chosen for this analysis since these cells harbor mutant BRAF, exhibit relatively low GPNMB surface expression (Tse et al., 2006), are relatively insensitive to growth-inhibition by CR011-vcMMAE, and are highly responsive to GPNMB induction by inhibitors of the ERK pathway (Figure 3A). Following pretreatment without or with a non-toxic dose of a MEK inhibitor, UACC62 cells were incubated with various doses of CR011-vcMMAE for 3 days after which time cultures were photographed (Figure 4A) and live cells were quantified by trypan blue dye exclusion (Figure 4B). The results of this experiment indicated that pretreatment with a MEK inhibitor sensitized melanoma cells to the growth-inhibitory activity of CR011-vcMMAE. For example, little growth-inhibition was mediated by CR011-vcMMAE (0.16 μg/mL) on UACC62 cells that were not pretreated with the MEK inhibitor, while strong growth-inhibition was mediated by the same dose of CR011-vcMMAE used on cells that were pretreated with the MEK inhibitor. Sensitization of UACC62 cells to CR011-vcMMAE by pretreatment with the MEK inhibitor was confirmed using another measure of cell viability (CellTiter-Glo assay which measures ATP; data not shown). In addition, a RAF inhibitor (553013) was also found to sensitize UACC62 cells to CR011-vcMMAE (data not shown). Pretreatment with a MEK inhibitor also sensitized SKMEL2 cells to CR011-vcMMAE (Figure 4C).

<table>
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Flow cytometry was performed on intact, non-permeabilized cells using the indicated primary monoclonal antibodies. The CR011 antibody utilized was not MMAE-conjugated, but is the same antibody used to generate CR011-vcMMAE. Results are reported as GeoMeans. Top: A375 (melanoma) or SF539 (glioblastoma) cells were treated with the indicated concentrations of MEKi (U0126), RAFKi (553013) or ERKi (FR180204) for 12, 24 or 48 h prior to analysis. Bottom left: SKMEL2 (melanoma) cells were treated with MEKi (U0126; 10 μM), imatinib (20 μM), p38i (p38 MAPKi SB202190; 50 μM), NH4Cl (ammonium chloride; 20 mM) or CLQ (chloroquine; 20 μM) for 48 h prior to analysis. Bottom right: WM2664 (melanoma) cells were treated with MON (monensin) or MMPi (GM6001) for 48 h prior to analysis.
2.5. Inhibition of the ERK signaling pathway enhances the expression of some, but not all, melanoma-associated targets

The induction of GPNMB in melanoma cell lines by a MEK inhibitor was compared to that of other melanoma-associated proteins by immunoblotting (Figure 5). Results indicated that GPNMB was the most consistently induced melanoma target of those examined. Some of the other melanoma-associated targets (MART-1, TYRP-2, TYRP-1, PMEL17) were also induced by MEK inhibition in some cell lines, while other targets either were not affected or even decreased (MAGEA1, MCSP) in response to MEK inhibition.

2.6. Identification of compounds that enhance GPNMB expression in melanoma and glioblastoma cell lines independently of NRAS/BRAF mutational status

In the screen for compounds capable of inducing GPNMB expression, several compounds that increased the expression of this protein in both melanoma and glioblastoma cell lines, regardless of the presence of NRAS or BRAF mutations, were identified. Compounds falling into this category included imatinib, p38 MAPK inhibitors, ammonium chloride (NH4Cl) and chloroquine. GPNMB was induced by these compounds in melanoma cell lines harboring mutations in NRAS (SKMEL2) or BRAF (A375, WM2664), as well as in melanoma cell line (MEWO) possessing wild-type NRAS/BRAF (Figure 6A). These compounds were also shown by flow cytometry to increase GPNMB surface-expression on SKMEL2 cells (Table 2, bottom left).
Figure 6 – Identification of drugs that increase the expression of GPNMB without inhibiting the ERK pathway. (A) Immunoblotting for GPNMB on whole-cell lysates harvested from various melanoma cell lines following exposure to the indicated compounds for 48 h. Compounds and concentrations were as follows: MEKi: U0126 (10 μM); imatinib (20 μM); p38i (p38 MAPKi SB202190; 50 μM); NH4Cl (ammonium chloride; 20 mM); CLQ (chloroquine; 20 μM). (B) Immunoblotting for GPNMB on whole-cell lysates harvested from various cell lines following exposure to the MEKi (U0126; 10 μM) or imatinib (20 μM) for 48 h. (C) Immunoblotting for GPNMB on whole-cell lysates harvested from various cell lines following exposure to the MEKi (U0126; 10 μM) or p38i (p38 MAPKi SB230580; 50 μM) for 48 h. (D) Immunoblotting for GPNMB on whole-cell lysates harvested from various cell lines following exposure to the protein synthesis inhibitors emetine (5 μg/mL) or cyclohexamide (20 μg/mL) in the presence or absence of NH4Cl (50 mM) for 1 h. (E) Immunoblotting for GPNMB and other melanoma-associated targets on whole-cell lysates harvested from various cell lines following exposure to emetine (5 μg/mL) or NH4Cl (20 mM) for 48 h. Information on the cell lines used in this figure is as follows: A375 and WM2664 are melanomas harboring mutations in BRAF, SKMEL2 is a melanoma harboring an NRAS mutation, MEWO is a melanoma wild-type for NRAS and BRAF, SF539 is a glioblastoma wild-type for NRAS and BRAF, and XF498 is a glioblastoma for which the mutational status of NRAS/BRAF is unknown (see Table 1). An antibody to ERK was used as a loading control and in some cases an antibody to phospho-ERK was used to determine the level of ERK activation.
A close examination of the effects of imatinib shows that this compound induced robust GPNMB expression in a variety of melanoma and glioblastoma cell lines without inhibiting phospho-ERK (Figure 6B). The inhibition of p38 MAPK similarly induced GPNMB expression in both melanoma and glioblastoma cell lines (albeit with some variability), but in contrast to imatinib, compounds that inhibited p38 MAPK decreased phospho-ERK levels in melanomas harboring mutations in NRAS (A375, WM2664) but not in the non-melanoma cell line examined (SF539 glioblastoma) that possesses wild-type NRAS/BRAF (Figure 6C).

Ammonium chloride increases the pH of lysosomes/endo- somes, thereby reducing the activity of the proteases residing in these organelles. We therefore considered the possibility that this compound increased GPNMB expression by increasing the half-life of this protein. To examine this possibility, cells were treated with a protein synthesis inhibitor (cyclohexamide or emetine) in the presence or absence of ammonium chloride, followed by immunoblotting for GPNMB (Figure 6D). Cells treated for just 1 h with a protein synthesis inhibitor in the absence of ammonium chloride expressed little or no GPNMB, indicating that this protein has a relatively short half-life. However, when ammonium chloride was included along with the protein synthesis inhibitor, GPNMB was once again readily detectable, thus supporting the hypothesis that ammonium chloride increased the expression of GPNMB via protein stabilization.

To examine whether a short half-life and stabilization by ammonium chloride are common attributes of melanoma-associated targets, a comparison of the effects of protein synthesis inhibition and ammonium chloride on the expression of GPNMB as well as two other melanoma-associated targets (MTF, MCAM) was performed (Figure 6E). The results of this experiment showed that GPNMB possessed a shorter half-life and was more highly induced by ammonium chloride than were the two other melanoma-associated targets.

2.7. Identification of compounds that inhibit GPNMB shedding

Shedding of membrane proteins is a common phenomenon that may affect ADC activity. To investigate potential GPNMB shedding, conditioned media collected from melanoma cell lines was immunoblotted for GPNMB (Figure 7A). Results showed that a protein which migrated slightly faster than the slowest migrating ~130 kDa cell-associated GPNMB species was readily detectable in the conditioned media of GPNMB-expressing melanoma cell lines (WM2664, UACC62). Shed GPNMB was also readily detected in conditioned media harvested from several other melanoma and glioblastoma cell lines examined, although a very low level of shed GPNMB was found in conditioned media harvested from A375 melanoma cells (data not shown), a finding which is consistent with the low basal level of GPNMB expression by these cells. Next, the ability of various compounds to influence GPNMB shedding was examined, and two compounds that decreased or eliminated GPNMB shedding were identified (monensin and the metalloprotease inhibitor GM6001; Figures 7A,B). Monensin also decreased or eliminated the slowest migrating cell-associated ~130 kDa GPNMB species, and increased the expression of the faster migrating ~110 kDa GPNMB species. Both monensin and GM6001 increased GPNMB surface expression on melanoma cells (Table 2, bottom right). These results indicate that GPNMB shedding occurs and can be decreased through pharmacological intervention.

3. Discussion

The main goal of this preclinical investigation was to examine whether the expression of GPNMB could be pharmacologically induced in cancer cells, thereby potentially sensitizing cancer cells to the effects of CR011-vcMMAE, and our results demonstrate that this is indeed the case.

Inhibitors of the ERK pathway induced GPNMB expression in melanoma cell lines harboring NRAS or BRAF mutations, but not in a melanoma cell line which possessed wild-type NRAS/BRAF or in non-melanoma cancer cell lines possessing wild-type or mutant NRAS/BRAF. A possible explanation for these findings is that GPNMB is often subjected to partial transcriptional repression in melanoma cells possessing a constitutively activated ERK pathway, and inhibitors of the ERK pathway relieve this repression. Our timecourse data (Figure 3B) is consistent with ERK inhibitors inducing GPNMB expression at the transcriptional level, as is microarray data generated by Shields et al. (2007) in which GPNMB transcript levels were found to be significantly increased in BRAF-mutant melanoma cell lines following exposure to a MEK inhibitor. The observed lack of GPNMB induction by ERK pathway inhibitors in non-melanoma cell lines harboring mutations in BRAF (HT29 colon carcinoma) or NRAS (HT1080 fibrosarcoma) in our study may indicate that such cell types lack crucial transcription factors required for GPNMB expression. It should be noted that although GPNMB may be transcriptionally repressed as a consequence of constitutive ERK activation, the fact that at least some expression of GPNMB transcript and protein is detected in most melanoma cell lines harboring NRAS or BRAF mutations indicates that this transcriptional repression is generally incomplete.

There are a number of reports demonstrating the induction of genes other than GPNMB in response to inhibitors of the ERK pathway. For example, the aforementioned microarray data generated by Shields et al. (2007) lists a number of genes that are transcriptionally increased in BRAF-mutant melanoma cells following exposure to a MEK inhibitor. One of these genes, TYRP-2, encodes a protein involved in normal melanogenesis, and our results show that TYRP-2 is induced at the protein level following exposure of melanoma cell lines to a MEK inhibitor. Others have shown that melanoma cell lines exhibit evidence of phenotypic differentiation in response to inhibition of the ERK pathway, concomitant with increased expression of proteins which play a role in normal melanogenesis (Englaro et al., 1998; Kono et al., 2006; Koo et al., 2002), and that melanoma progression is associated with reduced expression of differentiation-associated genes (Ryu et al., 2007). Thus, a constitutively activated ERK pathway, which is present in most melanomas as a consequence of NRAS or BRAF mutation, may actively participate in the maintenance of a dedifferentiated state in melanoma cells by suppressing the expression of differentiation-associated genes. A similar scenario has
recently been described for thyroid cancer where a constitutively activated ERK pathway in tumor cells was associated with the loss of expression of iodide-metabolizing genes, and whose expression was restored, with therapeutic implications, following inhibition of the ERK pathway (Liu et al., 2007).

While the precise function of GPNMB in normal melanocytes is unknown, evidence suggests that this protein is required for normal melanosome function (Anderson et al., 2002). Thus, our finding that inhibitors of the ERK pathway specifically induce GPNMB expression in melanomas harboring mutations in NRAS or BRAF may represent another example of a differentiation-associated gene that is repressed in cancers possessing a constitutively activated ERK pathway, and inducible in response to the pharmacological inhibition of this signaling pathway. We propose that it may be possible to therapeutically exploit these findings for the treatment of malignant melanomas harboring NRAS or BRAF mutations by using inhibitors of the ERK pathway (to induce GPNMB expression) in combination with the GPNMB-targeting ADC, CR011-vcMMAE. The results obtained in the “proof-of-principle” tumor cell growth-inhibition experiment performed in the present investigation support this approach. There is already an FDA-approved inhibitor of the ERK pathway (sorafenib, which inhibits RAF) and a number of others are in clinical development (Gray-Schopfer et al., 2007; Roberts and Der, 2007). It is interesting to note that inhibitors of the ERK pathway have previously been shown to sensitize tumor cells to antibody-based therapy (anti-EGFR) in preclinical studies (Benvenuti et al., 2007), and that the combination of an ERK pathway inhibitor (sorafenib) with an anti-EGFR antibody (cetuximab) is currently being examined in a clinical trial.

In addition to inhibitors of the ERK pathway, we also found that GPNMB was strongly induced by imatinib, an FDA-approved inhibitor of multiple tyrosine kinases (c-Kit, PDGFR, Bcr-Abl). However, in contrast to what was found with inhibitors of the ERK pathway, imatinib induced GPNMB expression in both melanoma and glioblastoma cell lines regardless of NRAS/BRAF mutational status, and did so without inhibiting the ERK pathway. Thus, the combination of imatinib and CR011-vcMMAE is potentially useful for the treatment of both

Figure 7 – Identification of drugs that inhibit GPNMB shedding by tumor cells. (A) Immunoblotting for GPNMB on whole-cell lysates (P) and supernatants (S) harvested from WM2664 and UACC62 melanoma cell lines following exposure to the indicated compounds for 48 h. Compounds and concentrations were as follows: MEKi: U0126 (10 μM); imatinib (20 μM); NH₄Cl (ammonium chloride; 20 mM); CLQ (chloroquine; 20 μM); MON (monensin; 330 nM); MMPi (metalloprotease inhibitor GM6001; 25 μM). (B) Immunoblotting for GPNMB on whole-cell lysates (P) and supernatants (S) harvested from the WM2664 melanoma cell line following exposure to the indicated compounds/ concentrations for 48 h. The vehicle for MON (monensin) was ethanol and the vehicle for the MMPi (GM6001) was DMSO. An antibody to ERK was used as a loading control for whole-cell lysates.
brane-anchored metalloproteases, particularly ADAM10 and "sheddases", and members of the ADAMs family of membrane proteins (Bonifacino and Traub, 2003). Thus, the possibility that imatinib increases GPNMB expression through increased protein stability due to altered sorting and/or processing as a consequence of changes in the phosphorylation status of GPNMB deserves consideration. Imatinib is currently being examined in a clinical trial for the treatment of melanoma in patients whose tumors harbor c-KIT mutations.

Like imatinib, inhibitors of p38 MAPK increased the expression of GPNMB in both melanoma and glioblastoma cell lines regardless of NRAS/BRAF mutational status and did so without inhibiting the ERK-pathway in cells possessing wild-type NRAS/BRAF. However, in contrast to imatinib, p38 MAPK inhibitors did inhibit the ERK pathway in melanoma cells harboring BRAF mutations. Thus, while an inhibition of the ERK pathway provides a potential mechanism by which p38 MAPK inhibitors induce GPNMB expression in melanomas possessing mutant BRAF, this cannot be the sole mechanism by which these compounds increase the expression of GPNMB. Of interest, p38 MAPK has been shown to influence the downregulation and trafficking of the EGFR and p38 MAPK inhibitors enhanced the stability of this receptor (Frey et al., 2006). Thus, the possibility that p38 MAPK inhibition similarly increases GPNMB expression through enhanced protein stability deserves consideration. There are currently a number of p38 MAPK inhibitors under clinical investigation (Roberts and Der, 2007).

Other compounds which induced GPNMB expression in the present investigation were ammonium chloride and chloroquine, which are both lysosomotropic weak bases. Since these compounds neutralize the acidic environment of endocytic vesicles thereby causing decreased activity of resident acidic proteases, it is possible that GPNMB expression is induced by these compounds as a consequence of reduced GPNMB degradation. Our finding that GPNMB protein has a short half-life which is extended in the presence of ammonium chloride is consistent with this hypothesis. Since the activity of CR011-vcMMAE is dependent upon functional endosome/lysosome-mediated proteolytic activity to facilitate dissociation of antibody/toxin moieties following ADC internalization, the benefit of this drug combination may be best realized by sequentially treating cancer cells first with a lysosomotropic agent, which is expected to enhance the activity of CR011-vcMMAE, although this remains to be determined.

A straightforward mechanistic rationale for using the CR011-vcMMAE in combination with compounds that increase the surface expression of GPNMB on tumor cells is that this would allow for more efficient tumor-targeting of CR011-vcMMAE, which would in turn translate into enhanced anti-tumor activity by this ADC. However, additional mechanisms independent of effects on GPNMB expression can also be envisioned as potentially contributing to a favorable outcome upon using CR011-vcMMAE in combination with these other drugs. For example, metastatic malignant melanoma does not respond well to single-agent chemotherapy and available evidence suggests that there may be a therapeutic benefit from simultaneously targeting multiple signaling pathways in this malignancy (Smalley et al., 2006). Thus, the combined use of CR011-vcMMAE (whose auristatin-based cytoxic moiety targets tubulin) together with a compound that targets a different pathway (such as an inhibitor of the ERK pathway) might be therapeutically beneficial due to a simultaneous attack on two different pathways. Consistent with this possibility, auristatin has previously been shown to synergize with other anticancer agents to induce tumor growth inhibition (Mohammad et al., 1998). Also, since inhibition of the ERK pathway has been shown to decrease the expression of P-glycoprotein (Katayama et al., 2007), it is possible that using CR011-vcMMAE in combination with an
inhibitor of the ERK pathway would enhance the anticancer activity of CR011-vcMMAE by increasing the intracellular retention of the MMAE drug moiety within tumor cells. Finally, the relatively long half-life of antibody-based drugs such as CR011-vcMMAE may be particularly advantageous for use in combination due to dosing/scheduling considerations.

In summary, a number of compounds that increase the expression and/or decrease the shedding of GPNMB have been identified, and the results of a proof-of-principal experiment has shown that such compounds can be used to enhance the growth-inhibitory activity of CR011-vcMMAE towards cancer cell lines. These studies support the evaluation of CR011-vcMMAE used in combination with other drugs for the treatment of metastatic malignant melanoma and glioblastoma. Whether any of the compounds identified in this study can be used to increase the therapeutic window of CR011-vcMMAE in cancer patients remains to be determined.

4. Experimental procedures

4.1. Cell lines and reagents

Cancer cell lines were of human origin and were obtained from the American Type Culture Collection (Manassas, VA) and from the National Cancer Institute (Bethesda, MD) and cultured in RPMI-1640 supplemented with 10% FBS (growth media). Sources of reagents were as follows: RAFKi (553013), MEKi (U0126), MEKi (PD98059), MEKi (444939), ERKi (FR180204), p38 MAPKi (SB202190), p38 MAPKi (SB203580), aurora kinase inhibitor (189404), JNK inhibitor (420119), MMPi (FR180204), p38 MAPKi (SB202190), p38 MAPKi (SB203580), aurora kinase inhibitor (189404), JNK inhibitor (420119), MMPi (GM6001) were from EMD Chemicals (San Diego, CA). ERKi (A6355), RAFKi: (GW5074), ammonium chloride (NH4Cl), chloroquine, monensin, cyclohexamide and emetine were from Sigma (Saint Louis, MO). HSP90i (geldanamycin) was from AG Scientific (San Diego, CA) and imatinib (Gleevec) was purchased from a pharmacy.

4.2. Immunoblotting

Cells were seeded in 6-well tissue culture dishes at a subconfluent density and allowed to attach overnight. The following day, cells were treated with the compounds diluted in growth media, as indicated in the figures/legends. For harvesting cell lysates, media was removed, cells were briefly washed with serum-free media and then 1 × Tris-glycine SDS sample buffer (Invitrogen, Carlsbad, CA) supplemented with 10 mM dithiothreitol (Sigma) was added directly to the wells. Cell lysates were then collected and boiled for 10 min. For preparing conditioned media, drug treatments were performed in a minimum volume of growth media (1.5 mL/well). Following 48 h of incubation, conditioned media was collected, briefly microfuged, mixed with an equal volume of 2 × Tris-glycine SDS sample buffer (Invitrogen) supplemented with 20 mM dithiothreitol (Sigma) and boiled for 10 min. For cell lysates, samples were quantified and an equal quantity of protein/lane was resolved on 4–20% gradient polyacrylamide gels (Invitrogen) and then transferred to nitrocellulose filters (Invitrogen). The volume of conditioned media loaded/lane was adjusted based on the protein concentration determined in the respective cell lysates. Immunoblotting was performed using standard procedures and the following primary antibodies: MART1 (sc-20032), TYRP2 (sc-25544), TYRP1 (sc-25543), PMEL17 (sc-15010), MAGEA1 (sc-20033), MCSP (sc-20162), MTF (sc-26651), MCAM (sc-18942), pp-ERK (sc-7976) and ERK (sc-94) (preceding antibodies were purchased from Santa Cruz Biotechnologies, Santa Cruz, CA), GPNMB (AF2550; R & D Systems, Minneapolis MN), actin (A5060; Sigma). Following incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies, enhanced chemiluminescence (GE Healthcare, Chalfont St. Giles, United Kingdom) was used for detection.

4.3. Flow cytometry

Cells were seeded in 6-well tissue culture dishes at a subconfluent density and allowed to attach overnight. The following day, cells were treated as indicated in the figures/legends, harvested with versene (Invitrogen), washed with PBS and incubated with primary antibody (10 μg/mL) diluted in staining buffer (PBS pH 7.4, 4% FBS, 0.1% sodium azide) for 30 min on ice. Primary antibodies utilized were CR011 (human IgG2 monoclonal antibody raised to the extracellular domain of human GPNMB (Tse et al., 2006)) and an unrelated human IgG2 isotype control monoclonal antibody. After incubation with primary antibody, samples were washed with staining buffer and then incubated with secondary antibody (PE-conjugated anti-human antibody; 1:100) for 30 min on ice. After incubation, samples were washed with staining buffer and examined on a Becton Dickinson FACSCalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA). Data analysis was performed with Becton Dickinson Cell Quest software and the geometric mean fluorescence intensity (GeoMean) was determined for each sample.

4.4. In vitro growth-inhibition/cytotoxicity assay

For UACC62, cells were seeded in tissue culture dishes at a subconfluent density and allowed to attach overnight. The following day, cells were treated with or without U0126 (1 μM) diluted in growth media and incubated for 48 h. After incubation, cells were washed, harvested, counted and seeded in growth media in 6-well tissue culture dishes in the absence or presence of CR011-vcMMAE at various concentrations for 72 h. CR011-vcMMAE is a GPNMB-targeting ADC which has been previously described (Tse et al., 2006). After incubation, photomicrographs were taken and adherent cells were trypsinized, mixed with trypan blue dye and counted on a haemocytometer. For SKMEL2, cells were seeded in 96-well plates after U0126 treatment and assayed after 72 h using the CellTitert-Glo assay according to the manufacturer’s instructions (Promega, Madison, WI).

REFERENCES


