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From existing therapies to novel targets: a current view on melanoma

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1. ABSTRACT

Identifying new drugs and targets for melanoma therapy is critical, considering that melanoma, the most dangerous form of skin cancer, is resistant to currently available therapeutics. Much work has been focused on finding novel drugs and exploring different treatment options that could increase the overall survival of patients. In our laboratory we have developed mouse models to study melanoma. We discovered that aberrant expression of metabotropic glutamate receptor 1 (Grm1) in melanocytes promotes melanoma development *in vivo*. Grm1 is a seven transmembrane domain G-protein coupled receptor that is normally expressed and functional in the central nervous system. The natural ligand of Grm1 is glutamate. Signaling by the major neurotransmitter glutamate has been well characterized in neuronal cells; however glutamate signaling in other tissues is not well understood. We demonstrated that Grm1 signaling in melanoma cells is mediated by the Ras/Raf/MEK/ERK pathway, one of the major pathways previously shown to be activated in human melanoma cells. Based on these earlier studies and results from our recent work, we predict that inhibition of Grm1 signaling and its downstream cascade may potentially provide new, effective therapies for melanoma patients. In this review, we propose several attractive targets.

2. INTRODUCTION

Melanoma, the most dangerous form of skin cancer, is characterized by uncontrolled growth of melanocytes. Melanocytes are pigment cells that reside at the basal layer of the epidermis and produce melanin to protect the skin from UV damage. Although only 4% of all skin cancers are melanoma, approximately 80% of deaths among skin cancer patients are attributed to this disease (1). In the year 2005, around 60,000 new cases of melanoma are expected with approximately 8,000 deaths in the United States (1). The overall lifetime risk of developing melanoma is one in 81 for women, and one in 57 for men. Furthermore, melanoma is one of the most common cancers among people under the age of forty (1). There are four major types of melanoma: superficial spreading, nodular, lentigo maligna, and acral, as well as less usual types such as ocular and mucosal (2). Different types of melanoma are likely due to differences in molecular mechanisms underlying the disease; the most common type is superficial spreading melanoma, which accounts for 80% of all melanomas (2). Studies on the causes of melanoma have led to the identification of several risk factors, including the presence of congenital or dysplastic nevi, genetics (light skin, light hair color, poor tanning ability, etc.), UV radiation exposure, family history, and

immunosuppression (1). UV radiation exposure as a factor in melanoma development remains a center for controversy, as a mechanism has not been elucidated (3). Development of melanoma is most likely due to the combination of several factors, including the tumor microenvironment (3).

Physicians generally are guided by the ABCD rule, which is Asymmetry, Border irregularity, Color variance, and Diameter (4), in examining suspicious moles. In addition, efforts to educate individuals about the dangers of skin cancer, risk factors and warning signs, have been important in assisting physicians to detect, diagnose and treat melanoma early, thereby increasing the overall survival of patients. Patients with localized tumors have a 5-year survival rate of 98% (1). However, if distant metastasis occurs, the 5-year survival rate falls to 16% (1). Histopathology of the tumor determines the prognosis for the patient. Some prognostic markers for poor survival rates include thickness, ulceration, location of melanoma, lymph node involvement, and poor overall health status of the patient (4). Melanoma thicker than 4mm in diameter is associated with nodal and distant metastasis (4). In the early 1990s, a new surgical technique known as sentinel node biopsy emerged (5). This method permits the identification and removal of the particular lymph nodes to which tumors drain. Currently, this technique remains the most accurate staging indicator for melanoma (6). Comparison between those patients receiving sentinel node biopsy, and those in the "wait and watch" set, showed that the sentinel-node group had a 26% less chance of recurrence of melanoma (7).

3. TREATMENTS

3.1. Interferon alpha

The most effective treatment option for melanoma is surgical resection. Patients with tumors thicker than 4mm, or with positive sentinel node indicating infiltration of tumor cells, have medium to high risk for recurrent or metastatic melanoma. It is recommended that these patients undergo systemic adjuvant therapy after removal of primary tumors. The only adjuvant therapy approved by the Food and Drug Administration (FDA) for melanoma with high risk of recurrence, is interferon alpha (4). Interferons, of which there are several types, are naturally produced by cells to protect themselves from viral infections. Once bound to their receptors, interferons activate downstream signaling cascades. The classical pathway involved in interferon signaling is signal transducers and activators of transcription (STAT). STAT proteins are transcription factors that translocate to the nucleus when activated, and regulate expression of gene products including those that are involved in apoptosis and inhibition of cell growth (8). Several studies pointed to the ability of interferon to inhibit tumor cell proliferation; therefore, it has been used as a therapeutic agent for several cancers including melanoma (8). Interferon was initially advanced as an option for melanoma treatment due to its ability to inhibit growth of B16 murine melanoma cells implanted in mice (9). It was observed that, in hematopoietic cells, long-term treatment with interferon alpha induces cell cycle arrest at G_0/G_1, and short-term treatment inhibits the activation of cell signaling pathways, including the MAPK (Mitogen Activated Protein Kinase) pathway (10). Studies of several human melanoma cell lines showed that both interferons alpha and beta induced apoptosis through caspases, Fas/FasL, and TNF-alpha related apoptosis inducing ligand (TRAIL), with interferon beta being more efficient (11). In addition to induction of apoptosis, interferon also inhibits angiogenesis (12).

Clinical trials of low or intermediate doses of interferon on melanoma patients demonstrated no survival benefits, although meta-analysis indicated a 13% reduction in the odds of recurrence in some cases with low-dose interferon treatment (13). Several clinical trials were performed in Europe with maximum tolerated dose of interferon in melanoma patients. One trial by Eastern Cooperative Oncology Group (ECOG) that used high-dose interferon alpha-2b administered intravenously and subcutaneously (E1684), resulted in prolonged relapse-free and overall survival (14). This trial was followed by E1690, which included both high-dose and low-dose interferon treatments (15). Although relapse-free 5 year survival was improved in the high-dose group, the 5 year overall survival was not improved, possibly due to confounding factors such as a high-number of crossover of patients from observation group to high-dose group (16). Low-dose interferon treatment did not improve relapse-free survival or overall survival (16). After updating disease-status and survival, several trials were pooled to analyze data. For trial E1684, after follow-up of 12.6 years, statistical significance was observed in relapse-free survival, but not in overall survival (16). Taken together, the pooled data revealed that there are advantages for high-risk patients to be treated with interferon due to improvement in relapse-free survival, despite lack of significance for overall survival (16). The significance of interferon treatments remains controversial due to yet another pooled analysis of nine trials that revealed no advantage in disease-free survival or overall survival (17). The response-rate is not substantial enough to warrant high-dose interferon treatments, especially since such options produce extremely unpleasant side effects (18). Side effects of interferon treatment include flu-like symptoms, fatigue, diarrhea, and depression, thus requiring careful monitoring by administering physicians (13). Since the effectiveness of interferon adjuvant therapy on patients with intermediate to high-risk melanoma remains unclear, additional therapeutic options for better relapse-free and overall survival would benefit melanoma patients.

3.2. Interleukin-2

Interleukin-2 (IL-2) is an immune-modulating glycoprotein. One of the functions of IL-2 is the activation of immune cells such as natural killer cells. This activity has generated interest in the use of IL-2 in cancer immunotherapy (19). Regression of metastatic tumors has been shown with IL-2 in renal cell carcinoma (20). Clinical response is measured in terms of complete response, partial response, and no response. In general, complete response represents regression of tumors followed by no growth for 6 months; partial response represents 50% reduction of tumors; and no response means no change (21). The first

randomized clinical trial for intermediate or high risk melanoma patients was conducted with subcutaneous injection of low-dose IL-2 in combination with interferon alpha-2B (22). An overall survival benefit was not found for patients who received the combination therapy, when compared to observation group, therefore this clinical trial was unsuccessful. Treatment of patients with high-dose IL-2 alone has been approved by the FDA for metastatic melanoma, although results have been disappointing with only 16% of patients showing a response (23). Treatment with IL-2 causes severe side effects, including, but not limited to, hypotension, myocarditis, transient renal insufficiency, and capillary-leak syndrome (24). There have been attempts to alter dose levels and change routes of administration in clinical trials, to diminish these severe side effects and to improve response-rate. Recently, intra-lesional injection of IL-2 was done in patients with skin and soft-tissue metastasis: 62.5% of patients had complete response and another 21% showed partial response (21). With intra-lesionally delivered IL-2, patients tolerated this route of administration well with minimal side effects (21). For melanoma patients with metastasis who did not respond to current therapeutic regimens, a new immunotherapy was tested. This immunotherapy consisted of chemotherapy, followed by re-introduction of the patient's own activated lymphocytes in combination with high-dose IL-2 adjuvant therapy (25). About 51% of the patients showed improvement, from complete responses (8.6%) to partial responses (43%) with manageable side effects.

3.3. Others

Compounds such as dacarbazine and cisplatin, among others, have been used in clinical trials for treatment of stage IV (metastatic) melanoma, in hopes of better response-rate and prolonged survival of patients. Dacarbazine, an alkylating agent, showed the highest responses among selected chemotherapeutic drugs (26). After combining data gathered through several clinical trials over the years, Eggermont and Kirkwood reported that dacarbazine alone showed similar or better responses, compared to when dacarbazine was used in combination with other drugs (26). In a phase III clinical trial of stage IV melanoma patients, no differential survival benefit was shown between patients treated with dacarbazine alone, and patients treated with the Dartmouth regimen (dacarbazine, cisplatin, carmustine, and tamoxifen) (27).

In addition to the quest for novel and more effective drugs, many groups are actively pursuing improvements in gene therapy for the treatment of melanoma. The ideal way to correct a lost or defective gene would be to replace it, in order to restore proper cellular function. In melanoma, tumor suppressor genes such as PTEN, or cell cycle regulators such as $p16^{Ink4A}$, have been found to be lost in many cases; therefore they represent good candidates for replacement by gene therapy. However, gene therapy has been proven to be a simple concept with numerous complex problems in targeting, delivery, dosage, toxicity, and immune responses (28). The first successful gene therapy trial was with children suffering severe combined immunodeficiency (SCID); failing other treatments, such patients are required to stay within a germ-free environment. They develop SCID due to a defective cytokine receptor gamma chain, and replacement of the defective gene by gene therapy was touted as freeing children from their confinement. However, more recent reports indicate that some of the children so treated, later developed leukemia (29). Another gene therapy trial resulted in the death of a patient with ornithine transcarbamylase deficiency, just a few days after treatment (30). This case, highly publicized in 1999, brought attention to the limitations of gene therapy, including the safety and toxicity of delivery vehicles. Despite such limited successes in treating patients with gene therapy, investigators continue to improve techniques, and to devise safer ways to deliver and replace gene(s) for effective restoration of their normal functions. For melanoma or other skin diseases, one of the major problems of gene therapy is proper targeting to the skin. One way to deliver genes to the skin is by electroporation (31). Electroporation, which opens transient pathways into cells by means of short electric pulses, has been used to deliver exogenous DNA into human skin xenografts (31). In that study, electroporation greatly enhanced delivery of DNA compared to intradermal injection of DNA alone. In addition, the expression of a given gene was sustained for longer than 15 days, which was the maximum time point measured. This new technical improvement in electroporation increases the feasibility for the future use of gene therapy for the treatment of melanoma by delivering active reagents to the skin.

Despite numerous uncertainties underlying the mechanisms of actions, and the recurrence and persistence of metastases after treatments with interferon alpha and IL-2, these two options continue to be the most promising management techniques for overall survival of melanoma. Taken together, the necessity to discover novel, and more relevant therapeutic targets remains a top priority. Below, we will attempt to select several attractive targets for the treatment of melanoma, based on published literature and results from our laboratory.

4. METABOTROPIC GLUTAMATE RECEPTOR 1 (GRM1)

Animal models have evolved as indispensable tools to study the initiation, progression, and treatments of many different types of human disease. Animal model systems also provide means for understanding the molecular mechanisms underlying a particular disorder and provide opportunities to identify putative targets, discover and develop new drugs, and treatments. In the last 15 years, an increasing number of animal models have been developed and used to study a diverse array of cancers including melanoma. Our laboratory has developed a transgenic mouse model (TG-3) that is predisposed to spontaneous melanoma development. Onset of melanoma in TG-3 occurs with high penetrance, short latency and high metastatic potential (32-34). Spontaneous development of melanoma and tumor progression in TG-3 mimics and recapitulates the evolution of melanoma as seen in humans, from benign lesions to metastasis (32, 33). In TG-3, we showed that insertional mutagenesis by a

transgene resulted in the appearance of pigmented lesions which subsequently were confirmed to be melanoma by histopathology (34, 35). Extensive molecular studies revealed that the transgene had integrated into intron 3 of metabotropic glutamate receptor 1 (Grm1), resulting in ectopic expression of Grm1 in melanocytes and leading to tumor development (36). In order to distinguish whether the aberrant expression of Grm1 in tumors was a cause or a consequence of melanoma, a new transgenic line (E line) was constructed. It was engineered to express Grm1 cDNA under the regulation of a melanocyte-specific promoter, dopachrome tautomerase (DCT). This new E transgenic line displayed similar onset and progression of melanoma as observed for TG-3. Taken together, results from these studies demonstrated that aberrant expression of Grm1 in melanocytes is sufficient to induce melanoma development *in vivo* (36).

Grm1 is a seven-transmembrane-domain, G-protein-coupled receptor (GPCR), normally expressed and functional in the central nervous system. The natural ligand for Grm1 is glutamate, a major neurotransmitter. To date, eight metabotropic glutamate receptors have been discovered and they are classified into three groups according to sequence homology and pharmacological profiles. Grm1 and Grm5 belong to group I metabotropic glutamate receptors. Group I receptors, when stimulated, are preferentially coupled to $G_{q/11}$ leading to phospholipase C (PLC) activation, and give rise to two second messengers: diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP3). DAG, in turn, activates protein kinase C (PKC) and IP3 leads to Ca^{2+} release from the endoplasmic reticulum, intracellular calcium storage. Involvement of GPCR signaling in tumor development, especially in melanoma, was reviewed recently (37). GPCRs such as endothelin receptor (ETRB) have been implicated to be important in tumor cell proliferation (37). A specific inhibitor to ETRB, BQ788, induced apoptosis in melanoma cells *in vitro* and growth arrest in xenografts of human melanoma cells in nude mice (38). Cell signaling mediated by Grm1 has been extensively studied in neuronal cells, as well as in heterologous cells transfected with Grm1. In the heterologous systems, activation of Grm1 by its natural ligand glutamate, or its agonist L-quisqualate, leads to activation of extracellular-signal regulated kinases (ERK) (39, 40). This activation is inhibited by PD 098059, a MAPK/ERK kinase (MEK) inhibitor, implicating involvement of MEK/ERK in Grm1 signaling (39).

Activation of MAPK pathway has been demonstrated to be an important event in human melanoma. Constitutively activated MAPK has been detected in several human melanoma cell lines and biopsies (41). In our laboratory, several TG-3 tumor-derived cell lines were established. We showed these cells to be transformed and tumorigenic (42). We confirmed Grm1 in these cells to be functional as demonstrated by the accumulation of IP3 in the presence of Grm1-agonist. Furthermore, we also showed the activation of MEK and ERK by Grm1-agonist (manuscript submitted). The specificity of Grm1 signaling in IP3 accumulation and activation of MEK/ERK was demonstrated by suppression of both, by Grm1-specific antagonist LY367385 (manuscript submitted). Additionally, dominant-negative mutants of Grm1 (43) were used to further verify Grm1 signaling in these mouse melanoma cells (manuscript submitted).

Normally, Grm1 acts as a regulator of synaptic transmission. There are several pharmacological agents available to block Grm1 signaling. These antagonists have been shown to be valuable in elucidating the physiological functions of Grm1 in neuronal cells. LY367385 [(+)-2-methyl-4-carboxyphenylglycine] is a specific competitive antagonist of Grm1 (44); CPCCOEt [(-)-(1aS, 7aS)-(2-hydroxyimino-1a,2-dihydro-1H-7-oxa-cyclopropa-[b]naphthalene-7a-carboxylic acid ethyl ester) is a noncompetitive antagonist (45); and BAY 36-7620 [(3a*S*, 6a*S*)-6a-naphtalen-2-ylmethyl-5-methyliden-hexahydro-cyclopental[*c*]furan-1-on] is also a noncompetitive antagonist of Grm1 with inverse agonist activity (46). Inverse agonists are antagonists that inhibit both agonist-induced activation and constitutive activation of receptors. Homer proteins belong to a family of the PDZ domain-containing proteins that bind to the intracellular C-terminus of group I metabotropic glutamate receptors and regulate their signaling by linking them to inositol trisphosphate receptors (47, 48). Overexpression of group I metabotropic glutamate receptors (Grm1 and Grm5) resulted in constitutive activation of receptors in heterologous systems (49). Ango and colleagues reported that expression of Homer1a can be induced in cerebellar granule cells unlike other Homer proteins (50, 51). This dominant negative form of Homer (Homer1a) was shown to interfere with the regulatory interaction between Grm1 and Homer3, leading to the constitutive activation of endogenous Grm1; this constitutive activation is inhibited by BAY 36-7620, an inverse agonist (50, 51). Based on these earlier studies, we hypothesize that constitutive activation of Grm1 may be required for the maintenance of melanocyte transformation and melanoma development. Recent studies showed that noncompetitive antagonist with inverse agonist activity such as BAY 36-7620 can inhibit both agonist-induced and constitutive activity of Grm1 (50, 51), thus BAY 36-7620 may be useful for suppression of tumor progression.

5. PROLIFERATION PATHWAY

We have potentially pinpointed MAPK as one of the pathways activated in response to Grm1 stimulation; however molecules that participate in Grm1-to-ERK cascade as well as downstream targets of activated ERK remain to be identified. As stated earlier, results from our studies showed that treatment of TG-3 tumor cells with Grm1-agonist, L-quisqualate, resulted in activation of MEK and ERK (manuscript submitted), which are two of the integral components of the Ras/Raf/MEK/ERK pathway. This pathway has been shown by others to be one of the most important pathways in melanoma cell proliferation (41, 52).

5.1. Ras

Ras is a membrane-bound G-protein that conveys a wide variety of extracellular signals to specific

intracellular signaling pathways. There are three major isoforms of Ras: K-Ras, N-Ras and H-Ras. Upon activation by extracellular signals, Ras-specific guanine-nucleotide-exchange factors (GEFs) promote the exchange of GDP to GTP, activating Ras. Ras has been known to link receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) to downstream effectors such as Raf (MEKK) through Grb2, an adaptor protein, and son of sevenless (SOS), a guanine nucleotide exchange factor (53). Several mutants of Ras, which lack GTP hydrolase activity, have been shown to be most effective in promoting Ras-dependent signaling (53). Depending on cell type or receptors involved, Ras-dependent signaling pathways may be important in mediating cell differentiation or proliferation (54). MAPK, specifically Raf/MEK/ERK cascade, is one of the downstream targets of activated Ras that leads to cell proliferation (53).

In human melanoma, about 15% of tumors and cell lines were reported to carry N-Ras mutations (55). Previous studies showed the involvement of an activating mutant of N-Ras in promoting cell proliferation; therefore, mutated N-Ras has been recognized as a prime target for therapy. Ras is only active when it is bound to the plasma membrane. This is achieved through prenylation, in particular farnesylation, an addition of a 15-carbon group. Prevention of Ras signaling has been accomplished through inhibitors of farnesylation, blocking the membrane localization of Ras and interfering with oncogenic Ras signaling cascade (53). For example, an inhibitor of farnesyl transferase, SCH66336, inhibited B16 melanoma cell growth by inducing cell cycle arrest at G1 and inactivating retinoblastoma (RB) protein (52). Cisplatin is a DNA damaging agent that induces apoptosis in cancer cells (56); however some melanoma cells have been shown to be resistant to cisplatin. When used in combination with SCH66336, enhanced cisplatin-induced apoptosis was detected in melanoma cells (52). Since the actions of farnesylation inhibitors are not restricted to Ras, additional studies are needed to address general cytotoxic effects. Farnesyl thiosalicylic acid is a Ras antagonist that inhibits attachment of Ras to membrane, thereby promoting degradation of Ras. It has been shown to have anti-tumor activities by inducing apoptosis of Colo853 and B16 melanoma cells (57, 58). Farnesyl thiosalicylic acid thus may be a better therapeutic agent than farnesyl transferase inhibitor, due to its higher specificity for Ras and lower general cytotoxicity (58). As an alternative, inhibition of constitutive Ras signaling may also be accomplished by the use of antisense oligonucleotides or RNA interference (RNAi). Suppression of mutant protein expression via either method can be achieved with higher specificity than by pharmacological agents. Mutation of N-Ras at codon 61 is the most frequent Ras activating-mutation detected in human melanomas (53). Small interfering RNA (siRNA), targeted to the Q61R activating mutation in human melanoma cells, resulted in both suppression of downstream activation of ERK and induction of apoptosis (59).

5.2. Raf

Raf protein is one of the downstream targets of Ras. In general, activated Raf phosphorylates MEK, and activated MEK, in turn, phosphorylates ERK. There are three Raf isoforms: A-Raf, B-Raf and C-Raf. C-Raf, also known as Raf-1, is ubiquitously expressed in all tissues. A-Raf is highly expressed in ovary, but also detected in several other tissues at varying levels. However, B-Raf expression is restricted mostly to neuronal tissues (60). Transforming activity of Ras was impaired in a heterologous system (COS-M6) by dominant-negative mutants of C-Raf (61). Although C-Raf has been extensively studied, the regulation of Raf proteins, redundancy of their functions and interactions among different Raf isoforms, generally are not well understood (62). Depletion of A-Raf and C-Raf by siRNA did not inhibit the activation of MEK and ERK, while depletion of B-Raf by siRNA inhibited MEK and ERK phosphorylation in melanoma cells (63, 64). On the other hand, in human melanoma cells that harbor activating Ras mutations, or in Ras-transformed mouse melanocytes, activation of MEK and ERK was not affected by depletion of either B-Raf or C-Raf (64). These observations suggest Ras signaling may be mediated through proteins other than B-Raf or C-Raf, or functional redundancy between B-Raf and C-Raf. Taken together, results from these studies showed that depletion of one of the Raf isoforms is not sufficient to inhibit downstream targets either in Ras-transformed cells, or in cells harboring mutant Ras.

The most common mutation of B-Raf (V600E) has been detected in 66% of human melanomas (65). Subsequent studies by Dong *et al.* also have found that approximately 70% of nevi contain B-Raf mutations, while only 10% of radial growth phase (RGP) melanoma, or early stage melanoma, carried this B-Raf mutation. These findings imply that perhaps most nevi with B-Raf mutations may not progress to the next stage in melanoma development (66, 67), and that B-Raf may be involved in the initiation rather than the progression of melanoma. However, others have reported higher rates of B-Raf mutations in early as well as later stage melanomas. Goydos *et al.* showed that 55% of primary melanomas, 67% of regional metastases, 40% of nodal metastases, and 75% of distant metastases carry a detectable V600E mutation (68). Nonetheless, B-Raf is an important potential target for melanoma therapy since it is involved in a high percentage of melanomas, and also plays a pivotal role in the proliferation of melanoma cells. Normal melanocytes require supplementation with the phobol ester TPA (12-o-tetradecanoyl phorbol-13-acetate) for growth, and loss of this requirement is one of the indicators of melanocyte transformation (69). When normal mouse melanocytes (Melan-a) were transfected with mutated B-Raf (V600E), stable clones selected no longer required the presence of TPA for growth (64). Inhibition of this B-Raf mutant by siRNA led to inhibition of MEK phosphorylation, while inhibition of C-Raf did not (70). Cyclin D1, a key protein in cell cycle progression was also shown to be a downstream target of activated MAPK. When mutant B-Raf was transfected into normal human epidermal melanocytes, an increase in cyclin D1 expression was observed; melanoma cells which harbored mutant B-Raf displayed constitutive activation of cyclin D1 (71). Inhibition of the downstream target of B-Raf, MEK, by

U0126 abolished cyclin D1 expression, and direct targeting of cyclin D1 by siRNA was shown to inhibit DNA synthesis in mutant B-Raf melanoma cells (71).

In related efforts to inhibit mutant B-Raf using RNAi, conflicting reports have emerged. One group reported that siRNA specific for mutant B-Raf induced apoptosis in a human melanoma cell line (70); whereas another group suggested that apoptosis induced by inhibition of B-Raf by siRNA is not a general response, but rather cell line-dependent (72). In any case, both groups do agree that RNAi successfully targeted to mutant B-Raf would provide an excellent therapeutic target due to its specificity and its inhibition of growth and invasion of melanoma cells.

BAY 43-9006 is a general inhibitor particularly potent against both B- and C-Raf proteins and vascular endothelial growth factor receptor (VEGFR2). It is effective against both wild-type and mutant B-Rafs. BAY 43-9006 was shown to inhibit the growth of melanoma cells *in vitro* and *in vivo*; however, no apoptosis was detected, implying the possible requirement of combination therapies for optimal use against tumor cells (63). Clinical trials using BAY 43-9006 are ongoing to assess the effectiveness of this compound in clinical setting (73). Since BAY 43-9006 is also a general inhibitor, more specific inhibitors to mutant B-Raf are being actively pursued by several investigators.

The antibiotic geldanamycin displays potent anti-tumor activity; its mechanism of action was shown to operate through inhibition of heat-shock protein 90 (Hsp90) (74). Consequently, Hsp90 has emerged as a new molecular target for cancer treatment. Hsp90 is a chaperone involved in ensuring proper folding and conformational maintenance of proteins, properties which are critical for the function and stability of a given protein (75). Client proteins of Hsp90 include wild-type and mutant B-Rafs, which has been demonstrated by co-immunoprecipitation in extracts from B-Raf transfected cells (76). A derivative of geldanamycin,17-(Allylamino)-17-demethoxy-geldanamycin (17-AAG), has similar potency with less general toxicity, when compared to geldanamycin. 17-AAG has been shown to inhibit tumor growth in a human melanoma xenograft model (77), and it is currently in phase I/II clinical trials for melanoma (7).

5.3. MEK/ERK

U0126 and PD 098059 are specific inhibitors of MEK1 and MEK2, the downstream targets of activated Raf. Urokinase plasminogen activator (uPA), colleagenase-1 (MMP-1), and matrix metalloproteinase-9 (MMP-9), are proteins known to be involved in tumor cell invasion and have been demonstrated to be elevated in melanoma cells (78, 79). Treatment of melanoma cells with either U0126 or PD 098059 results in a decrease in levels of uPA, MMP-1, and MMP-9 as well as inhibition of cell growth (79-81). One group showed that pretreatment of cisplatin-resistant melanoma cells with the MEK inhibitor PD 098059, followed by cisplatin treatment, results in apoptosis (82). These results imply that cisplatin might be more effective in treating melanoma when used in combination with drugs that inhibit cell growth without inducing apoptosis, such as SCH66336, the inhibitor of farnesyl transferase (see above).

The MAPK pathway plays a pivotal role in mediating signal transduction cascades regulating cell proliferation, including the induction of transcription factors that promote cell growth. The potential of such transcription factors as targets for melanoma therapy has been the topic of intense study. Activation of B-Raf in melanoma cells induces the expression of the transcription factor, Brn-2 (83). Downregulation of Brn-2 by RNAi in human melanoma cells, results in inhibition of [^{3}H] thymidine incorporation indicating a reduction in DNA synthesis (83). Some other downstream targets of activated MAPK pathways have been identified; they include the cAMP response element binding protein (CREB). Overexpression of CREB and a related protein, activating transcription factor 1 (ATF-1), has been observed in melanoma. Building upon earlier reports using monoclonal antibodies to disrupt the activity of ATF-1, an anti-ATF-1 single chain antibody fragment was engineered and shown to effectively inhibit ATF-1 activity, leading to the suppression of both tumorigenicity and metastasis of human melanoma cells (84).

6. OTHER TARGETS OF MELANOMA THERAPY

Like Ras and Raf, Akt was first identified as a proto-oncogene. The Akt kinases have since been acknowledged to have a significant role in signal transduction pathways starting at the cell surface, and regulating various downstream cellular functions. Briefly, Akt is activated when extracellular stimuli activate PI3K (phosphoinositide 3-kinase), and its activity is reduced by the action of a phosphatase, the PTEN tumor suppressor gene. (85). Increase in the activated form of Akt has been associated with melanoma progression, as well as poor survival of patients (86). Deregulation of the Akt pathway is reported to occur in about 40-65% of melanomas, and is attributed to genetic amplifications causing overexpression of Akt, and also to loss of the negative regulator PTEN (87). Elevated levels of active Akt were observed in cell lines isolated from progressively later stages of melanoma (87). Increasing activated Akt leads to a decrease in apoptosis, and enhances tumor cell survival. One of the downstream targets of the Akt pathway is nuclear factor kappa B (NF-kappaB). NF-kappaB is an important transcription factor which is involved in cell survival, as well as inflammation (88). In several melanoma cell lines, NF-kappaB was shown to be constitutively activated leading to expression of several anti-apoptotic proteins and enhanced tumor cell survival (89). When a proteasome inhibitor, bortezomib, was used to interfere with NF-kappaB activity, an increase in the apoptotic response to chemotherapy, as well as a reduction in melanoma tumor growth, was reported (90).

Melanoma is refractory to standard, currently available therapeutic regimens, including chemotherapy, radiotherapy and immunotherapy. It has been proposed that

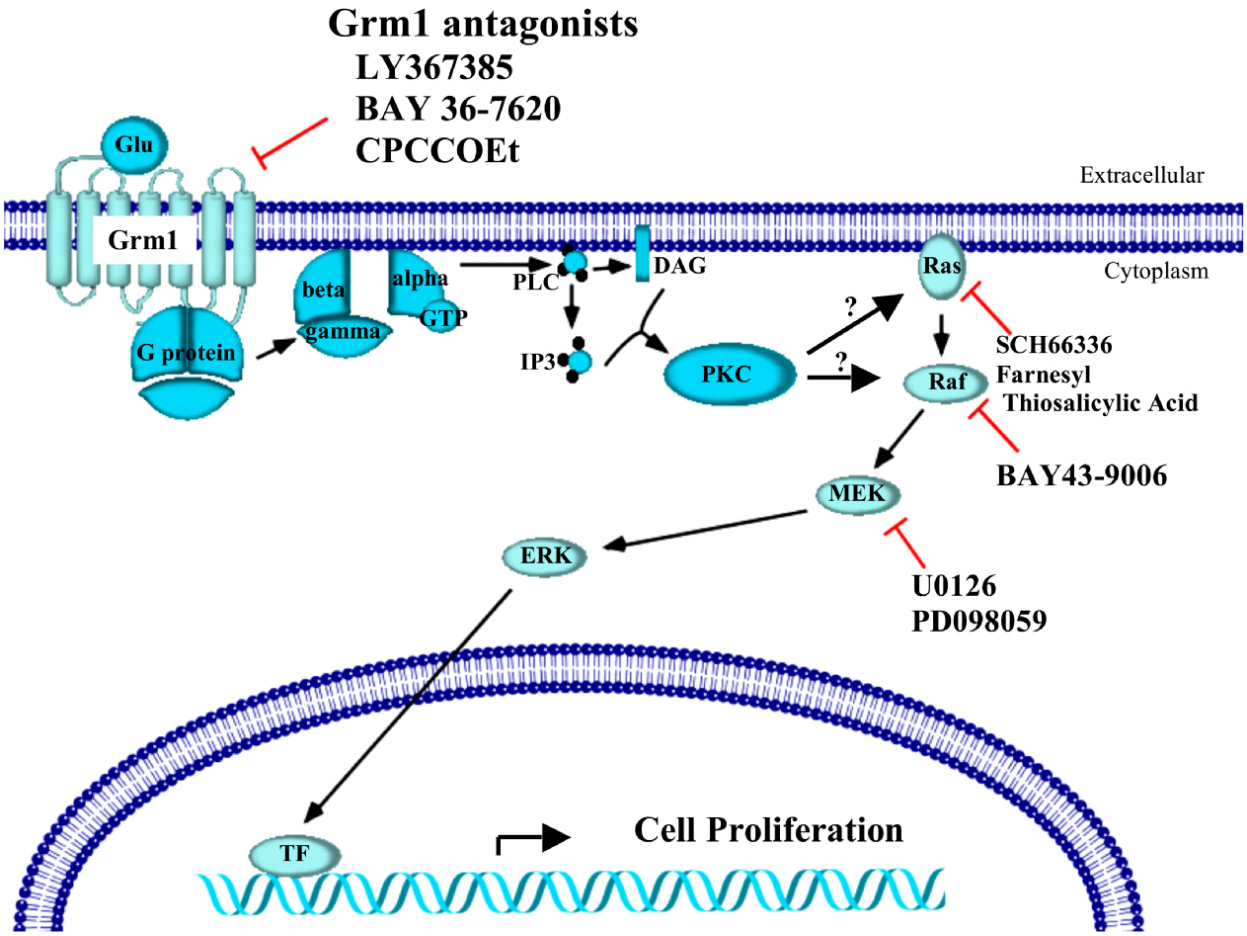

Figure 1. Metabotropic glutamate receptor 1 and its signaling cascade. Metabotropic glutamate receptor 1 (Grm1, also known as mGluR1) is a seven-transmembrane domain, G-protein coupled receptor. It has been reported to signal through $G_{q/11}$, leading to the production of diacylglycerol (DAG) and inositol triphosphate (IP3). Accumulations of these second messengers lead to the activation of the Ras/Raf/MEK/ERK pathway and activation of transcription factors (TF) such as cAMP response element binding protein (CREB). These signaling molecules can be hampered by pharmacological agents, as well as by RNA interference. Inhibition of this signaling pathway led to decrease in melanoma cell proliferation.

this resistance to conventional therapy may be related to the particular resistance of melanoma cells to apoptosis, which in turn suggested a link to the expression of genes involved in anti-apoptotic responses, such as B-cell lymphoma derived protein 2 (Bcl-2) and Bcl-xL (91). In fact, it has been shown that melanoma cells can be sensitized to apoptosis in response to antisense oligonucleotides or siRNA to Bcl-2 and related proteins (92, 93), suggesting antisense Bcl-2 can act as a chemosensitizing agent in melanoma therapy (94). Indeed, promising results have been reported in clinical trials where patients were treated with Bcl-2 antisense oligonucleotides in combination with chemotherapeutic agents such as dacarbazine. With such combined treatment, 11 patients achieved complete response, whereas only 2 patients showed complete response when treated with dacarbazine alone (94). Combination therapy not only increased apoptosis of tumor cells, but also increased overall survival of patients (95). These results emphasize the importance of targeting Bcl-2 and related proteins in the quest to modify the poor response of melanoma cells to conventional anti-cancer therapies.

7. PERSPECTIVES

Glutamate signaling has been well characterized in neuronal cells where glutamate is the major neurotransmitter. However, its signaling is not well understood in other tissues. The first reported participation of aberrant metabotropic glutamate receptor signaling in cancer, was the involvement of metabotropic glutamate receptor 1 in melanoma. This receptor and its signaling cascade are depicted in Figure 1, with potential check points indicated. Interference at specific points within the signaling pathways should inhibit melanoma cell proliferation. These prospective targets for interruption and their inhibitors are summarized in Table 1. The state of investigation into each of the inhibitory compounds ranges from *in vitro* experimentation, through animal models, to testing as therapeutic drugs in clinical trials. More recently,

Table 1. Potential Target Genes of Melanoma Therapy

Target Gene	Therapeutics	Mode of Action	References
Grm1	LY367385	Competitive Antagonist	44
	CPCCOEt	Non-competitive Antagonist	45
	BAY 36-7620	Non-competitive Antagonist with Inverse Agonist Activity	46
Ras	SCH66336	Farnesyl Transferase Inhibitor	52
	Farnesyl Thiosalicylic Acid	Antagonist	57, 58
	siRNA	RNA interference	59
B-Raf	BAY 43-9006	Raf Kinase Inhibitor	63
	17-AAG	Inhibitor of Hsp90 (one of Hsp90's client proteins is mutant B-Raf)	77
	siRNA	RNA interference	70-72
MEK	U0126, PD 098059	Inhibitor	79-81
Brn-2	siRNA	RNA interference	83
ATF-1	Monoclonal Antibody	Inhibitory Antibody	84
NF-kappaB	Bortezomib	Proteasome Inhibitor	90
Bcl-2	siRNA or Antisense Oligonucleotides	RNA interference or Antisense	92-94

another metabotropic receptor, metabotropic glutamate receptor 4, was reported to be overexpressed in colorectal cancer (96). The anticancer potential of ionotropic glutamate receptor antagonists has been examined by others; in cancers such as breast, colon, astrocytoma and lung, treatment of tumor cells by antagonists of ionotropic glutamate receptors resulted in inhibition of tumor cell proliferation and decreases in tumor cell motility (97). These observations, as well as data from our mouse models, underscore the importance of glutamate signaling in tumor development and progression. It will be of significant interest to further explore the intricate signaling network of metabotropic and ionotropic glutamate receptors in cancer.

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Abbreviations: Grm1: metabotropic glutamate receptor 1; MEK: MAPK/ERK kinase; ERK: extracellular-signal regulated kinases; MAPK: mitogen activated protein kinase; NF-kappaB: nuclear factor kappa B; Bcl-2: B-cell lymphoma derived protein 2; TF: transcription factor; IL-2: Interleukin-2; DCT: dopachrome tautomerase; GPCR: G-protein-coupled receptor; PLC: phospholipase C; DAG: diacylglycerol; IP3: inositol 1, 4, 5-triphosphate; RNAi: RNA interference; siRNA: small interfering RNA; Hsp90: heat-shock protein 90; CREB: cAMP response element binding protein; ATF-1: activating transcription factor 1; PI3K: phosphoinositide 3-kinase

Key Words: Metabotropic glutamate receptor, Melanoma, Cell Proliferation, MAPK, Grm1, Review

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