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Review

New Therapeutic Agents in Uveal Melanoma

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Abstract. *Uveal melanoma is the most common primary intraocular malignant tumour in adults. Five, ten and fifteen years after primary tumour treatment, up to 25%, 34% and 50% of patients may develop metastases, respectively. There are only a few systemic therapies that have been approved for uveal melanoma, all with doubtful efficacy. As the molecular knowledge over cancer has improved, new therapies are being developed. Several drugs, such as bortezomib, celecoxib, dacarbazine, anti-angiogenic agents (such as bevacizumab, sorafenib and sunitinib), temsirolimus, mitogen-activated protein kinase kinase (MEK) inhibitors, ipilimumab and AEB071 are candidate drugs, and studies are underway to determine the therapeutic effects of these drugs in uveal melanoma.*

Epidemiology

Uveal melanoma is the most common primary intraocular malignant tumour in adults (incidence of 6 per million per year; lifetime risk, 1 in 2500). It differs from cutaneous melanoma in its incidence and biological behaviour (1-3). A north to south decreasing gradient of uveal melanoma incidence is observed among European populations, supporting a protective role of pigmentation (4), and not supporting a contributory role for sunlight.

Development of uveal melanoma has been associated with the presence of ocular lesions, such as choroidal naevi and ocular or oculodermal melanocytosis, and with cutaneous melanocytic lesions (familial atypical mole or cutaneous melanoma). A lightly-coloured iris also increases the risk. No association has been found with any dietary habits, or smoking or alcohol consumption (5-6).

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The survival rates of patients with uveal melanoma have not changed in the last century. Possible locations of metastases from melanoma include liver (90%), lung (24%) and bone (16%). After five and ten years of treatment of the primary tumour, up to 25% and 34% of patients develop metastases, respectively (6). Half of the patients with a large uveal melanoma die of their disease within 15 years after treatment of the primary tumour (3).

Monosomy of Chromosome 3 and the Inflammatory Phenotype

Specific clinical and histological factors show a correlation with survival, such as loss of one copy of chromosome 3, human leukocyte antigen (HLA) expression and macrophage infiltration (7). In fact, all of these prognostic factors are related, as tumours that carry only one chromosome 3 also exhibit the 'inflammatory phenotype' (7). This includes the presence of high numbers of macrophages and lymphocytes [with overexpression of cytokines and molecules such as cyclooxygenase-2 (COX-2)], high levels of HLA class I and II expression [Natural Killer (NK) cells are unable to lyse tumour cells with a high HLA class I expression that migrate through the blood stream, so that cells from highly malignant tumours expressing high class I levels manage to metastasize] and de-regulation of several inflammatory response genes (7-8). One possibility that may explain why loss of one chromosome 3 can lead to de-regulation of several inflammatory response genes is that a regulator of inflammation, peroxisome proliferator-activated receptor (PPAR)-γ, is located on this chromosome. PPARγ plays a role in regulating several inflammatory response genes, and loss of its activity may lead to an up-regulation of factors such as Nuclear Factor Kappa B (NF-κB) in tumour cells, creating an inflammatory phenotype (7). PPARγ negatively modulates Activating Protein-1 (AP-1) and NF-KB activity and this negative cross-talk is implicated in carcinogenesis and inflammation (8-9). There are few studies correlating the up-regulation of the NF-κB pathway and uveal melanoma, but one hypothesis is that loss of chromosome 3 leads to

inefficient suppression of the NF-KB pathway by PPARγ. As NF-KB is no longer negatively modulated by PPARγ, the NF-κB pathway is up-regulated. Dror *et al*. published data that supports this hypothesis. They reported higher expression of NF-KB transcription factor family genes [including Transcriptor Factor p65 *(RELA), NF-κB1, RELB, NF-κB2* and NF-κB-inducing Kinase *(NIK)*] in primary uveal melanoma and in liver metastases than in normal choroid samples (10). They concluded that the NF-κB1 and NF-κB2 pathways are active in both primary and metastatic uveal melanoma and that these pathways regulate metastatic cell proliferation and apoptosis (10).

Nuclear Factor-κB Pathway

NF-κB is part of one signalling pathway that contributes in various ways to normal and neoplastic proliferation. The most common form of NF-κB is a heterodimer composed of two subunits (p65 and p50). Usually, NF-κB is sequestered in the cytoplasm by a polypeptide named IκB (inhibitor of NF-κB). While being bound, signalling is shut down. However, in response to signals originating from a diverse array of sources, such as tumour necrosis factor-α and interleukin-1β (extracellular signals involved in the inflammatory response of the immune system), lipopolysaccharides, reactive oxygen species (ROS), anticancer drugs, and gamma irradiation, IκB becomes phosphorylated, and thus tagged for destruction. As a result, NF-κB is liberated from IκB and migrates to the nucleus, stimulating the activation of the expression of at least 150 target genes (9).

In a tumour cell, NF-κB has important effects on cell survival and proliferation. Cell adhesion molecules such as E-selectin, Intracellular Adhesion Molecule 1 (ICAM-1) and Vascular Cell Adhesion Molecule 1 (VCAM-1) are also regulated by NF-κB and are involved in the development of angiogenesis and tumour metastasis. Once NF-κB arrives in the nucleus, it induces expression of genes encoding a number of key anti-apoptotic proteins, such as B-cell Lymphoma 2 *(BCL-2)* and Inhibitor of Apoptosis *(IAP-1)* and -2. NF-κB also works in a mitogenic way by inducing the expression of the Myelocytomatosis *(MYC)* and *cyclin D1* genes. Therefore, NF-κB can protect cancer cells from apoptosis and, at the same time, drive their proliferation. In cancer, this pathway is frequently found to be constitutively activated, and even in low-grade pre-malignant growths this pathway is often de-regulated.

As the NF-κB pathway is up-regulated in uveal melanoma (10), particularly in cases with monosomy of chromosome 3, where loss of PPARγ expression may disable negative modulation of NF-κB, malignant cells are able to escape apoptosis, proliferate and evade chemotherapy (11).

Like many other polyubiquitylated proteins, IκB is ultimately degraded in proteasomes. Hence, by inhibiting proteasome action, IκB should be protected from degradation, survive in the cytoplasm, and continue to sequester NF-κB, thereby blocking NF-κB nuclear translocation and activation of transcription (12). Proteasome inhibitors, such as bortezomib, may represent a new treatment option for many types of cancer, particularly in uveal melanoma, where blocking the NF-κB pathway would induce malignant cell apoptosis, inhibit tumour cell proliferation and sensitize them to chemotherapy.

Bortezomib: a Proteasome Inhibitor

Bortezomib is a tripeptide that binds the catalytic site of the 26S proteasome, with high specificity and affinity, inhibiting proteasome activity. The possibility that proteasome inhibitors can be useful in cancer was considered after studies showed that they induced apoptosis preferentially in transformed cells and were active against non-Hodgkin's lymphoma in an *in vivo* model (13, 14).

Proteasome inhibitors also interfere with degradation of cyclins and other cell-cycle regulatory proteins, inducing cell-cycle arrest (15). They induce a proapoptotic state by stabilizing proapoptotic proteins, such as p53 and Bcl-2-associated X Protein (BAX), while reducing levels of some antiapoptotic proteins, such as BCL-2 (15). Proteasome inhibitors also induce aggresome formation, endoplasmatic reticulum (ER) stress and the unfolded protein response (a cellular stress response related to the endoplasmic reticulum that leads to apoptosis) (15).

Chemotherapy has limited efficacy in uveal melanoma due to multiple mechanisms of resistance of melanoma cells to apoptosis, including BCL-2 overexpression, silencing of the apoptotic protease-activating factor-1 gene and activation of proliferative and antiapoptotic signalling pathways (16). In addition, the NF-κB pathway is activated in cancer cells treated with chemotherapy, playing a central role in the observed proliferation and resistance (16, 17). As proteasome inhibitors block the NF-κB pathway, the hypothesis of a synergic therapy, combining proteasome inhibitors with chemotherapy, has been proposed. Horton *et al*. reported impressive results with temozolomide combined with bortezomib in a human melanoma xenograft model (18). They carried out a phase I trial of bortezomib with temozolide in patients with advanced melanoma, observing inhibition of proteasome activity for a limited time in peripheral blood mononuclear cells; however, they were unable to show consistent effects on NF-κB activation (16). Other recent publications describe trials combining bortezomib with other therapies: i) in a phase I trial in brain tumors, Kubicek *et al*. combined bortezomib with temozolomide and irradiation, and reported that bortezomib administered at its typical systemic dose (1.3 mg/m^2), is well-tolerated and that the combination was safe when used in the treatment of central nervous system

malignancies (19); ii) in a phase I trial, Dees *et al*. combined bortezomib with pegylated liposomal docorubicin or doxorubicin in therapy of solid tumours, reporting that this combination was safe and merits further investigation (20); iii) in another phase I trial, LoConte *et al*. combined bortezomib with doxorubicin in patients with advanced cancer, recommending a phase II trial (21); iv) van Waes *et al*. combined bortezomib with reirradiation in patients with squamous cell carcinoma of the head and neck, reporting that bortezomib induced detectable differences in NF-κB localization, apoptosis, and NF-κB-modulated genes and cytokines in tumor and serum in association with tumor reduction (22).

Use of Bortezomib in Cancer Therapy

In multiple myeloma, the NF-κB pathway is the most important pathogenic pathway. Bortezomib is already approved for patients with multiple myeloma in combination with melphalan and prednisone, for the treatment of patients with previously untreated multiple myeloma who are not eligible for high-dose chemotherapy with a bone marrow transplant (23). Bringhen *et al*. reported that a once-weekly and twice-weekly bortezomib schedule (combined with melphalan and prednisone), resulted in 3-year progression-free survival rates of 46% and 39%, and complete response rates of 23% and 27%, respectively (24). Bortezomib is indicated as monotherapy for the treatment of progressive multiple myeloma in patients who have received at least one prior therapy and who have already undergone or are unsuitable for bone marrow transplantation (23). Two open-label, phase II trials (SUMMIT and CREST) established the efficacy of bortezomib, reporting at least a minimal response of 50% in CREST (25) and 35% in SUMMIT (26). The open-label phase III APEX trial reported that the median time to disease progression was longer with bortezomib than with dexamethasone (27). With the bortezomib regimen, more patients achieved partial response (38% *vs*. 18%) and complete response (6% *vs*. 1%), and the overall one-year survival rate was higher (80% *vs*. 66%) (28).

Bortezomib is also approved by the United States Food and Drug Administration (FDA) for the treatment of mantle cell lymphoma. The PINNACLE trial, an open-label phase II study, reported an overall response rate of 31%, with 8% complete response. The median duration of response was 9.3 months in responding patients and 15.4 in patients with complete response (29). Moreau *et al*. recorded the incidence of grade 2 or greater peripheral neuropathy as being 24% for the subcutaneous (*s.c.*) route compared with 41% for intravenous (*i.v.*); grade 3 or higher occurred in 6% when administered subcutaneously *vs*. 16% for IV administration (30); the FDA approved administration of bortezomib by the *s.c*. route in January 2012.

Fanucchi *et al*. reported an 8% response rate in patients with non-small cell lung cancer receiving single-agent bortezomib (31), while Kondagunta *et al*. reported a response rate of 11% and 38% of patients with stable disease with bortezomib in a phase II trial of patients with metastatic renal cell carcinoma (32). Bortezomib improved the treatment of glioblastoma with neural stem cells engineered to express membrane-bound TNF-related apoptosis-inducing ligand (TRAIL) (NSCs-mTRAIL), demonstrating that the combined treatment is a potent cell-based approach for the treatment of glioma (33).

Addition of bortezomib to fluvestrant enhances its efficacy by taking advantage of the ability of fluvestrant to promote cytoplasmic aggregates of the estrogen receptor (ER), suggesting that this novel combination may be effective in breast carcinomas that are ER-positive but estrogen independent (34).

In contrast, no tumour responses were seen in recent studies with bortezomib in metastatic melanoma and colorectal cancer (35, 36). This is surprising and indicates that more research is required.

Bortezomib and Immunotherapeutic Strategies

Immunotherapeutic strategies using adoptive tumor-specific T-cell transfer aim to mount a powerful antitumour cytolytic T-lymphocyte (CTL) response, aiming at cell lysis by activation of the apoptotic machinery (37). However, tumour cells often escape apoptotic pathways, enabling their survival despite CTL attack. As proteasome inhibitors enhance the activation of apoptotic pathways, bortemozib was tested by Seeger *et al*. to sensitize melanoma cells towards adoptive CTL attack, and the results were quite impressive: i) bortezomib enhanced the susceptibility of established melanoma cells towards redirected CTL attack, while tumour cell lysis was not due to direct cytotoxic effects of bortezomib; ii) bortezomib sensitized melanoma cells to CTLs by enhancing the proapoptotic response of mitochondria to cytolytic effector functions, including caspase-8 and granzyme B; iii) bortezomib treatment resulted in the accumulation of BCL2 member proteins, including myeloid cell leukemia 1 (MCL-1), and BCL-2 homology 3 (BH3)-only proteins, p53 up-regulated modulator of apoptosis (PUMA), BH3 interacting-domain death agonist (BID), and NADPH oxidase activator (NOXA) [NOXA potentiates the release of mitochondrial second mitochondria-derived activator of caspases (SMAC), including caspase-8 and granzyme B]; iv) bortezomib treatment enhanced redirected T-cell responses in primary melanoma cells (38).

Bortezomib and Inflammation

Another enzyme that has garnered increasing interest in cancer prevention is COX-2, due to epidemiological, experimental, pathological and clinical evidence that

suggests that non-steroidal anti-inflammatory drugs (NSAIDs) possess anticancer properties (39-41). Prostaglandins, particularly prostaglandin E2, appear to be important in oncogenesis due to their effects on cellular adhesion, immune surveillance and apoptosis (42). As carcinomas have been shown to overexpress prostaglandins, inhibition of their synthesis by blocking COX-2 appears to protect against oncogenesis in many tissue types including breast, colon, esophageal, lung and skin (39, 40). Inhibition of COX-2 is thought to promote apoptosis of cancer cells through inhibition of the NF-κB pathway (43). NF-κB is also a positive regulator of COX-2 expression in response to various cytokines and growth factors (8), so the combination of a COX-2 inhibitor with a proteasome inhibitor should lead to a new synergistic inhibition of the NF-κB pathway. Minami *et al*. found that combining the NSAID sulindac with bortezomib was synergistic *in vitro* on colon cancer cell lines, and *in vivo* in a xenograft model (44). Kim *et al*. reported that the COX-2 inhibitor celecoxib induced apoptosis independently of its COX-2 effects using NF-κB as a probable target (45). Celecoxib is already approved by the FDA as a secondary treatment among patients with familial adenomatous polyposis (FAP), as it reduces the number of polyps.

Other Immunotherapeutic Strategies

The FDA and European Medicines Agency (EMA) have recently approved ipilimumab for the treatment of metastatic cutaneous melanoma. Ipilimumab is a fully human monoclonal antibody (IgG1) that blocks CTL-associated antigen 4 (CTLA-4), an immune checkpoint molecule that down-regulates pathways of T-cell activation, and promotes antitumor immunity (46, 47). In a phase III study, Hodi *et al*. reported that ipilimumab leads to improved survival in metastatic cutaneous melanoma (48). Danielli *et al*. evaluated the efficacy and the safety of ipilimumab, administered at 10 mg/kg intravenously on weeks 1, 4, 7 and 10, with maintenance doses administered every 12 weeks from week 24, in pre-treated patients with metastatic uveal melanoma. The results indicate that ipilimumab is potentially indicated for uveal melanoma, and that it should be further investigated in other clinical trials (49). However, one must be aware of the potentially severe autoimmune side-effects of ipilimumab.

Angiogenesis in Uveal Melanoma

In uveal melanoma, levels of vascular endothelial growth factor (VEGF) are significantly elevated in patients with metastatic disease compared to patients without metastases (50). VEGF plays an important role in angiogenesis, by regulating the proliferation and migration of endothelial cells. Uveal melanoma cells invade surrounding blood vessels, and circulate systemically to a new location, which in uveal melanoma is usually the liver. A local factor that plays a role in liver homing may be the expression of insulin growth factor-1 receptor (IGF-1R); this receptor is up-regulated in uveal melanoma and carries a poor prognosis (51). IGF-1 is involved in cell proliferation and is principally produced by the liver, explaining the preferential growth of uveal metastasis in the liver (52). Additionally, IGF-1 has been shown to stimulate secretion of VEGF in retinal pigment epithelial (RPE) cells and IGF-1 signaling may also stimulate tumor angiogenesis in uveal melanoma liver metastases (53).

Anti-angiogenesis therapy has not yet been used for the standard treatment of primary uveal melanoma or related metastatic diseases, although the intravitreal application of bevacizumab has been successfully applied in non-oncologic neovascularization diseases such as macular degeneration and proliferative diabetic retinopathy. Still, there has been extensive research into the effect of anti-angiogenic agents such as bevacizumab on uveal melanoma cells *in vitro* and in animal models (50, 54). Yang *et al*. studied the effect of bevacizumab on the growth and number of hepatic micrometastases in a mouse model of ocular melanoma: systemic bevacizumab suppressed primary ocular melanoma growth and the formation of hepatic micrometastases in a dose-dependent manner. In addition, bevacizumab reduced the level of VEGF in the culture media of two human uveal melanoma cell lines (54). Bevacizumab is being tested in patients with cutaneous melanoma (55). A recent clinical trial examined the response of intravitreal bevacizumab in causing a clinically significant reduction of uveal melanoma tumor size (ClinicalTrials.gov Identifier: NCT00596362), but no results have been published yet. Bortezomib has been shown to trigger a dose-dependent inhibition of VEGF and interleukin-6 (IL-6) secretion by the multiple myeloma patient-derived endothelial cells, and reverse transcriptase-PCR confirmed drug-related down-regulation of VEGF, IL-6, insulin-growth factor-I, angiopoietin 1 and angiopoietin 2 transcription, through an unknown mechanism in multiple myeloma (56). One may speculate that bortezomib is able to inhibit angiogenesis in uveal melanoma.

Uveal melanoma cells express v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (C-KIT or CD117). Sunitinib, a multi-targeted receptor targeted (RTK) kinase inhibitor against VEGFR, FMS-like tyrosine kinase 3 (FLT-3), C-KIT and platelet-derived growth factor receptor (PDGFR), was tested in a phase I study, achieving one partial response and seven cases of stable disease, with an overall clinical benefit rate of 80% (57). Sunitinib and sorafenib [a small molecular inhibitor of several tyrosine protein kinases and rapidly accelerated fibrosarcoma (RAF) kinases], are currently

being tested in phase II clinical trials. Minor *et al*. reported one complete remission for 15 months and two partial responses (of one- and seven-months' duration) in four evaluable patients with acral, mucosal or cumulative sun-damaged skin melanoma and KIT mutations (58). Yeramian *et al*. reported that bortezomib synergistically enhanced the sunitinib-induced growth arrest in sunitinib-sensitive cells in metastatic cutaneous melanoma cell lines, suggesting that melanoma cells harbouring an activated RTK may be clinically responsive to pharmacological RTK inhibition by sunitinib; a strategy combining sunitinib and bortezomib may provide therapeutic benefit (59). Sorafenib has been tested in a xenograft model in which uveal melanoma cell line 92.1 was dorsally injected subcutaneously: Mangiameli *et al*. demonstrated inhibition of tumour growth ($p \leq 0.0035$) and fewer metastases after sorafenib treatment (33% *vs*. 60%) (60). Recently, a phase III trial which compared treatment of metastatic (not including uveal) melanoma patients (n=823) with carboplatin, paclitaxel and with either sorafenib or placebo did not demonstrate a difference in overall survival: the median overall survival for the sorafenib-treated group was 11.1 months (95% CI and 10.3-12.3) and for the placebo group 11.3 months (95% confidence interval, CI=9.7-12.3) (50).

Mitogen-activated Protein Kinase Kinase (MEK) Inhibitors

Most melanomas have activating mutations in the mitogen-activated protein kinase (MAPK) pathway involving guanine nucleotide-binding protein G(q) subunit alpha (*GNAQ*) and guanine nucleotide-binding protein subunit alpha-11 (*GNA11*) in uveal melanoma (61, 62). These mutations render melanoma cells independent of the normal RTK-mediated pathway regulation, and constitutively drive melanoma cells to oncogenic proliferation and survival. As *GNAQ* and *GNA11* seem to be important in the development of uveal melanoma, inhibition of mitogen-activated protein kinase kinase (MEK) (MEK is a kinase enzyme which phosphorylates MAPK) may be a new way to treat metastatic uveal melanoma. *GNAQ* knockdown, as well as treatment with U0126 MEK inhibitor, resulted in inhibition of MAPK signalling and loss of viability of melanocytes (61). Kirkwood *et al*. performed a phase II, open-label, multicenter, randomized, parallel-group trial, comparing the MEK1/2 inhibitor selumetinib, as monotherapy, with temozolomide and reported no significant difference in progression-free survival between patients with unresectable stage III/IV cutaneous melanoma unselected for v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*)/ neuroblastoma RAS viral oncogene homolog (*NRAS*) mutations (63). Von Euw *et al*. reported that the MEK inhibitor TAK733 has antitumor properties in cutaneous and uveal melanoma cell lines with different oncogenic mutations (64).

Conclusion

Inhibitors of the proteasome using an agent such as bortezomib may be new therapeutic agents in cancer, especially in those types of cancer that have overexpression of the NF-κB pathway (such as uveal melanoma). Despite the fact that patients with metastatic melanoma treated with bortezomib exhibit no tumour responses in a phase II trial, we now have data suggesting that bortezomib as monotherapy or combined with celecoxib, prednisone or an alkylating agent, such as temozolomide or dacarbazine, may be effective as it sensitizes melanoma cells to chemotherapy, induces a proapoptotic state, aggresome formation, endoplasmatic reticulum stress and the unfolded protein response. It also promotes cell cycle arrest and sensitizes melanoma cells towards adoptive CTLs.

Patients with metastasized uveal melanoma have a poor prognosis. Therefore, new therapies are needed. Bortezomib and proteasome inhibitors are promising drugs to combat uveal melanoma. Other agents have been tested, but are far from becoming standard therapy. New studies are being carried out to verify the possibility of the use of new therapeutics in uveal melanoma, such as the international AEB071 phase I study [ClinicalTrials.gov Identifier: NCT01430416 (this study is already recruiting patients)], which will test a protein kinase C (PKC) inhibitor, and the SECIRA-UM study [EudraCT Number: 2011-004200-38 (open in the NKI-AvL in Amsterdam, the Netherlands, since April, 2012)], which will test the combination of CTLA-4 blockade and radiofrequency ablation in patients with metastasized uveal melanoma.

As new therapeutics are needed and there are many new promising drugs to combat uveal melanoma, new studies with combinations of drugs should be carried out, to verify if bortezomib, combined with celecoxib, prednisone, temozolomide, dacarbazine, anti-angiogenic agents, such as bevacizumab, sorafenib, sunitinib, cetuximab, panitumumab, erlotinib, transtuzumab or temsirolimus, MEK inhibitors or ipilimumab, can lead to new ways of treating uveal melanoma.

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