Towards the Targeted Therapy of Melanoma

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Abstract: Novel anti-cancer treatments use knowledge about the underlying biology of the tumor to find suitable molecular targets. The recent years have seen great advances in understanding the biology of melanoma. In the current review we discuss the most promising molecular targets for melanoma and suggest possible strategies for overcoming resistance.

Keywords: Melanoma, melanocyte, BRAF, PI3K, therapy, cancer, proteasome.

THE FAILURE OF CHEMOTHERAPY IN MELANOMA

Despite years of research, the prognosis for disseminated melanoma remains dismal with average survival rates of 6-10 months [1]. All major chemotherapy drugs, immunotherapies and radiotherapies have failed in large-scale clinical trials. The only agent currently approved for the treatment of metastatic melanoma is the alkylating agent dacarbazine (DTIC) (structure given in (Fig. **1**)). Responses to DTIC are poor, with clinical response rates of 5-10% and cure rates of 1% [2]. Other regimens, combining DTIC with cisplatin, vinblastine, tamoxifen or carmustine show no clinical benefit over DTIC alone [3]. Clearly melanoma therapy is in a parlous state and improvements are urgently needed.

Unlike many other cancers, drug resistance in melanoma is not acquired following drug therapy and is present even in untreated lesions. The "intrinsic" drug resistance of melanoma most likely stems from the phenotype of the parental melanocytes, which are well suited to resist the effects of DNA damage following ultraviolet (UV) light exposure.

In addition to this intrinsic resistance, melanoma cells acquire further mutations and constitutive activity in cell signaling pathways such as phosphoinositide 3 (PI3)-Kinase, mitogen activated protein (MAP) kinase and nuclear factor kappa B (NFκB) which contribute towards enhanced cell survival. Together these acquired mutations and enhanced cell signaling work together to enhance the survival of tumor cells following chemotherapeutic insult. The idea is emerging that targeting these pathways using small molecule inhibitors – so-called targeted therapy - in combination with classical chemotherapy could be a viable strategy for overcoming therapeutic resistance of melanoma. Our greater understanding of the underlying biochemical processes underpinning melanoma development and progression have identified new potential therapeutic targets. In the following review we will discuss the rationale for targeting various signaling pathways involved in melanoma survival and whether using these targeted therapies in combination with cytotoxic agents would be a viable therapeutic strategy in this currently untreatable disease.

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THE PROMISE OF TARGETED THERAPY

There is much to be learned about targeted therapy by considering the example of the multiple kinase inhibitor imatinib mesylate (Gleevec), which has revolutionized the treatment of chronic myeloid leukemia (CML) and gastro intestinal stromal tumors (GIST) [4]. The growth and survival of both CML and GIST are driven almost entirely by mutations in defined kinases; the Bcr-Abl kinase fusion in the case of CML and either point mutations in c-kit or PDGF-α in the case of GIST [4]. Inhibition of these critical kinases by Gleevec is directly correlated with the ability of the drug to block tumor growth and progression [4]. The lesson here is very clear; target the critical kinase responsible for tumor initiation and maintenance with a small molecule inhibitor, the lesions will melt away and the patient will be cured. Ongoing genome-wide screens are revealing that many cancers may have an "Achilles heel" equivalent to Bcr-Abl in CML. The critical question is whether the kinase mutations identified from these genomic screens occur early or late in life of the cancer and if these mutations are responsible for tumor initiation or maintenance. The key to the success of Gleevec in CML is the fact that Bcr-Abl mutation is an early initiating event in this disease. Targeting an alternate kinase, such as Flt-3, which is more important in tumor maintenance than in initiation is clinically a less successful strategy than targeting Bcr-Abl [5].

There are also caveats to the idea of treating a cancer with an inhibitor to one kinase or signaling pathway. Tumors are notoriously genetically unstable and undergo rapid mutation. Once selection pressure, in the form of a kinase inhibitor, is applied to the tumor system mutations rapidly accumulate in the drug binding domain of the targeted kinase and pharmacological activity is lost. In the case of Gleevec, patients receiving the drug initially go into remission but then eventually relapse as drug-binding mutations in Bcr-Abl are acquired [6].

BRAF: MELANOMA'S ANSWER TO BCR-ABL

The search for melanoma's answer to Bcr-Abl has generated some promising initial results. A recent genome-wide screen performed at the Sanger Institute has revealed that 66% of melanomas harbor activating mutations in one of the Raf isoforms, BRAF [7]. The mutation, which is a result of a mis-sense mutation leading to the substitution of glutamate to valine (V600E), directly activates the MAP

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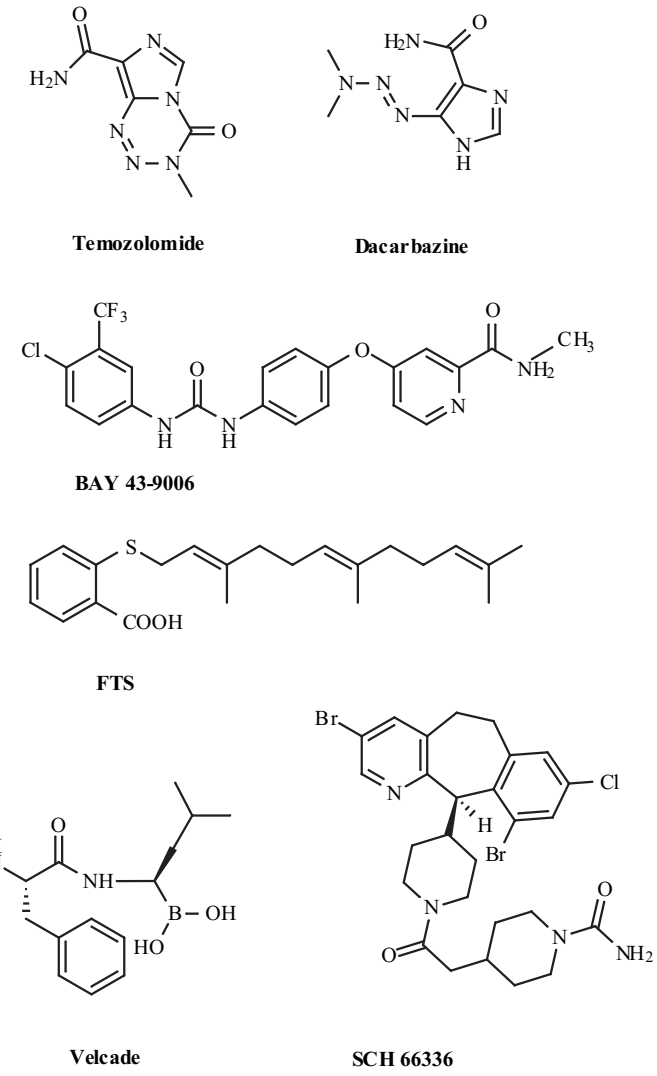

Fig. (1). Structures of compounds either being used or assessed for future treatment of melanoma. Temozolomide and DTIC are chemotherapy agents currently used in the clinical treatment of melanoma. FTS and SCH 66336 target (among things) MAP kinase signaling and have shown some *in vitro* activity against melanoma. Velcade and BAY 43-9006 are currently undergoing clinical assessment as possible melanoma therapies.

kinase pathway in melanoma [7]. Recent structural biology studies have shown that the V600E mutation, which accounts for over 80% of reported BRAF mutations, destabilizes the inactive conformation and shifts the equilibrium to the active conformation which then activates the MAP kinase cascade [8], a family of serine/threonine protein kinases [9]. Typically, activation of the MAP kinase pathway occurs through activated growth factor receptors transmitting their signals through the small GTPase Ras. In its GTP-bound state, Ras interacts with and activates the Raf family of serine/threonine protein kinases [10], which in turn phosphorylates and activates extracellular-signal regulated kinase (ERK) 1 and ERK2. Upon activation, the ERKs either phosphorylate cytoplasmic targets or migrate to the nucleus where they activate a number of transcription factors such as Elk-1 or c-Fos [11].

In normal cells proliferation is tightly regulated at a number of cell-cycle check points. At these points there is a convergence of many signals generated either internally or externally which determine whether a cell remains quiescent or undergoes division. One of the critical points of this regulation is at the G1 phase of the cell cycle. Cancer cells are characterized by uncontrolled growth arising from signals that overcome the G1 checkpoint and force the cells through the cell cycle. The MAP kinase pathway is a key regulator of this checkpoint as it allows the cell to pass through the G1 phase of the cell cycle [12-13]. It also worth noting that high MAP kinase activity is not restricted to proliferative effects and it also enhances invasion, migration and cell survival, which all contribute to the oncogenic phenotype [9]. The likely role of MAP kinase activity in chemoresistance and enhanced cell survival is outlined in Fig. (**2**).

The *in vitro* evidence for mutant BRAF being a critical oncogene in melanoma is compelling. All melanoma cell lines have high MAP kinase activity and their growth can be blocked by small molecule inhibitors of the downstream kinase MEK [14-15]. There is also evidence that other drugs which block the MAP kinase pathway, such as the Ras inhibitor farnesyl thiosalicylic acid (FTS) and the farnesyl transferase inhibitor SCH66336, also block melanoma

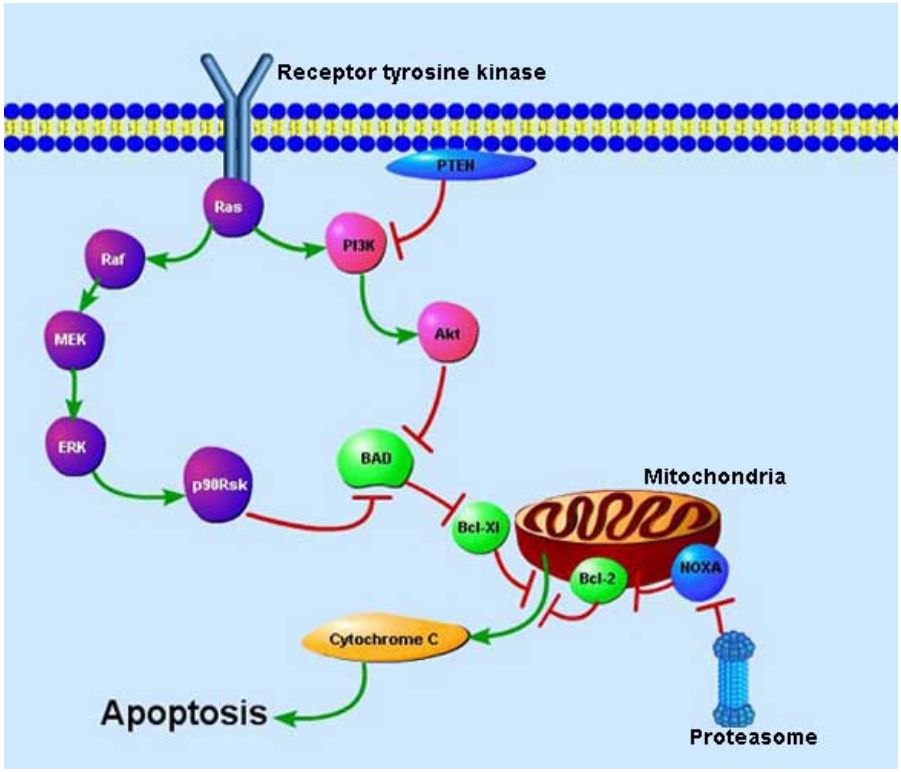

Fig. (2). Signaling scheme showing how constitutive activity in the MAP kinase and PI3-Kinase/Akt pathways directly inhibits apoptosis and increase cell survival in melanoma. Constitutive activity in the MAP kinase pathway arises from either activating mutations in N-Ras, BRAF or by activation of receptor tyrosine kinases. The increased activity in the PI3-kinase pathway arises from either loss of the negative regulator PTEN or through constitutive receptor tyrosine kinase activity. Positive regulations are shown in black, and inhibitory regulations are shown in red.

growth *in vitro* [14, 16] (Fig. **1**). Other studies have shown that BRAF can transform immortalized mouse melanocytes [17], and that selective abrogation of BRAF, using RNAi, reduces melanoma cell growth and induces apoptosis [18]. The *in vivo* evidence for the involvement of BRAF in melanoma is more difficult to interpret. It has been reported that between 21-80% of congenital nevi also harbor the same V600E BRAF mutations as melanoma [19]. Whereas nevi (which are melanocytic hyperplasias) sometimes give rise to melanoma, this is a very rare event and a majority of melanomas arise *de novo*, with no obvious precursor lesion. Currently it is unclear whether BRAF is the key initiating mutation in melanoma, or is involved in tumor maintenance at a later stage. Microarray studies on early passage cell lines from melanoma lesions have revealed there is a BRAF mutational signature, which is also associated with loss of the cyclin dependent kinase inhibitor p16/ARF [20]. The argument for BRAF co-operating with a host of other genetic factors is supported by studies that show that BRAF mutations are often present in nevi, then lacking in early-stage melanomas only to re-appear in more advanced tumors [21]. There is also evidence that the presence of the V600E mutation is correlated with a poor clinical outcome, particularly when found in metastatic lesions [22]. With the evidence accumulating for BRAF being a viable therapeutic target in melanoma, many pharmaceutical companies have compounds in development which inhibit BRAF activity. One such compound, BAY 43-9006 (Fig. **1**), is already showing clinical activity in phase II melanoma trials and is discussed in more detail below.

PI3K & AKT3

The MAP kinase cascade is only one of many signaling pathways with constitutive activity in melanoma. During tumor development, one of the key things the nascent cancer cell must do is escape from the control of its local environment. Alteration of the tissue microenvironment for a normal, non-transformed cell will lead to survival signals being blocked - causing the cell to undergo a specialized form of cell death known as anoikis [23]. The most important of these survival signals are transduced through both the MAP kinase pathway and by another pathway, PI3-kinase/Akt [23-24]. Constitutive activity in the PI3-kinase/Akt pathway is known to suppress apoptosis and anoikis in cancer cells (Fig. **2**). The Akt family consists of three members, Akt1-3 [25], which exhibit a different pattern of activation depending upon cell type. Although activating mutants of Akt have not been identified in melanoma cells [26], they are known to have constitutive Akt activity, with 43%-50% of melanomas having selective constitutive activity in Akt3 [27]. Inhibition of Akt in melanoma, using either PI3-kinase inhibitors or selective RNAi to Akt3, both reduced growth and induced apoptosis [27-28]. Again, like the MEK/ERK pathway, there is a suggestion that the observed constitutive activity is the result of autocrine growth factors. In particular, the insulin-like growth factor (IGF)-I, is known to aid the growth of early-stage melanoma cells, at least partly through activity of PI3-kinase/Akt [29]. Another possible mechanism for the increased PI3-kinase/Akt activity observed in melanoma comes from the

decreased activity of upstream regulators of this pathway. One of the most critical regulators of Akt, is the phosphatase and tensin homologue (PTEN), which degrades the products of PI3-kinase, therefore preventing the activation of Akt [30]. PTEN is located on chromosome 10, a site of major deletions in 30-60% of melanomas [31-32]. Studies have revealed that PTEN is lost in up to 30% of melanoma cell lines [33] but in only 10% of human tumor material [34-35]. Functionally, loss of PTEN in melanoma has been shown to upregulate Akt activity, the end consequence being reduced apoptosis rates and enhanced cell survival [36]. Akt affects cell survival through a number of mechanisms. The Akt-mediated phosphorylation of the FOXO family of transcription factors targets them for proteasomal degradation and therefore reduces levels of their pro-apoptotic targets, such as BIM and Fas Ligand [37]. In a similar manner, Akt phosphorylates and inactivates pro-apoptotic proteins such as Bcl-2 and Bad [38] (Fig. **2**).

There are many reasons for presuming that Akt inhibition would be a viable therapeutic strategy in melanoma. Firstly, melanoma cells and human melanoma lesions have been demonstrated to have high constitutive Akt activity. Secondly, the PI3K/Akt pathway occupies a central role in down regulating the apoptotic response. Indeed, loss of PTEN in breast cancer patients (which leads to enhanced PI3K/Akt activity) is predictive of resistance to the ErbB2 antibody, Herceptin [39]. The structural similarity of Akt to other protein kinases, such as protein kinase A, has long hampered drug development. However, these technical challenges are being overcome and a number of Akt inhibitors have been developed which sensitize tumor cells to apoptotic stimuli [40]. Most of the current Akt inhibitors have dual-specificity for Akt1 and Akt2 and it is not known whether there is sufficient structural difference between the isoforms to allow the development of Akt3-selective drugs.

NFκB

Nuclear factor kappa B (NFκB) is a heterodimeric transcription factor, largely composed of 50 and 65 kDa subunits of the Rel family. It occupies a pivotal point in many survival and growth pathways and can be activated through either the PI3K/Akt or the MEK/ERK pathways [41]. Constitutive activation of the NFκB pathway has been identified in many tumor types including pancreatic, breast, colon and melanoma [42-45]. In quiescent cells, NFκB is located in the cytoplasm through the suppressive effects of a family of inhibitory proteins called IκB (Fig. **3**). Following stimulation by cytokines, such as TNF-α, IL-1 or LPS, the NH_2 terminus of IκB is phosphorylated by the high molecular weight IκB kinase (IKK) complex. Once phosphorylated the inhibitory IκB is poly-ubiquitinated by a specific ubiquitin ligase belonging to the SCF (Skp-1/Cul/F box) family called β-TrCP and targeted for degradation by the 26S proteasome (Fig. **3**). After NFκB is freed of its inhibitory IκB subunit it migrates to the nucleus and activates the transcription of genes involved in driving cell growth and survival.

In melanoma, NFκB is constitutively active and linked to increased expression of cyclin D1, the anti-apoptotic

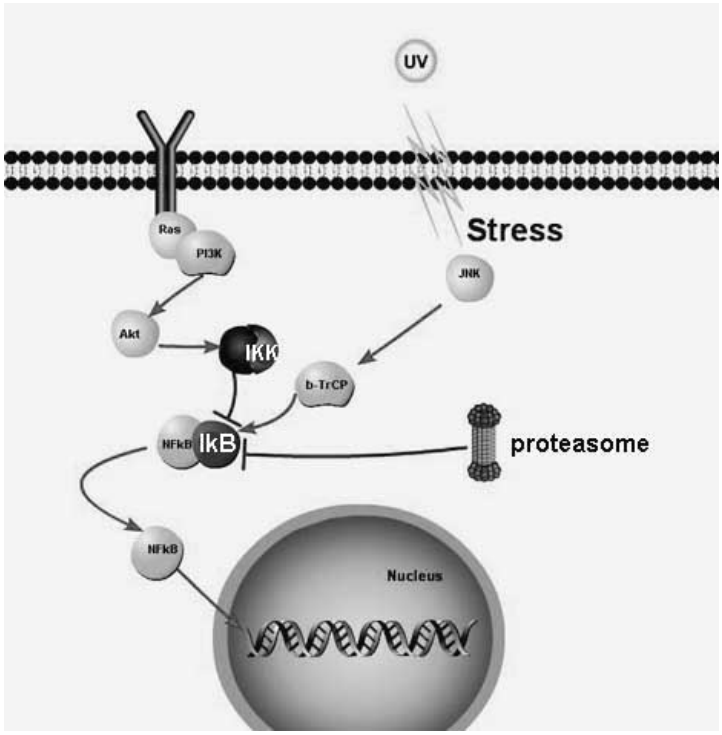

Fig. (3). Signaling scheme showing the role of the NFκB pathway in the suppression of apoptosis in melanoma. The NFκB is usually complexed in the cytoplasm through interaction with the inhibitory subunit IκB. The negative regulation of IκB is released via phosphorylation by IKK (IκB kinase) and subsequent poly-ubiquitination by the SCF ubiquitin ligase β-TrCP, leading to its destruction in the 26S proteasome. Once free of this negative regulation, the NFκB is free to migrate to the nucleus where it regulates gene transcription and cell survival.

factor TRAF2, Mel-CAM and the pro-angiogenic cytokine GRO [46]. The role of increased NFκB activity in metastatic melanoma is demonstrated by studies showing that transfection of IκBα reduces subcutaneous tumor growth and metastatic spread of human melanoma in nude mice [47]. There is also evidence that constitutive NFκB activity contributes towards radiosensitivity in melanoma, as shown in studies where cells are transfected with dominant-negative IκBα construct [48]. Other studies have shown that NFκB activity is upregulated in melanoma cells following doxorubicin treatment, suggesting that it may be also involved in drug resistance [49]. Recent work has demonstrated that in melanoma, loss of E-cadherin expression is linked to upregulation of NFκB activity [50], which may implicate NFκB in the early stages of melanoma development. The cadherin switch, whereby E-cadherin expression is lost and N-cadherin expression upregulated, is one of the early critical events whereby malignant melanocytes escape from local keratinocyte control [51].

THE PROTEASOME

Cell signaling can also be regulated at the level of protein homeostasis. One strategy that a cancer cell can use to downregulate pro-apoptotic signals following chemotherapeutic insult is to target the effectors of these pathways for proteolytic degradation via the proteasome (Fig. **4**). The active role of the proteasome in driving tumorigenic behavior is in contrast to the classical idea that the proteasome is the cell's "trash can", mainly involved in the degradation of mis-folded or damaged proteins. Proteins that are targeted for destruction are tagged by the attachment of chains of ubiquitin molecules by ubiquitin (E1) activating, conjugating (E2) and ubiquitin (E3) ligases [52].

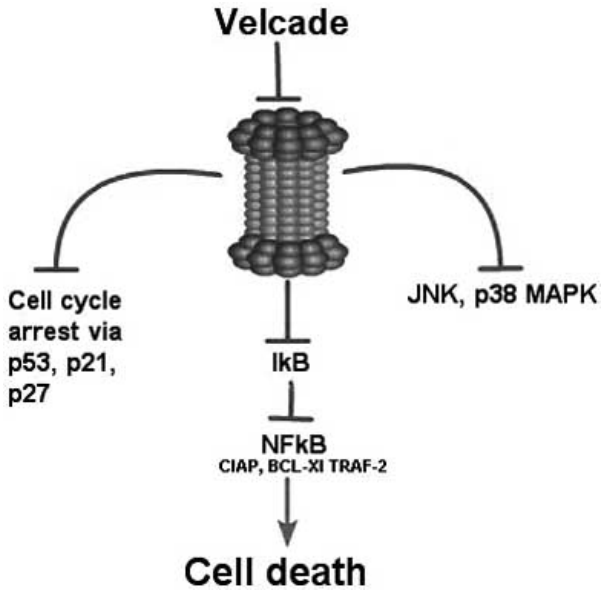

Fig. (4). Mechanisms of action of the proteasome inhibitor Velcade (Bortezomib). In tumor cells, the 26S proteasome is responsible for degradation of cell cycle inhibitors such as p21, p27 and p53 and the negative regulator of NFκB – IκB. Through blocking the proteolytic activity of the 26S proteasome, Velcade is able to reduce cell growth and increase apoptosis.

After ubiquitination the proteins are transported to the proteasome, a vast 2.5mDa complex which unfolds the protein and cleaves it into small peptide fragments.

Interest in the proteasome as a therapeutic target in cancer has been fueled by the recent approval of the proteasome inhibitor bortezomib (Velcade) for the treatment of multiple myeloma (MM) [53] (Fig. **1, 4**). There is evidence that proteasome inhibition may work in other cancers with pre-clinical studies showing Velcade's efficacy against ovarian, lung, prostate and pancreatic cancer [54-57]. One possible explanation for the therapeutic activity of Velcade in tumors is linked to the requirement for rapid protein turnover in fast-growing cancer cells. However this argument is rather confounded by the efficacy of Velcade in MM, where the cells proliferate slowly. In melanoma Velcade treatment has been shown to inhibit NFκB activity, and reduce cell growth *in vitro* [58]. Interestingly, Velcade synergizes with temozolomide (a more soluble analog of DTIC) in human melanoma xenografts [58], providing the rationale for using Velcade to overcome drug resistance. It seems that Velcade may synergize with chemotherapy through modulation of the apoptotic response. Recent studies have shown that Velcade treatment upregulates levels of the pro-apoptotic BH3-domain protein Noxa in human melanoma cells [59]. These effects seem to be highly selective for melanoma as equivalent studies on melanocytes showed no upregulation of Noxa or apoptosis [59].

So far the encouraging *in vitro* work of proteasome inhibition in melanoma has not been borne out in the clinic, with Velcade failing to show any clinical benefit in phase II trials of advanced melanoma [60]. At this stage it seems unlikely that Velcade will ever be used as a monotherapy for melanoma. However, its potential synergistic activity with established anti-cancer drugs makes it a good candidate for further drug-combination trials.

IMPROVING MELANOMA THERAPY – INHIBIT MULTIPLE PATHWAYS OR COMBINE TARGETED THERAPY WITH CHEMOTHERAPY?

As we have discussed, melanoma cells have a complex biology with activity in many pathways responsible for regulating proliferation, survival and invasion. Choosing the correct pathways to block is of critical importance. As metastatic melanomas have activity in multiple signaling pathways, targeting one single pathway may not be an option, as there is likely to be functional redundancy. There is evidence that targeting multiple signaling pathways may act synergistically to kill cancer cells. Recent work has shown that constitutive MAP kinase activity in epithelial carcinoma cells targets key pro-apoptotic molecules to the proteasome, providing the paradigm for targeting both the MAP kinase pathway and the proteasome simultaneously [61].

Another approach may be to combine targeted therapy, which blocks cell signaling pathways, with more traditional chemotherapy drugs. Blocking the pathways which are responsible for enhanced survival may be sufficient to tip the balance away from resistance towards cell death. There is already clinical evidence that this may be a good therapeutic strategy in melanoma. Although the putative Raf-kinase

inhibitor, BAY 43-9006 had little clinical activity in melanoma as a single agent, it was found to give robust responses when used in combination with carboplatin and taxol. The underlying biochemistry of these clinical responses are currently unclear as BAY 43-9006 is not a specific Raf inhibitor and has a broad-spectrum of activity against other kinases including Flt-3, vascular endothelial growth factor receptor (VEGFR)-2, platelet derived growth factor (PDGF), and c-Kit [62]. It is entirely possible that the clinical responses seen to BAY 43-9006 in melanoma are the result of its broad spectrum of anti-kinase activity. Evidence for the clinical benefits of the off-target effects of BAY 43-9006 come from clinical studies in renal cell carcinoma (RCC) (which do not harbor BRAF mutations). In this instance, BAY 43-9006 gave good clinical responses in RCC, which are thought to be due to the anti-angiogenic effects of the drug [63]. Indeed, there is evidence from melanoma xenograft models that BAY 43-9006 may also target tumor angiogenesis [64]. The clinical responses seen with the BAY 43-9006 and chemotherapy combination represents a real therapeutic breakthrough in melanoma therapy. Further study is now required to ascertain the underlying biology responsible for this and whether using alternate drug combinations can improve upon these initial results. One key question that still needs to be addressed is whether the clinical activity of BAY 43-9006 is the result of BRAF inhibition or the broad spectrum kinase activity of this drug. We will come closer to answering this question as other pharmaceutical companies bring their more selective BRAF inhibitors to the clinic. Certainly, the early clinical evidence from the BAY 43-9006 trial provides the rationale for using targeted therapies in melanoma to overcome resistance to more classical therapies.

At this juncture it is uncertain whether we will be able to recreate the success of Gleevec in melanoma. It is highly possible that the unique combination of the initiating mutation in Bcr-Abl with the simpler, hematological nature of leukemia may play to the strengths of targeted therapy. Clearly there is more complexity in the host-tumor response of solid tumors, such as melanoma, which also involves stromal cells, the immune system and the vasculature. It is however certain that through an enhance understanding of the biology of melanoma, and targeting key survival pathways it will be possible to use chemotherapy more effectively and dramatically improve the outcome and prognosis for a vast majority of melanoma patients.

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