

## Adjuvant Melanoma Treatment Strategies

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The following table categorizes suggested adjuvant measures for melanoma control with respect to their likely utility as retardants of cancer growth and spread, and as adjuvants to chemotherapy. Suggested dose schedules are provided for many of these agents; these are provisional and may change in light of future research. This is intended as a menu of options, from which patients can select to create a personal cancer control program, preferably with the guidance of a sympathetic physician or health scientist; no one could be expected to use *all* of these measures, and it is unlikely that all of them will ultimately prove to confer important benefit. Moreover, every cancer is unique, and measures which help some patients with colorectal cancer may not help others. Abstracts of research pertinent to each of these suggested measures are appended below.

“IGF-I Reduction” refers to lifestyle measures which keep blood levels of the hormones IGF-I and insulin relatively low; a whole-food plant-based (vegan) diet can be helpful in this regard. A fast of several days duration prior to and during chemotherapy markedly lowers IGF-I activity, and may lessen chemotherapy side effects while possibly improving response. Metformin and berberine have very similar activities (activation of the enzyme AMPK), so one or the other can be used; metformin is a prescription drug, whereas berberine is a nutraceutical. In patients whose cancers harbor the common BRAF V600E mutation, it may be prudent to use these agents in conjunction with vemurafenib (Zelboraf) therapy. I.v. ascorbate can be used alone or as an adjuvant to chemotherapy; so far, little or no published literature pertains to its use in melanoma.

Note that many of these agents are prescription drugs, and hence require the active cooperation and approval of your doctor. Use of spirulina at the same time as chemotherapy or i.v. ascorbate is not recommended. GcMAF (macrophage activating factor) must be administered by subcutaneous injection; although it does not have drug approval, it can be obtained by mail-order from Europe or Japan. Importantly, these measures should be considered as adjuvants to, *not substitutes for*, recommended surgery, chemotherapy regimens, or other therapies employed by your doctor.

	Growth Control	Chemo Adjuvant	Prescription Drug	Suggested Dose
IGF-I Reduction	X	X		
Metformin or	X	X	X	500 mg 3 times daily
Berberine	X	X		500 mg 2-3 times daily
Fish Omega-3	X	X		2-5 g daily
Green Tea Catechins	X	X		250 mg twice daily
Melatonin	X	X		10-20 mg at bedtime

Vitamin D	X			5,000-10,000 IU daily
Spirulina	X	--	\	15-30 g daily
Salsalate	X	X	X	1500 mg 2-3 times daily
Tocotrienols	X	X		125 mg twice daily
Low-Dose Naltrexone	X		X	3-5 mg before bedtime
GcMAF	X			100 ng i.m.once weekly
I.V. Ascorbate	X	X	X	
Valproate	X	X		
Nelfinavir		X	X	
Hydroxychloroquine	X	X	X	
Itraconazole	X	X	X	
Met Cyclophosphamide	X		X	

### IGF-I Reduction - Plant-based Diet/Fasting

Fontana L, Weiss EP, Villareal DT, Klein S, Holloszy JO. Long-term effects of calorie or protein restriction on serum IGF-1 and IGFBP-3 concentration in humans. *Aging Cell* 2008 October;7(5):681-7.

Abstract: Reduced function mutations in the insulin/IGF-I signaling pathway increase maximal lifespan and health span in many species. Calorie restriction (CR) decreases serum IGF-1 concentration by ~40%, protects against cancer and slows aging in rodents. However, the long-term effects of CR with adequate nutrition on circulating IGF-1 levels in humans are unknown. Here we report data from two long-term CR studies (1 and 6 years) showing that severe CR without malnutrition did not change IGF-1 and IGF-1 : IGFBP-3 ratio levels in humans. In contrast, total and free IGF-1 concentrations were significantly lower in moderately protein-restricted individuals. Reducing protein intake from an average of 1.67 g kg(-1) of body weight per day to 0.95 g kg(-1) of body weight per day for 3 weeks in six volunteers practicing CR resulted in a reduction in serum IGF-1 from 194 ng mL(-1) to 152 ng mL(-1). These findings demonstrate that, unlike in rodents, long-term severe CR does not reduce serum IGF-1 concentration and IGF-1 : IGFBP-3 ratio in humans. In addition, our data provide evidence that protein intake is a key determinant of circulating IGF-1 levels in humans, and suggest that reduced protein intake may become an important component of anticancer and anti-aging dietary interventions

Santonocito C, Paradisi A, Capizzi R et al. Insulin-like growth factor I (CA) repeats are associated with higher melanoma's Breslow index but not associated with the presence of the melanoma. A pilot study. *Clin Chim Acta* 2008 April;390(1-2):104-9.

Abstract: BACKGROUND: IGF-I-(CA) repeats have been previously analysed in few types of cancer and the results, although discordant in different studies, showed possible associations between cancer and

IGF-I(CA)(19) repeats. Aim of this pilot study was to detect a possible association between some of the IGF-I(CA) repeats and the presence of malignant melanoma and its Breslow index. METHODS: Two hundred patients affected with cutaneous malignant melanoma and 100 control healthy subjects were analysed for IGF-I(CA) repeats by fragment analysis sequencing and, partially, confirmed by direct sequencing. RESULTS: A significant association of IGF-I(CA)(19) repeats was observed with melanoma higher Breslow indices ( $P < 0.001$ ), while no association between melanoma patients and the different genotypes of IGF-I(CA) was found. The above mentioned association was confirmed after Bonferroni's correction for multiple comparisons and also by logistic regression analysis adjusted for age, sex and BMI variables. A slight, significant difference ( $P = 0.03$ ) was observed for serum IGF-I values in IGF-I(CA)(19)-positive or IGF-I(CA)(19)-negative subjects. DISCUSSION: The association observed for IGF-I(CA)(19) and malignant melanoma is in keeping with similar results obtained in prostate or breast cancers, suggesting that this type of repeat may be directly or indirectly important in controlling cancer induction and its severity

Hilmi C, Larribere L, Deckert M et al. Involvement of FKHRL1 in melanoma cell survival and death. *Pigment Cell Melanoma Res* 2008 April;21(2):139-46.

Abstract: Melanoma is a highly aggressive tumour characterized by a strong resistance to apoptotic stimuli that give rise to a selective advantage for tumour progression and metastasis formation. Therefore, it is of paramount importance to better understand the mechanisms involved in this resistance to apoptosis. In this report, we focused our attention on FKHRL1, a member of the forkhead family of transcription factors, which controls expression of genes involved in cell cycle progression and apoptosis. In melanoma cells, we show that IGF1, which exerts pro-survival properties, induces the phosphorylation and nuclear exclusion of FKHRL1 in a PI3K/AKT-dependent pathway. Moreover, we observe that over-expression of a non-phosphorylatable mutant of FKHRL1 (FKHRL1-TM), constitutively localized to the nucleus, promotes apoptotic cell death of melanoma cells. Finally, we find that FKHRL1-TM decreases the expression of survivin, a member of the inhibitor of apoptosis protein and that survivin re-expression partially rescues the deleterious effects of FKHRL1. Taken together, these findings reveal, in melanoma cells, that endogenous FKHRL1 is a downstream target of the PI3K/AKT pathway and suggest that the phosphorylation of this transcription factor may be involved in the pro-survival effects of growth factors such as IGF1. On the other hand, forced nuclear localization of FKHRL1 decreases melanoma cell growth and may serve as a therapeutic strategy against melanoma

Lee JT, Brafford P, Herlyn M. Unraveling the mysteries of IGF-1 signaling in melanoma. *J Invest Dermatol* 2008 June;128(6):1358-60.

Abstract: The inherent ability of a cell to undergo apoptosis governs a number of developmental processes essential to proper mammalian development. Into adulthood, the pathways that potentiate the apoptotic response are extremely diverse and finely regulated to prevent potential diseases. Of these, cancer is often associated with loss of an apoptotic response. Hanahan and Weinberg (2000) list evasion of apoptosis as a hallmark feature acquired during neoplastic transformation. The impact of this event is dramatic on several levels; avoidance of apoptosis not only prevents programmed cell death in an array of cell types but also promotes chemotherapeutic resistance during anticancer regimens

Wang HJ, Li D, Yang FY, Tashiro S, Onodera S, Ikejima T. Oridonin induces human melanoma A375-S2 cell death partially through inhibiting insulin-like growth factor 1 receptor signaling. *J Asian Nat Prod Res* 2008 July;10(7-8):787-98.

Abstract: Our previous studies indicated that oridonin, a diterpenoid isolated from *Rabdosia rubescens*, induced human melanoma A375-S2 cell apoptosis. In this study, we investigated whether the proapoptotic effect of oridonin on A375-S2 cells would depend on an interference with function of the

insulin-like growth factor 1 (IGF-1) receptor, a plasma membrane receptor critical for the survival or antiapoptotic ability in melanoma cells. We found that IGF-1 receptor (IGF-1R) signaling was a potential survival pathway against a low concentration of 20 micromol/L oridonin-induced apoptosis in A375-S2 cells. The activation of Ras or its downstream effector p38 mitogen-activated protein kinase (p38 MAPK) was shown to be necessary for IGF-1-mediated protection, but the activation of phosphatidylinositol-3-OH kinase (PI3 kinase) or extracellular signal-regulated kinase (ERK) did not correlate with the regulation of survival. However, in the presence of 40 micromol/L (IC<sub>50</sub> at 24 h) oridonin, A375-S2 cells could not be protected by IGF-1 from apoptosis, accompanied by a severe impairment of IGF-1R expression. Therefore, we concluded that the proapoptotic activity of oridonin was partially attributed to its repression of IGF-1R signaling. In addition, p53 was supposed to be a pivotal transducer of proapoptotic and survival signaling pathway in this system

Girnita A, All-Ericsson C, Economou MA et al. The insulin-like growth factor-I receptor inhibitor picropodophyllin causes tumor regression and attenuates mechanisms involved in invasion of uveal melanoma cells. *Acta Ophthalmol* 2008 November;86 Thesis 4:26-34.

Abstract: PURPOSE: Uveal melanoma has a high mortality rate due to a high incidence of metastasis (up to 50%) which preferentially occurs in the liver. Conventional chemotherapy being the only therapeutic option today against metastatic uveal melanoma, has not proved to be effective. Therefore, new molecular targets important for malignant phenotype of uveal melanoma have to be found to design efficient pharmacologic agents. EXPERIMENTAL DESIGN: We previously reported data indicating that the insulin-like growth factor-1 receptor (IGF-IR) is a metastasis predictor as well as a therapeutic target for uveal melanoma. In the present study, we made use of the cyclolignan picropodophyllin (PPP), which is an inhibitor of the IGF-IR. RESULT: We showed that PPP efficiently block growth and viability of uveal melanoma cells in cultures and causes tumor regression in xenografted mice. In addition, treatment with PPP inhibited several mechanism involved in metastasis, including tumor cells adhesion to extracellular matrix proteins, activity and expression of matrix metalloproteinase 2, and cell migration as well as invasion through basement membranes and endothelial cell layer. Furthermore, PPP significantly delayed established of uveal melanoma tumor and drastically reduced the incidence of liver metastasis in mice. CONCLUSIONS: Our data suggest that IGR-IR is crucial for growth and survival as well as invasion and metastasis of uveal melanoma cells. Targeting this receptor may therefore comprise a strategy to treat ongoing disease (today incurable) as well as a strategy to prevent development of metastases in patients with primary disease

Lee C, Safdie FM, Raffaghello L et al. Reduced levels of IGF-I mediate differential protection of normal and cancer cells in response to fasting and improve chemotherapeutic index. *Cancer Res* 2010 February 15;70(4):1564-72.

Abstract: Inhibitors of the insulin-like growth factor-I (IGF-I) receptor have been widely studied for their ability to enhance the killing of a variety of malignant cells, but whether IGF-I signaling differentially protects the host and cancer cells against chemotherapy is unknown. Starvation can protect mice, but not cancer cells, against high-dose chemotherapy [differential stress resistance (DSR)]. Here, we offer evidence that IGF-I reduction mediates part of the starvation-dependent DSR. A 72-hour fast in mice reduced circulating IGF-I by 70% and increased the level of the IGF-I inhibitor IGFBP-1 by 11-fold. LID mice, with a 70% to 80% reduction in circulating IGF-I levels, were protected against three of four chemotherapy drugs tested. Restoration of IGF-I was sufficient to reverse the protective effect of fasting. Sixty percent of melanoma-bearing LID mice treated with doxorubicin achieved long-term survival whereas all control mice died of either metastases or chemotherapy toxicity. Reducing IGF-I/IGF-I signaling protected primary glia, but not glioma cells, against cyclophosphamide and protected mouse embryonic fibroblasts against doxorubicin. Further, *S. cerevisiae* lacking homologs of IGF-I signaling proteins were protected against chemotherapy-dependent DNA damage in a manner that could be

reversed by expressing a constitutively active form of Ras. We conclude that normal cells and mice can be protected against chemotherapy-dependent damage by reducing circulating IGF-I levels and by a mechanism that involves downregulation of proto-oncogene signals

Molhoek KR, Shada AL, Smolkin M et al. Comprehensive analysis of receptor tyrosine kinase activation in human melanomas reveals autocrine signaling through IGF-1R. *Melanoma Res* 2011 August;21(4):274-84.

Abstract: Melanomas depend on autocrine signals for proliferation and survival; however, no systematic screen of known receptor tyrosine kinases (RTKs) has been performed to identify which autocrine signaling pathways are activated in melanoma. Here, we performed a comprehensive analysis of 42 RTKs in six individual human melanoma tumor specimens as well as 17 melanoma cell lines, some of which were derived from the tumor specimens. We identified five RTKs that were active in almost every one of the melanoma tissue specimens and cell lines, including two previously unreported receptors, insulin-like growth factor receptor 1 (IGF-1R) and macrophage-stimulating protein receptor (MSPR), in addition to three receptors (vascular endothelial growth factor receptor, fibroblast growth factor receptor, and hepatocyte growth factor receptor) known to be autocrine activated in melanoma. We show, by quantitative real time PCR, that all melanoma cell lines expressed genes for the RTK ligands such as HGF, IGF-1, and MSP. Addition of antibodies to either IGF-1 or HGF, but not to MSP, to the culture medium blocked melanoma cell proliferation, and even caused net loss of melanoma cells. Antibody addition deactivated IGF-1R and hepatocyte growth factor receptors, as well as mitogen-activated protein kinase signaling. Thus, IGF-1 is a new growth factor for autocrine driven proliferation of human melanoma in vitro. Our results suggest that IGF-1-IGF-1R autocrine pathway in melanoma is a possible target for therapy in human melanomas

Wu X, Zhou J, Rogers AM et al. c-Met, epidermal growth factor receptor, and insulin-like growth factor-1 receptor are important for growth in uveal melanoma and independently contribute to migration and metastatic potential. *Melanoma Res* 2012 April;22(2):123-32.

Abstract: Uveal melanoma (UM) has a high propensity to develop hepatic metastases. We sought to define the mechanisms required for preferential liver homing and to understand further the biologic behavior of this disease. The Met tyrosine kinase receptor and its ligand hepatocyte growth factor are expressed in hepatocytes. We therefore considered Met/hepatocyte growth factor signaling as a candidate migration/growth factor for UM cells. We further explored the relationship between c-Met and other growth factor receptors prevalent in the liver and their roles in UM metastatic potential. UM cell lines were evaluated for c-Met, epidermal growth factor receptor (EGFR), and insulin-like growth factor-1R (IGF-1R) expression by immunoblotting, and gene amplification by comparative genomic hybridization and fluorescence in-situ hybridization. High c-Met, phosphorylated c-Met, and EGFR expression were noted in two of nine cell lines, independent of IGF-1R levels. Knockdown of c-Met decreased proliferation of high c-Met-expressing UM cells but did not induce apoptosis. Selective inhibitors of EGFR and IGF-1R decreased proliferation and induced apoptosis in UM cells regardless of the expression levels of c-Met, EGFR, and IGF-1R. Although c-Met, EGFR, and IGF-1R play proliferative roles, EGFR and IGF-1R are also critical for UM cell survival. High c-Met/EGFR-expressing cell lines possessed the greatest migration potential. c-Met knockdown and selective inhibitors of c-Met, EGFR, and IGF-1R revealed independent contribution of these receptors to migration. UM can be categorized by levels of c-Met and EGFR expression which are associated with migratory/invasiveness responses to soluble factors present at high levels in the liver. This provides biologic relevance for UM clinical behavior with potential therapeutic implications

Hilmi C, Larribere L, Giuliano S et al. IGF1 promotes resistance to apoptosis in melanoma cells through an increased expression of BCL2, BCL-X(L), and survivin. *J Invest Dermatol* 2008 June;128(6):1499-

Abstract: IGF1 plays a key role in the development and growth of multiple tumors and in the prevention of apoptosis. In melanoma cells, IGF1 has been shown to mediate resistance to anoikis-induced apoptosis. However, the effect of IGF1 on other proapoptotic stimuli has never been reported. Further, the molecular mechanisms by which IGF1 mediates its prosurvival properties in melanoma cells remain unknown. Here, we demonstrate that IGF1 impairs the onset of tumor necrosis factor-related apoptosis-inducing ligand and staurosporine-induced apoptosis in melanoma cells expressing either wild-type or oncogenic B-Raf. Further, we show that IGF1 inhibits mitochondrial damage that occurs during apoptosis, thereby indicating that IGF1 acts at the level of mitochondria to mediate its antiapoptotic stimuli. Accordingly, IGF1 increases the mRNA levels and protein expression of antiapoptotic members of the BCL2 family--BCL2 and BCL-X(L)--and that of the inhibitor of apoptosis protein, survivin. Further, their specific silencing by small interfering RNA prevents the protective effect of IGF1. These findings therefore delineate the molecular mechanisms by which IGF1 mediates its prosurvival properties and provide a basis for clinical strategies designed to neutralize IGF1 or its target genes

Girnita A, All-Ericsson C, Economou MA et al. The insulin-like growth factor-I receptor inhibitor picropodophyllin causes tumor regression and attenuates mechanisms involved in invasion of uveal melanoma cells. *Clin Cancer Res* 2006 February 15;12(4):1383-91.

Abstract: PURPOSE: Uveal melanoma has a high mortality rate due to a high incidence of metastasis (up to 50%), which preferentially occurs in the liver. Conventional chemotherapy, being the only therapeutic option today against metastatic uveal melanoma, has not proved to be effective. Therefore, new molecular targets important for malignant phenotype of uveal melanoma have to be found to design efficient pharmacologic agents. EXPERIMENTAL DESIGN: We previously reported data indicating that the insulin-like growth factor-1 receptor (IGF-IR) is a metastasis predictor as well as a therapeutic target for uveal melanoma. In the present study, we made use of the cyclolignan picropodophyllin (PPP), which is an inhibitor of the IGF-IR. RESULTS: We showed that PPP efficiently blocks growth and viability of uveal melanoma cells in cultures and causes tumor regression in xenografted mice. In addition, treatment with PPP inhibited several mechanisms involved in metastasis, including tumor cell adhesion to extracellular matrix proteins, activity and expression of matrix metalloproteinase 2, and cell migration as well as invasion through basement membranes and endothelial cell layers. Furthermore, PPP significantly delayed establishment of uveal melanoma tumors and drastically reduced the incidence of liver metastasis in mice. CONCLUSIONS: Our data suggest that IGF-IR is crucial for growth and survival as well as invasion and metastasis of uveal melanoma cells. Targeting this receptor may therefore comprise a strategy to treat ongoing disease (today incurable) as well as a strategy to prevent development of metastases in patients with primary disease

Neudauer CL, McCarthy JB. Insulin-like growth factor I-stimulated melanoma cell migration requires phosphoinositide 3-kinase but not extracellular-regulated kinase activation. *Exp Cell Res* 2003 May 15;286(1):128-37.

Abstract: Dysregulated signaling contributes to altered cellular growth, motility, and survival during cancer progression. We have evaluated the ability of several factors to stimulate migration in WM1341D, a cell line derived from an invasive human vertical growth phase melanoma. Basic fibroblast growth factor, hepatocyte growth factor, interleukin-8, and CCL27 each slightly increased migration. Insulin-like growth factor I (IGF-I), however, stimulated a 15-fold increase in migration. This response required the IGF-I receptor, which activates phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathways. Both pathways have been implicated in migration in a variety of cell types, but the signaling required for IGF-I-induced melanoma cell migration is not well defined. IGF-I-stimulated activation of MAPK/ERK signaling in WM1341D

cells was inhibited by U0126, but a 33-fold higher dose of U0126 was needed to inhibit IGF-I-stimulated cellular migration. In contrast, similar concentrations of either wortmannin or LY294002 were required to inhibit both IGF-I-induced PI3K activation and migration. These results indicate that IGF-I-stimulated migration of WM1341D cells requires PI3K activation but is independent of MAPK/ERK signaling. Determining the contributions of IGF-I signaling pathways to migration will help us to understand melanoma progression and may lead to new therapeutic targets of this highly metastatic cancer

Satyamoorthy K, Li G, Vaidya B, Kalabis J, Herlyn M. Insulin-like growth factor-I-induced migration of melanoma cells is mediated by interleukin-8 induction. *Cell Growth Differ* 2002 February;13(2):87-93.

Abstract: Successive events of growth factor-induced autocrine and paracrine activation promote tumor growth and metastasis. Insulin-like growth factor-I (IGF-I) stimulates melanoma cells to grow, survive, and migrate. Interleukin-8 (IL-8) is produced by melanoma cells and has been correlated with melanoma metastasis, but the biological functions of this cytokine have not been elucidated. We show here that IGF-I-induced migration of melanoma cells could be inhibited by neutralizing antibody against IL-8. IGF-I overexpression induced IL-8 production in melanoma cells, especially in biologically early melanomas by accelerating its transcription rate via activation of mitogen-activated protein kinase pathway. IGF-I treatment phosphorylated c-Jun and stimulated the binding of AP-1 but not NF-kappaB to the IL-8 promoter. These data identify IL-8 as a new target of IGF-I in melanoma and suggest that some of the biological functions of IGF-I are mediated by IL-8

Satyamoorthy K, Li G, Vaidya B, Patel D, Herlyn M. Insulin-like growth factor-1 induces survival and growth of biologically early melanoma cells through both the mitogen-activated protein kinase and beta-catenin pathways. *Cancer Res* 2001 October 1;61(19):7318-24.

Abstract: Melanoma cells produce growth factors for autocrine growth control and for stimulating fibroblasts and endothelial cells in the tumor stroma. Activated stromal fibroblasts can in turn secrete growth factors that support tumor growth. We studied this feedback from fibroblasts to melanoma cells by overexpressing insulin-like growth factor 1 (IGF-1) with an adenoviral vector. Melanoma cells do not produce IGF-1. IGF-1 enhanced survival, migration, and growth of cells from biologically early lesions, but not from biologically late primary or metastatic lesions. Early melanoma cells were activated by IGF-1 to phosphorylate Erk1 and -2 of the mitogen-activated protein kinase pathway. IGF-1 also activated Akt, inhibited its down-stream effector GSK3-beta, and stabilized beta-catenin. Late primary and metastatic melanoma cells did not respond to growth stimulation by IGF-1 because of a constitutive activation of the mitogen-activated protein kinase pathway and a higher level of stabilized beta-catenin. These studies demonstrate that fibroblast-derived growth factors from the tumor environment can provide the malignant cells with a positive feedback through multiple mechanisms but that this stimulation is required only for cells from early and not late stages of tumor progression

Kanter-Lewensohn L, Dricu A, Wang M, Wejde J, Kiessling R, Larsson O. Expression of the insulin-like growth factor-1 receptor and its anti-apoptotic effect in malignant melanoma: a potential therapeutic target. *Melanoma Res* 1998 October;8(5):389-97.

Abstract: The insulin-like growth factor-1 receptor (IGF-1R) and its possible protective effect on apoptotic cell death in malignant melanoma was analysed in four commercial melanoma cell lines. Inhibition of N-linked glycosylation by tunicamycin, which has previously been shown to block the translocation of IGF-1R to the cell surface, blocked cell growth and/or induced cell death in these cell lines. Treatment with alphaIR-3, an antibody blocking the binding domain of IGF-1R, also resulted in growth arrest and/or apoptosis. We also analysed lymph node metastases of malignant melanoma by Western blotting and immunohistochemistry. All these cases were shown to express IGF-1R at the cell surface. In three cases of lymph node metastases we had access to both tumour specimens and cultured

cells. One of these exhibited a substantially higher expression of IGF-1R than the two other cases. The corresponding cell lines showed growth arrest and apoptosis following treatment with alphaIR-3. However, the two cell lines with low expression of IGF-1R were more sensitive in this respect. Furthermore, we demonstrated an inverse correlation between IGF-1R expression and the frequency of apoptotic cells in the tumour specimens. Our data suggest that IGF-1R is crucial for the viability of malignant melanoma cells in vitro as well as in vivo

Fleming MG, Howe SF, Graf LH, Jr. Expression of insulin-like growth factor I (IGF-I) in nevi and melanomas. *Am J Dermatopathol* 1994 August;16(4):383-91.

Abstract: Expression of IGF-I mRNA and protein was evaluated in pigmented lesions by in situ hybridization and immunohistochemistry. An IGF-I cDNA clone (phigf1) was subcloned into pBluescript KS II-. Both sense and antisense 35S riboprobes were prepared and used for in situ hybridization on formalin-fixed, paraffin-embedded specimens. Control hybridizations with a beta-actin probe were also performed. Grains were counted in 787-microns<sup>2</sup> melanocytic areas of sections hybridized with the antisense IGF-I probe. Seven common nevi contained a mean of 218 grains; nine dysplastic nevi, a mean of 463 grains; eight early primary melanomas, a mean of 402 grains; five advanced primary melanomas, a mean of 217 grains; and nine metastatic melanomas, a mean of 194 grains. The differences between common and dysplastic nevus, common nevus and early melanoma, early and advanced primary melanoma, and early primary melanoma and metastatic melanoma were statistically significant. Keratinocytes also expressed abundant IGF-I message. IGF-I protein was demonstrable by immunohistochemistry in melanocytes and keratinocytes. These results suggest that progression-associated variation occurs in the net expression of IGF-I mRNA in melanocytic tumors

Furlanetto RW, Harwell SE, Baggs RB. Effects of insulin-like growth factor receptor inhibition on human melanomas in culture and in athymic mice. *Cancer Res* 1993 June 1;53(11):2522-6.

Abstract: The role of the insulin-like growth factor (IGF) receptor in regulating the growth of melanoma cells was evaluated by examining the effect of antibody-mediated IGF receptor inhibition on the growth of four human melanoma cell lines in culture and as xenotransplants in athymic mice. All four cell lines expressed typical type I IGF receptors and an antibody to this receptor (alpha IR-3) inhibited [<sup>125</sup>I]IGF-I binding. However, the cell lines varied widely in their in vitro responsiveness to IGF-I and alpha IR-3: in the WM 373 and WM 852 cell lines, IGF-I stimulated cell replication and alpha IR-3 inhibited this response, whereas in the WM 239-A and WM 266-4 cell lines neither the growth factor nor the antibody affected growth. A wide variation was also observed in the effect of the antibody on the growth of the different cell lines as xenotransplants but this qualitatively correlated with the responses observed in vitro: alpha IR-3 treatment significantly inhibited the growth of the WM 373 and WM 852 xenotransplants but did not inhibit the growth of the WM 239-A or WM 266-4 xenotransplants and may even have had a slight stimulatory effect. These results indicate that the IGF receptor pathway is a functional regulator of the in vivo growth of some melanomas and that this is reflected in the activity of this pathway as determined in vitro. These findings suggest that therapies aimed at inhibiting the IGF pathway may be beneficial in treating some melanomas

Stracke ML, Engel JD, Wilson LW, Rechler MM, Liotta LA, Schiffmann E. The type I insulin-like growth factor receptor is a motility receptor in human melanoma cells. *J Biol Chem* 1989 December 25;264(36):21544-9.

Abstract: Insulin-like growth factors I and II (IGF-I and II) and insulin are chemotactic agents for the human melanoma cell line A2058. As shown in this report, the motility receptor mediating this response is the heterodimeric type I IGF receptor. These three factors are able to compete with <sup>125</sup>I-labeled IGF-I for binding to the cell surface with IC<sub>50</sub> values equal to approximately 2 (IGF-I), approximately 150

(IGF-II), and approximately 300 nM (insulin). Cross-linking of <sup>125</sup>I-IGF-I to the cell surface with disuccinimidyl suberate followed by analysis with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography reveals a 130-kDa protein (reduced) consistent with the alpha component of a type I receptor and a 38-kDa protein which does not bind insulin, and thus could be another IGF-I cell surface binding protein. The anti-IGF-I receptor monoclonal antibody (alpha IR-3) also competes with labeled IGF-I in binding experiments. In contrast, a control monoclonal antibody, matched to alpha IR-3 with respect to IgG subclass, has no significant effect on IGF-I binding. While alpha IR-3 inhibits the motility induced by IGF-I, IGF-II, and insulin, pertussis toxin (0.01-1.0 micrograms/ml) has no significant effect on the motility induced by the insulin-like growth factors or insulin on this cell line. Therefore, the type I IGF receptor appears to mediate a highly potent pertussis toxin-insensitive motility response to IGF-I, IGF-II, and insulin. In contrast, motility induced by the autocrine motility factor, a cytokine produced by the A2058 cells, is not affected by alpha IR-3 but is extremely sensitive to pertussis toxin. When mixtures of autocrine motility factor and IGF-I are employed to induce chemotaxis, the resulting motility is greater than that induced by either agent alone. These data indicate that motility in this melanoma cell line can be initiated through multiple receptors that stimulate the cells by separate transduction pathways. This capability to respond to multiple stimuli could enhance the metastatic potential

## Metformin or Berberine

Martin MJ, Hayward R, Viros A, Marais R. Metformin accelerates the growth of BRAF V600E-driven melanoma by upregulating VEGF-A. *Cancer Discov* 2012 April;2(4):344-55.

Abstract: The antidiabetic drug metformin has antitumor activity in a variety of cancers because it blocks cell growth by inhibiting TORC1. Here, we show that melanoma cells that are driven by oncogenic BRAF are resistant to the growth-inhibitory effects of metformin because RSK sustains TORC1 activity even when AMP-activated protein kinase (AMPK) is activated. We further show that AMPK targets the dual-specificity protein phosphatase DUSP6 for degradation and this increases ERK activity, which then upregulates the VEGF-A protein. Critically, this drives angiogenesis and accelerates the growth of BRAF-driven tumors in mice. Unexpectedly, however, when VEGF signaling is inhibited, instead of accelerating tumor growth, metformin inhibits tumor growth. Thus, we show that BRAF-driven melanoma cells are resistant to the antigrowth effects of AMPK and that AMPK mediates cell-autonomous and cell-nonautonomous effects that accelerate the growth of these cells in vivo. Significance: Metformin inhibits the growth of most tumor cells, but BRAF-mutant melanoma cells are resistant to metformin in vitro, and metformin accelerates their growth in vivo. Unexpectedly, VEGF inhibitors and metformin synergize to suppress the growth of BRAF-mutant tumors, revealing a combination of drugs that may be effective in these patients

Tomic T, Botton T, Cerezo M et al. Metformin inhibits melanoma development through autophagy and apoptosis mechanisms. *Cell Death Dis* 2011;2:e199.

Abstract: Metformin is the most widely used antidiabetic drug because of its proven efficacy and limited secondary effects. Interestingly, recent studies have reported that metformin can block the growth of different tumor types. Here, we show that metformin exerts antiproliferative effects on melanoma cells, whereas normal human melanocytes are resistant to these metformin-induced effects. To better understand the basis of this antiproliferative effect of metformin in melanoma, we characterized the sequence of events underlying metformin action. We showed that 24 h metformin treatment induced a cell cycle arrest in G0/G1 phases, while after 72 h, melanoma cells underwent autophagy as demonstrated by

electron microscopy, immunochemistry, and by quantification of the autolysosome-associated LC3 and Beclin1 proteins. In addition, 96 h post metformin treatment we observed robust apoptosis of melanoma cells. Interestingly, inhibition of autophagy by knocking down LC3 or ATG5 decreased the extent of apoptosis, and suppressed the antiproliferative effect of metformin on melanoma cells, suggesting that apoptosis is a consequence of autophagy. The relevance of these observations were confirmed in vivo, as we showed that metformin treatment impaired the melanoma tumor growth in mice, and induced autophagy and apoptosis markers. Taken together, our data suggest that metformin has an important impact on melanoma growth, and may therefore be beneficial in patients with melanoma

Janjetovic K, Harhaji-Trajkovic L, Misirkic-Marjanovic M et al. In vitro and in vivo anti-melanoma action of metformin. *Eur J Pharmacol* 2011 October 15;668(3):373-82.

**Abstract:** The in vitro and in vivo anti-melanoma effect of antidiabetic drug metformin was investigated using B16 mouse melanoma cell line. Metformin caused a G(2)/M cell cycle arrest associated with apoptotic death of melanoma cells, as confirmed by the flow cytometric analysis of cell cycle/DNA fragmentation, phosphatidylserine exposure and caspase activation. Metformin-mediated apoptosis of melanoma cells was preceded by induction of oxidative stress and mitochondrial membrane depolarization, measured by flow cytometry in cells stained with appropriate fluorescent reporter dyes. The expression of tumor suppressor protein p53 was increased, while the mRNA levels of anti-apoptotic Bcl-2 were reduced by metformin, as revealed by cell-based ELISA and real-time RT-PCR, respectively. Treatment with metformin did not stimulate expression of the cycle blocker p21, indicating that p21 was dispensable for the observed cell cycle arrest. The activation of AMP-activated protein kinase (AMPK) was not required for the anti-melanoma action of metformin, as AMPK inhibitor compound C completely failed to restore viability of metformin-treated B16 cells. Metformin induced autophagy in B16 cells, as demonstrated by flow cytometry-detected increase in intracellular acidification and immunoblot-confirmed upregulation of autophagosome-associated LC3-II. Autophagy inhibitors ammonium chloride and wortmannin partly restored the viability of metformin-treated melanoma cells. Finally, oral administration of metformin led to a significant reduction in tumor size in a B16 mouse melanoma model. These data suggest that anti-melanoma effects of metformin are mediated through p21- and AMPK-independent cell cycle arrest, apoptosis and autophagy associated with p53/Bcl-2 modulation, mitochondrial damage and oxidative stress

Niehr F, von EE, Attar N et al. Combination therapy with vemurafenib (PLX4032/RG7204) and metformin in melanoma cell lines with distinct driver mutations. *J Transl Med* 2011;9:76.

**Abstract:** **BACKGROUND:** A molecular linkage between the MAPK and the LKB1-AMPK energy sensor pathways suggests that combined MAPK oncogene inhibition and metabolic modulation of AMPK would be more effective than either manipulation alone in melanoma cell lines. **MATERIALS AND METHODS:** The combination of the BRAF inhibitor vemurafenib (formerly PLX4032) and metformin were tested against a panel of human melanoma cell lines with defined BRAF and NRAS mutations for effects on viability, cell cycle and apoptosis. Signaling molecules in the MAPK, PI3K-AKT and LKB1-AMPK pathways were studied by Western blot. **RESULTS:** Single agent metformin inhibited proliferation in 12 out of 19 cell lines irrespective of the BRAF mutation status, but in one NRASQ61K mutant cell line it powerfully stimulated cell growth. Synergistic anti-proliferative effects of the combination of metformin with vemurafenib were observed in 6 out of 11 BRAFV600E mutants, including highly synergistic effects in two BRAFV600E mutant melanoma cell lines. Antagonistic effects were noted in some cell lines, in particular in BRAFV600E mutant cell lines resistant to single agent vemurafenib. Seven out of 8 BRAF wild type cell lines showed marginally synergistic anti-proliferative effects with the combination, and one cell line had highly antagonistic effects with the combination. The differential effects were not dependent on the sensitivity to each drug alone, effects on cell cycle or signaling pathways. **CONCLUSIONS:** The combination of vemurafenib and metformin tended to have

stronger anti-proliferative effects on BRAFV600E mutant cell lines. However, determinants of vemurafenib and metformin synergism or antagonism need to be understood with greater detail before any potential clinical utility of this combination

MacKenzie MJ, Ernst S, Johnson C, Winkquist E. A phase I study of temsirolimus and metformin in advanced solid tumours. *Invest New Drugs* 2012 April;30(2):647-52.

Abstract: The purpose of this phase I trial was to establish the maximum tolerated dose and define the dose-limiting toxicities of a combination of temsirolimus and metformin. Patients with advanced solid tumours who had exhausted standard treatment options were eligible. Treatment included weekly intravenous temsirolimus and daily oral metformin. Eleven patients were enrolled. Dose-limiting toxicities were observed in all patients at the initial dose level of 25 mg weekly of temsirolimus and metformin 500 mg po BID. At dose level -1, 2 of 8 patients experienced dose-limiting toxicities. Toxicities included grade 4 pneumonitis, persistent grade 3 fatigue, and thrombocytopenia requiring dose delays. The maximum tolerated dose (level -1) was 20 mg temsirolimus weekly and 500 mg po daily of metformin. One patient with head and neck cancer experienced a partial response. Five patients had stable disease including a patient with melanoma who had stable disease for 22 months

Woodard J, Platanius LC. AMP-activated kinase (AMPK)-generated signals in malignant melanoma cell growth and survival. *Biochem Biophys Res Commun* 2010 July 16;398(1):135-9.

Abstract: Extensive studies over the years have shown that the AMP-activated kinase (AMPK) exhibits negative regulatory effects on the activation of the mammalian target of rapamycin (mTOR) signaling cascade. We examined the potential involvement of AMPK in the regulation of growth and survival of malignant melanoma cells. In studies using the AMPK activators AICAR or metformin, we found potent inhibitory effects of AMPK activity on the growth of SK-MEL-2 and SK-MEL-28 malignant melanoma cells. Induction of AMPK activity was also associated with inhibition of the ability of melanoma cells to form colonies in an anchorage-independent manner in soft agar, suggesting an important role of the pathway in the control of malignant melanoma tumorigenesis. Furthermore, AICAR-treatment resulted in malignant melanoma cell death and such induction of apoptosis was further enhanced by concomitant statin-treatment. Taken together, our results provide evidence for potent inhibitory effects of AMPK on malignant melanoma cell growth and survival and raise the potential of AMPK manipulation as a novel future approach for the treatment of malignant melanoma

Kim HS, Kim MJ, Kim EJ, Yang Y, Lee MS, Lim JS. Berberine-induced AMPK activation inhibits the metastatic potential of melanoma cells via reduction of ERK activity and COX-2 protein expression. *Biochem Pharmacol* 2012 February 1;83(3):385-94.

Abstract: Berberine is clinically important natural isoquinoline alkaloid that affects various biological functions, such as cell proliferation, migration and survival. The activation of AMP-activated protein kinase (AMPK) regulates tumor cell migration. However, the specific role of AMPK on the metastatic potential of cancer cells remains largely unknown. The present study investigated whether berberine induces AMPK activation and whether this induction directly affects mouse melanoma cell migration, adhesion and invasion. Berberine strongly increased AMPK phosphorylation via reactive oxygen species (ROS) production. 5-Aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR), a well-known AMPK activator, also inhibited tumor cell adhesion and invasion and reduced the expression of epithelial to mesenchymal transition (EMT)-related genes. Knockdown of AMPK $\alpha$  subunits using siRNAs significantly abated the berberine-induced inhibition of tumor cell invasion. Furthermore, berberine inhibited the metastatic potential of melanoma cells through a decrease in ERK activity and protein levels of cyclooxygenase-2 (COX-2) by a berberine-induced AMPK activation. These data were confirmed using specific MEK inhibitor, PD98059, and a COX-2 inhibitor, celecoxib. Berberine- and AICAR-

treated groups demonstrated significantly decreased lung metastases in the pulmonary metastasis model in vivo. Treatment with berberine also decreased the metastatic potential of A375 human melanoma cells. Collectively, our results suggest that berberine-induced AMPK activation inhibits the metastatic potential of tumor cells through a reduction in the activity of the ERK signaling pathway and COX-2 protein levels

Hamsa TP, Kuttan G. Berberine inhibits pulmonary metastasis through down-regulation of MMP in metastatic B16F-10 melanoma cells. *Phytother Res* 2012 April;26(4):568-78.

Abstract: The present study demonstrated the potential antimetastatic and antiinvasive effect of berberine using both in vivo mouse lung metastasis and in vitro models. Administration of berberine resulted in significant suppression of B16F-10 melanoma induced tumor nodule formation and enhanced the survival of tumor-bearing mice. Berberine treatment also decreased various biochemical parameters associated with lung metastasis. These inhibitory actions may be due to the significant suppression of several signaling molecules such as ERK1/2, NF-kappaB, ATF-2 and CREB involved in the transcription signaling pathways for MMP gene expression. It could also inhibit the migration and invasion of highly metastatic murine melanoma cells in a dose-dependent manner in vitro. The results clearly show that berberine could significantly inhibit experimental lung metastasis produced by intravenous injection of B16F-10 melanoma cells and this effect could be linked to the down-regulation of metastasis-related signaling molecules

Burgeiro A, Gajate C, Dakir eH, Villa-Pulgarin JA, Oliveira PJ, Mollinedo F. Involvement of mitochondrial and B-RAF/ERK signaling pathways in berberine-induced apoptosis in human melanoma cells. *Anticancer Drugs* 2011 July;22(6):507-18.

Abstract: The natural isoquinoline alkaloid berberine exhibits a wide spectrum of biological activities including antitumor activity, but its mechanism of action remains to be fully elucidated. Here, we report that berberine induced apoptosis in human melanoma cells, through a process that involved mitochondria and caspase activation. Berberine-induced activation of a number of caspases, including caspases 3, 4, 7, 8, and 9. Pan-caspase inhibitor, z-VAD-fmk, and caspase-8 and caspase-9 inhibitors prevented apoptosis. Berberine also led to the generation of the p20 cleavage fragment of BAP31, involved in directing proapoptotic signals between the endoplasmic reticulum and the mitochondria. Treatment of SK-MEL-2 melanoma cells with berberine induced disruption of the mitochondrial transmembrane potential, release of cytochrome c and apoptosis-inducing factor from the mitochondria to the cytosol, generation of reactive oxygen species (ROS), and a decreased ATP/ADP ratio. Overexpression of bcl-xL by gene transfer prevented berberine-induced cell death, mitochondrial transmembrane potential loss, and cytochrome c and apoptosis-inducing factor release, but not ROS generation. N-acetyl-L-cysteine inhibited the production of ROS, but did not abrogate the berberine-induced apoptosis. Inhibition of extracellular signal-regulated kinase (ERK) phosphorylation, by using the mitogen-activated protein kinase/ERK kinase inhibitor PD98059, and reduction of B-RAF levels by silencing RNA induced cell death of SK-MEL-2 cells, and diminished the berberine concentration required to promote apoptosis. These data show that berberine-induced apoptosis in melanoma cells involves mitochondria and caspase activation, but ROS generation was not essential. Our results indicate that inhibition of B-RAF/ERK survival signaling facilitates the cell death response triggered by berberine

Singh T, Vaid M, Katiyar N, Sharma S, Katiyar SK. Berberine, an isoquinoline alkaloid, inhibits melanoma cancer cell migration by reducing the expressions of cyclooxygenase-2, prostaglandin E(2) and prostaglandin E(2) receptors. *Carcinogenesis* 2011 January;32(1):86-92.

Abstract: Melanoma is the leading cause of death from skin disease due, in large part, to its propensity to metastasize. We have examined the effect of berberine, an isoquinoline alkaloid, on human melanoma cancer cell migration and the molecular mechanisms underlying these effects using melanoma cell lines,

A375 and Hs294. Using an in vitro cell migration assay, we show that over expression of cyclooxygenase (COX)-2, its metabolite prostaglandin E(2) (PGE(2)) and PGE(2) receptors promote the migration of cells. We found that treatment of A375 and Hs294 cells with berberine resulted in concentration-dependent inhibition of migration of these cells, which was associated with a reduction in the levels of COX-2, PGE(2) and PGE(2) receptors (EP2 and EP4). Treatment of cells with celecoxib, a COX-2 inhibitor, or transient transfection of cells with COX-2 small interfering RNA, also inhibited cell migration. Treatment of the cells with 12-O-tetradecanoylphorbol-13-acetate (TPA), an inducer of COX-2 or PGE(2), enhanced cell migration, whereas berberine inhibited TPA- or PGE(2)-promoted cell migration. Berberine reduced the basal levels as well as PGE(2)-stimulated expression levels of EP2 and EP4. Treatment of the cells with the EP4 agonist stimulated cell migration and berberine blocked EP4 agonist-induced cell migration activity. Moreover, berberine inhibited the activation of nuclear factor-kappa B (NF-kappaB), an upstream regulator of COX-2, in A375 cells, and treatment of cells with caffeic acid phenethyl ester, an inhibitor of NF-kappaB, inhibited cell migration. Together, these results indicate for the first time that berberine inhibits melanoma cell migration, an essential step in invasion and metastasis, by inhibition of COX-2, PGE(2) and PGE(2) receptors

Serafim TL, Oliveira PJ, Sardao VA, Perkins E, Parke D, Holy J. Different concentrations of berberine result in distinct cellular localization patterns and cell cycle effects in a melanoma cell line. *Cancer Chemother Pharmacol* 2008 May;61(6):1007-18.

Abstract: PURPOSE: Natural products represent a rich reservoir of potential small molecule inhibitors exhibiting antiproliferative and tumoricidal properties. An example is the isoquinoline alkaloid berberine, which is found in plants such as goldenseal (*Hydrastis canadensis*). Studies have shown that berberine is able to trigger apoptosis in different malignant cell lines, and can also lead to cell cycle arrest at sub-apoptotic doses. A particularly interesting feature of berberine is the fact that it is a fluorescent molecule, and its uptake and distribution in cells can be studied by flow cytometry and epifluorescence microscopy. To test the relationships between berberine uptake, distribution and cellular effect in melanoma cells, K1735-M2 mouse and WM793 human melanoma cells were treated with different concentrations of berberine, and alterations in cell cycle progression, DNA synthesis, cell proliferation, and cell death measured. METHODS: Cell proliferation was measured by sulforhodamine B assays, cell death by flow cytometry, berberine uptake and distribution by laser scanning confocal microscopy and flow cytometry, cell cycle progression by flow cytometry, and DNA synthesis, M-phase, and mitochondrial effects by immunolabeling and epifluorescence microscopy methods. RESULTS: In these melanoma cell lines, berberine at low doses (12.5-50  $\mu$ M) is concentrated in mitochondria and promotes G1 arrest. In contrast, higher doses (over 50  $\mu$ M) result in cytoplasmic and nuclear berberine accumulation, and G2 arrest. DNA synthesis is not markedly affected by low doses of berberine, but 100  $\mu$ M is strongly inhibitory. Even at 100  $\mu$ M, berberine inhibits cell growth with relatively little induction of apoptosis. CONCLUSION: Berberine displays multiphasic effects in these malignant cell lines, which are correlated with the concentration and intracellular distribution of this alkaloid. These results help explain some of the conflicting information in the literature regarding the effects of berberine, and suggest that its use in clinical development may be more as a cytostatic agent than a cytotoxic compound

Petti C, Vegetti C, Molla A et al. AMPK activators inhibit the proliferation of human melanomas bearing the activated MAPK pathway. *Melanoma Res* 2012 October;22(5):341-50.

Abstract: Raf/MEK/ERK signaling can inhibit the liver kinase B1-AMP-activated protein kinase (LKB1-AMPK) pathway, thus rendering melanoma cells resistant to energy stress conditions. We evaluated whether pharmacological reactivation of the AMPK function could exert antitumor effects on melanoma cells bearing this pathway constitutively active because of a mutation in NRAS or BRAF genes. Nine melanoma cell lines were treated with the AMPK activators 5-aminoimidazole-4-carboxamide-ribonucleoside (AICAR) and phenformin. The activation of AMPK enzymatic activity, phosphorylation

of AMPK and acetyl-CoA carboxylase kinase, in-vitro proliferation, cell cycle, and in-vivo growth of xenografts in nude mice were evaluated. AICAR and phenformin promoted phosphorylation and enzymatic activity of AMPK, as well as phosphorylation of the AMPK downstream target acetyl-CoA carboxylase. Drug treatment of either BRAF-mutant or NRAS-mutant melanomas, at doses not inducing cell death, was accompanied by a dose-dependent decrease in melanoma cell proliferation because of cell cycle arrest in either the G0/G1 or the S phase, associated with an increased expression of the p21 cell cycle inhibitor. Melanomas isolated from subcutaneously implanted mice, 25 days from treatment with AICAR, showed increased staining of the senescence-associated marker beta-galactosidase, high p21 expression, and evidence of necrosis. Altogether, these results indicate that pharmacological activators of AMPK-dependent pathways inhibit the cell growth of melanoma cells with active Raf/MEK/ERK signaling and provide a rationale for further investigation on their use in combination therapies

Chen MB, Shen WX, Yang Y, Wu XY, Gu JH, Lu PH. Activation of AMP-activated protein kinase is involved in vincristine-induced cell apoptosis in B16 melanoma cell. *J Cell Physiol* 2011 July;226(7):1915-25.

Abstract: The molecular basis for induction of apoptosis in melanoma cells by vincristine remains unknown. Here we tested the potential involvement of AMP-activated protein kinase (AMPK) in this process. We found for the first time that vincristine induces AMPK activation (AMPK $\alpha$ , Thr 172) and Acetyl-CoA carboxylase (ACC, Ser 79) (a downstream molecular target of AMPK) phosphorylation in cultured melanoma cells in vitro. Reactive oxygen species (ROS) dependent LKB1 activation serves as the upstream signal for AMPK activation. AMPK inhibitor (compound C) or AMPK $\alpha$  siRNA knockdown inhibits vincristine induced B16 melanoma cell apoptosis, while AMPK activator 5-aminoimidazole-4-carboxamide-1-beta-ribose (AICAR) enhances it. AMPK activation is involved in vincristine induced p53 phosphorylation and stabilization, the latter is known to mediate melanoma cell apoptosis. Further, activation of AMPK by vincristine inhibits mTOR Complex 1 (mTORC1) in B16 melanoma cells, which serves as another important mechanism to induce melanoma cell apoptosis. Our study provides new insights into understanding the cellular and molecular mechanisms of vincristine induced cancer cell death/apoptosis. We suggest that combining AMPK activator AICAR with vincristine may have potential to be used as a new therapeutic intervention against melanoma

Esteve-Puig R, Canals F, Colome N, Merlino G, Recio JA. Uncoupling of the LKB1-AMPK $\alpha$  energy sensor pathway by growth factors and oncogenic BRAF. *PLoS ONE* 2009;4(3):e4771.

Abstract: BACKGROUND: Understanding the biochemical mechanisms contributing to melanoma development and progression is critical for therapeutical intervention. LKB1 is a multi-task Ser/Thr kinase that phosphorylates AMPK controlling cell growth and apoptosis under metabolic stress conditions. Additionally, LKB1(Ser428) becomes phosphorylated in a RAS-Erk1/2-p90(RSK) pathway dependent manner. However, the connection between the RAS pathway and LKB1 is mostly unknown. METHODOLOGY/PRINCIPAL FINDINGS: Using the UV induced HGF transgenic mouse melanoma model to investigate the interplay among HGF signaling, RAS pathway and PI3K pathway in melanoma, we identified LKB1 as a protein directly modified by HGF induced signaling. A variety of molecular techniques and tissue culture revealed that LKB1(Ser428) (Ser431 in the mouse) is constitutively phosphorylated in BRAF(V600E) mutant melanoma cell lines and spontaneous mouse tumors with high RAS pathway activity. Interestingly, BRAF(V600E) mutant melanoma cells showed a very limited response to metabolic stress mediated by the LKB1-AMPK-mTOR pathway. Here we show for the first time that RAS pathway activation including BRAF(V600E) mutation promotes the uncoupling of AMPK from LKB1 by a mechanism that appears to be independent of LKB1(Ser428) phosphorylation. Notably, the inhibition of the RAS pathway in BRAF(V600E) mutant melanoma cells recovered the complex formation and rescued the LKB1-AMPK $\alpha$  metabolic stress-induced response, increasing apoptosis in cooperation with the pro-apoptotic proteins Bad and Bim, and the down-regulation of Mcl-1.

**CONCLUSIONS/SIGNIFICANCE:** These data demonstrate that growth factor treatment and in particular oncogenic BRAF(V600E) induces the uncoupling of LKB1-AMPKalpha complexes providing at the same time a possible mechanism in cell proliferation that engages cell growth and cell division in response to mitogenic stimuli and resistance to low energy conditions in tumor cells. Importantly, this mechanism reveals a new level for therapeutical intervention particularly relevant in tumors harboring a deregulated RAS-Erk1/2 pathway

Zheng B, Jeong JH, Asara JM et al. Oncogenic B-RAF negatively regulates the tumor suppressor LKB1 to promote melanoma cell proliferation. *Mol Cell* 2009 January 30;33(2):237-47.

**Abstract:** The LKB1-AMPK signaling pathway serves as a critical cellular sensor coupling energy homeostasis to cell growth, proliferation, and survival. However, how tumor cells suppress this signaling pathway to gain growth advantage under conditions of energy stress is largely unknown. Here, we show that AMPK activation is suppressed in melanoma cells with the B-RAF V600E mutation and that downregulation of B-RAF signaling activates AMPK. We find that in these cells LKB1 is phosphorylated by ERK and Rsk, two kinases downstream of B-RAF, and that this phosphorylation compromises the ability of LKB1 to bind and activate AMPK. Furthermore, expression of a phosphorylation-deficient mutant of LKB1 allows activation of AMPK and inhibits melanoma cell proliferation and anchorage-independent cell growth. Our findings provide a molecular linkage between the LKB1-AMPK and the RAF-MEK-ERK pathways and suggest that suppression of LKB1 function by B-RAF V600E plays an important role in B-RAF V600E-driven tumorigenesis

### **Fish Omega-3s**

Bachi AL, Kim FJ, Nonogaki S et al. Leukotriene B4 creates a favorable microenvironment for murine melanoma growth. *Mol Cancer Res* 2009 September;7(9):1417-24.

**Abstract:** Chronic inflammation has long been associated with neoplastic progression. Our group had recently shown that the addition of a large number of apoptotic tumor cells to the tumor microenvironment induces a potent acute inflammatory reaction capable of promoting melanoma growth; however, primarily necrotizing cells do not cause such a reaction. Here, we show that potent inflammatory agents, such as lipopolysaccharide (LPS) and carrageenan, also promote growth of subtumorigenic doses of melanoma cells, having no effect on melanoma proliferation in vitro. Inhibition of 5-lipoxygenase (5-LOX) seems to have a pivotal role in this model because caffeic acid and MK886, a FLAP (5-LOX-activating protein) inhibitor, partially hindered tumor growth induced by apoptotic cells or LPS. Other enzymes of the arachidonic acid pathway, cyclooxygenase-1 and cyclooxygenase-2, seem to have no participation in this tumor promoter effect, as the inhibitor of both enzymes (indomethacin) did not alter melanoma growth. Leukotriene B4 (LTB4), the main product of the 5-LOX pathway, was able to induce growth of subtumorigenic inocula of melanoma cells, and a LTB4 receptor antagonist inhibited acute inflammation-associated tumor growth. Addition to the tumor inflammatory microenvironment of eicosapentaenoic acid, an omega3-polyunsaturated fatty acid with anti-inflammatory properties, or leukotriene B5, an eicosapentaenoic acid-derived leukotriene, significantly inhibited tumor development. These results give new insights to the mechanisms through which inflammation may contribute to tumor progression and suggest that LOX has an important role in tumor progression associated with an inflammatory state in the presence of apoptosis, which may be a consideration for apoptosis-inducing treatments, such as chemotherapy and radiotherapy

Mannini A, Kerstin N, Calorini L, Mugnai G, Ruggieri S. An enhanced apoptosis and a reduced angiogenesis are associated with the inhibition of lung colonisation in animals fed an n-3 polyunsaturated

fatty acid-rich diet injected with a highly metastatic murine melanoma line. *Br J Nutr* 2009 March;101(5):688-93.

Abstract: Both epidemiological and experimental studies indicate that dietary n-3 PUFA inhibit carcinogenesis and tumour growth. Metastatic diffusion has also been found to be affected in animals fed diets containing purified n-3 PUFA or fish oil. In the present study, we investigated whether the metastatic diffusion of a highly metastatic variant (F10-SR cells) isolated from the B16 melanoma F10 line was affected by feeding host animals a diet containing 5 % fish oil. In these animals, compared with those fed a diet containing 5 % maize oil, there was a reduced number of metastatic pulmonary colonies. The immunohistochemical analysis of appropriate markers revealed that the antimetastatic effect of dietary n-3 PUFA was not related to a reduction of proliferation, but rather to an enhanced apoptotic activity. The reduction of von Willebrand factor immunoreactivity found in pulmonary colonies of F10-SR cells grown in fish oil-fed animals indicates that a decrease of angiogenesis contributes to the antimetastatic effect of dietary n-3 PUFA. This conclusion stands in spite of the higher expression of vascular endothelial growth factor observed in pulmonary colonies grown in fish oil-fed animals

Xia S, Lu Y, Wang J et al. Melanoma growth is reduced in fat-1 transgenic mice: impact of omega-6/omega-3 essential fatty acids. *Proc Natl Acad Sci U S A* 2006 August 15;103(33):12499-504.

Abstract: An important nutritional question as to whether the ratio of omega-6 (n-6) to omega-3 (n-3) fatty acids plays a role in tumorigenesis remains to be clarified in well qualified experimental models. The recently engineered fat-1 mice, which can convert n-6 to n-3 fatty acids and have a balanced ratio of n-6 to n-3 fatty acids in their tissues and organs independent of diet, allow carefully controlled studies to be performed in the absence of potential confounding factors of diet and therefore are a useful model for elucidating the role of n-6/n-3 fatty acid ratio in tumorigenesis. We implanted mouse melanoma B16 cells into transgenic and WT littermates and examined the incidence of tumor formation and tumor growth rate. The results showed a dramatic reduction of melanoma formation and growth in fat-1 transgenic mice. The level of n-3 fatty acids and their metabolite prostaglandin E(3) (PGE(3)) were much higher (but the n-6/n-3 ratio is much lower) in the tumor and surrounding tissues of fat-1 mice than that of WT animals. The phosphatase and tensin homologue deleted on the chromosome 10 (PTEN) gene was significantly up-regulated in the fat-1 mice. In vitro experiments showed that addition of the n-3 fatty acid eicosapentaenoic acid or PGE(3) inhibited the growth of B16 cell line and increased the expression of PTEN, which could be partially attenuated by inhibition of PGE(3) production, suggesting that PGE(3) may act as an antitumor mediator. These data demonstrate an anticancer (antimelanoma) effect of n-3 fatty acids through, at least in part, activation of PTEN pathway mediated by PGE(3)

Denkins Y, Kempf D, Ferniz M, Nileshwar S, Marchetti D. Role of omega-3 polyunsaturated fatty acids on cyclooxygenase-2 metabolism in brain-metastatic melanoma. *J Lipid Res* 2005 June;46(6):1278-84.

Abstract: Cyclooxygenase-2 (COX-2) is important in the progression of epithelial tumors. Evidence indicates that omega-6 PUFAs such as arachidonic acid (AA) promote the growth of tumor cells; however, omega-3 fatty acids [eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] inhibit tumor cell proliferation. We investigated the effects of omega-3 PUFA on the expression and function of COX-2 in 70W, a human melanoma cell line that metastasizes to the brain in nude mice. We show that 1) tumor necrosis factor-alpha upregulates the expression of both COX-2 mRNA and prostaglandin E2 (PGE2) production, and 2) omega-3 and omega-6 PUFA regulate COX-2 mRNA expression and PGE2 production. AA increased COX-2 mRNA expression and prostaglandin production in omega-6-stimulated 70W cells. Conversely, COX-2 mRNA expression decreased in cells incubated with EPA or DHA. AA increased Matrigel invasion 2.4-fold, whereas EPA or DHA did not. Additionally, PGE2 increased in vitro invasion 2.5-fold, whereas exposure to PGE3 significantly decreased invasion. Our results demonstrate that incubation of 70W cells with either AA or PGE2 increased invasiveness, whereas

incubation with EPA or DHA downregulated both COX-2 mRNA and protein expression, with a subsequent decrease in Matrigel invasion. Taken together, these results indicate that omega-3 PUFA regulate COX-2-mediated invasion in brain-metastatic melanoma

Salem ML, Kishihara K, Abe K, Matsuzaki G, Nomoto K. N-3 polyunsaturated fatty acids accentuate B16 melanoma growth and metastasis through suppression of tumoricidal function of T cells and macrophages. *Anticancer Res* 2000 September;20(5A):3195-203.

Abstract: **BACKGROUND:** The antitumor effects of the n-3 polyunsaturated fatty acids (PUFAs) are still controversial and as yet undefined. **MATERIALS AND METHODS:** EPA-28, a fish oil enriched with n-3 PUFAs including eicosapentaenoic and docosahexaenoic acids, was administered subcutaneously into C57BL/6 mice before and after subcutaneous inoculation of B16 melanoma cells. The effects of EPA-28 on the antitumor activities of T cells and macrophages were investigated. **RESULTS:** The treatment of the mice with EPA-28 before and after the tumor inoculation enhanced the growth and metastasis of B16 melanoma and decreased the survival rate of the tumor-bearing mice. The treatment also decreased the number of CD4+ T cells in the spleen and tumor draining lymph nodes on day 14 after the tumor inoculation. Moreover, EPA-28 suppressed the antimelanoma cytolytic activity of T cells and macrophages of the tumor-bearing mice. **CONCLUSION:** The results suggest that EPA-28 treatment increased both the growth and metastasis of B16 melanoma cells by suppressing the cytolytic function of both T cells and macrophages

## Green Tea Polyphenols

Lemarie F, Chang CW, Blatchford DR et al. Antitumor activity of the tea polyphenol epigallocatechin-3-gallate encapsulated in targeted vesicles after intravenous administration. *Nanomedicine (Lond)* 2012 August 14.

Abstract: **Aim:** The therapeutic potential of epigallocatechin-3-gallate (EGCG), a green tea polyphenol with anticancer properties, is limited by its inability to specifically reach tumors following intravenous administration. The purpose of this study was to determine whether a tumor-targeted vesicular formulation of EGCG would suppress the growth of A431 epidermoid carcinoma and B16-F10 melanoma in vitro and in vivo. **Materials & methods:** Transferrin-bearing vesicles encapsulating EGCG were administered intravenously to mice bearing subcutaneous A431 and B16-F10 tumors. **Results:** The intravenous administration of EGCG encapsulated in transferrin-bearing vesicles resulted in tumor suppression in 40% of A431 and B16-F10 tumors. Animal survival was improved by more than 20 days compared with controls. **Conclusion:** Encapsulation of EGCG in transferrin-bearing vesicles is a promising therapeutic strategy. Original submitted 28 November 2011; Revised submitted 11 May 2012

Singh T, Katiyar SK. Green tea catechins reduce invasive potential of human melanoma cells by targeting COX-2, PGE2 receptors and epithelial-to-mesenchymal transition. *PLoS ONE* 2011;6(10):e25224.

Abstract: Melanoma is the most serious type of skin disease and a leading cause of death from skin disease due to its highly metastatic ability. To develop more effective chemopreventive agents for the prevention of melanoma, we have determined the effect of green tea catechins on the invasive potential of human melanoma cells and the molecular mechanisms underlying these effects using A375 (BRAF-mutated) and Hs294t (Non-BRAF-mutated) melanoma cell lines as an in vitro model. Employing cell invasion assays, we found that the inhibitory effects of green tea catechins on the cell migration were in the order of (-)-epigallocatechin-3-gallate (EGCG)>(-)-epigallocatechin>(-)-epicatechin-3-gallate>(-)-

gallo catechin>(-)-epica techin. Treatment of A375 and Hs294t cells with EGCG resulted in a dose-dependent inhibition of cell migration or invasion of these cells, which was associated with a reduction in the levels of cyclooxygenase (COX)-2, prostaglandin (PG) E(2) and PGE(2) receptors (EP2 and EP4). Treatment of cells with celecoxib, a COX-2 inhibitor, also inhibited melanoma cell migration. EGCG inhibits 12-O-tetradecanoylphorbol-13-acetate-, an inducer of COX-2, and PGE(2)-induced cell migration of cells. EGCG decreased EP2 agonist (butaprost)- and EP4 agonist (Cay10580)-induced cell migration ability. Moreover, EGCG inhibited the activation of NF-kappaB/p65, an upstream regulator of COX-2, in A375 melanoma cells, and treatment of cells with caffeic acid phenethyl ester, an inhibitor of NF-kappaB, also inhibited cell migration. Inhibition of melanoma cell migration by EGCG was associated with transition of mesenchymal stage to epithelial stage, which resulted in an increase in the levels of epithelial biomarkers (E-cadherin, cytokeratin and desmoglein 2) and a reduction in the levels of mesenchymal biomarkers (vimentin, fibronectin and N-cadherin) in A375 melanoma cells. Together, these results indicate that EGCG, a major green tea catechin, has the ability to inhibit melanoma cell invasion/migration, an essential step of metastasis, by targeting the endogenous expression of COX-2, PGE(2) receptors and epithelial-to-mesenchymal transition

Ellis LZ, Liu W, Luo Y et al. Green tea polyphenol epigallocatechin-3-gallate suppresses melanoma growth by inhibiting inflammasome and IL-1beta secretion. *Biochem Biophys Res Commun* 2011 October 28;414(3):551-6.

Abstract: Epigallocatechin-3-gallate (EGCG), the major polyphenolic component of green tea, has been demonstrated to possess anti-inflammatory, antioxidant, anti-mutagenic and anti-carcinogenic properties. The anti-melanoma effect of EGCG has been previously suggested, but no clear mechanism of action has been established. In this study, we demonstrated that EGCG inhibits melanoma cell growth at physiological doses (0.1-1 muM). In the search for mechanisms of EGCG-mediated melanoma cell suppression, we found that NF-kappaB was inhibited, and that reduced NF-kappaB activity was associated with decreased IL-1beta secretion from melanoma cells. Since inflammasomes are involved in IL-1beta secretion, we investigated whether IL-1beta suppression was mediated by inflammasomes, and found that EGCG treatment led to downregulation of the inflammasome component, NLRP1, and reduced caspase-1 activation. Furthermore, silencing the expression of NLRP1 abolished EGCG-induced inhibition of tumor cell proliferation both in vitro and in vivo, suggesting a key role of inflammasomes in EGCG efficacy. This paper provides a novel mechanism for EGCG-induced melanoma inhibition: inflammasome downregulation-->decreased IL-1beta secretion-->decreased NF-kappaB activities-->decreased cell growth. In addition, it suggests inflammasomes and IL-1beta could be potential targets for future melanoma therapeutics

Nihal M, Roelke CT, Wood GS. Anti-melanoma effects of vorinostat in combination with polyphenolic antioxidant (-)-epigallocatechin-3-gallate (EGCG). *Pharm Res* 2010 June;27(6):1103-14.

Abstract: PURPOSE: Melanoma is an aggressive neoplasm with a propensity for metastases and resistance to therapy. Previously, we showed that (-)-epigallocatechin-3-gallate (EGCG), the major polyphenolic antioxidant present in green tea, resulted in a significant decrease in the viability and growth of melanoma and induction of apoptosis via modulation of the cki-cdk-cyclin network and Bcl2 family proteins. Epigenetic regulation of gene transcription by histone deacetylase (HDAC) inhibitors is gaining momentum as a novel cancer therapy. SAHA-suberoylanilidene hydroxamic acid Zolinza (vorinostat) is the first HDAC inhibitor approved by the U.S. FDA. In this study, we determined if vorinostat alone or in combination with EGCG imparts anti-proliferative effects against human melanoma cells. METHODS: Employing human melanoma cell lines A-375, Hs-294T and G-361, we determined the effect of vorinostat and/or EGCG on 1) growth/viability and colony formation, 2) apoptosis, and 3) the critical molecules involved in cell cycle and apoptosis regulation. RESULTS: Our data demonstrated that the anti-proliferative effects of vorinostat were greater than or similar to those of EGCG among the cell lines

tested. Furthermore, relative to monotherapy, the combination treatment resulted in significantly greater inhibition of cell proliferation, increased apoptosis, activation of p21, p27 and caspases (3, 7 and 9) and Bax as well as down-regulation of cdk2, cdk4, cyclin A, NF-kappaB protein p65/RelA and Bcl2 protein and transcript. CONCLUSIONS: Our preclinical findings suggest that combination therapy with EGCG and vorinostat may be beneficial for the management of human melanoma

Nihal M, Ahsan H, Siddiqui IA, Mukhtar H, Ahmad N, Wood GS. (-)-Epigallocatechin-3-gallate (EGCG) sensitizes melanoma cells to interferon induced growth inhibition in a mouse model of human melanoma. *Cell Cycle* 2009 July 1;8(13):2057-63.

Abstract: Melanoma incidence has increased over the last few decades and metastatic melanoma is one of the hardest malignancies to treat. Thus, novel approaches are needed for an effective management of melanoma. Interferon-alpha2b (IFN), an immunomodulatory cytokine commonly used in melanoma treatment, has shown marginal efficacy and often results in discontinuation of therapy due to toxicity. We earlier demonstrated that epigallocatechin-3-gallate (EGCG), the major polyphenolic constituent of green tea, caused cell cycle arrest and apoptosis of human melanoma cells via modulation in cki-cyclin-cdk machinery and Bcl-2 family proteins. This study was undertaken to determine if EGCG could enhance the anti-proliferative effects of IFN. In this study, we demonstrated that EGCG and/or IFN treatments to melanoma cells resulted in a marked (1) decrease in cell proliferation and colony formation ability, and (2) induction of apoptosis. Interestingly, the combination was found to be more effective than either of the agents alone. Further, the anti-proliferative effects of EGCG and/or IFN were accompanied with an increase in Fas protein levels and a decrease in nuclear factor NFkappaB/p65 in the nucleus as well as NFkappaB promoter activity. EGCG and/or IFN also resulted in an increase in Fas-L mediated apoptosis. Further, EGCG and/or IFN treatments resulted in a decrease in melanoma tumor growth and protein levels of proliferation marker PCNA, in athymic nude mice implanted with melanoma tumors. The combination of the two modalities demonstrated a better response than either of them alone. Our data suggest that EGCG could impart therapeutic advantage if used in conjunction with IFN

Ravindranath MH, Ramasamy V, Moon S, Ruiz C, Muthugounder S. Differential growth suppression of human melanoma cells by tea (*Camellia sinensis*) epicatechins (ECG, EGC and EGCG). *Evid Based Complement Alternat Med* 2009 December;6(4):523-30.

Abstract: We previously reported that catechins of green tea have different antiproliferative effects on cell lines derived from gender-dependent cancers; epicatechin 3-gallate (ECG) had the strongest inhibitory effect. In the present study, we examined the effects of epigallocatechin (EGC), epicatechin-gallate (ECG) and EGC 3-gallate (EGCG) on the viability, density, doubling time and cycle number of cell lines derived from melanoma metastasized to lymph nodes (MB-1133 and SE-0154) or distant organs (CH-0356, JK-0346, SA-1171, GE-0208, NS-1176 and LF-0023). These catechins have been documented to have no growth suppressive or apoptotic effects on normal melanocytes (Nihal et al., *Int J Cancer* 2005;114:513-21). EGCG (50 muM) showed greater inhibitory potency than EGC (50 muM) in SE-0154, NS-1176, GE-0208 and LF-0023 cell lines but the two catechins produced similar inhibitory effects in CH-0356, JK-0346 and SA-1171 cell lines. The IC(50) (50% inhibitory concentration) was lower for EGC than EGCG in MB-1133 and CH-0356 cells, higher for EGC than EGCG in GE-0208 cells and comparable (11-12 muM) for both the catechins in LF-0023 cells. When compared with EGC, the cytotoxic effect (% dead cell counts) and the suppression of the growth (change in cell number) of all melanoma cell lines tested were pronounced with EGCG. This investigation validates the hypothesis that anticancer action of the various catechins may vary with the type of malignancy and provides a model for tumor cell heterogeneity based on susceptibility and resistance of tumor cells to different green tea catechins. Therefore, this information is critical for undertaking chemopreventive or chemotherapeutic trials against melanoma and gender-based cancers

Michael A, Hedayati B, Dalglish AG. Disease regression in malignant melanoma: spontaneous resolution or a result of treatment with antioxidants, green tea, and pineapple cores? A case report. *Integr Cancer Ther* 2007 March;6(1):77-9.

Roomi MW, Roomi N, Ivanov V, Kalinovsky T, Niedzwiecki A, Rath M. Inhibition of pulmonary metastasis of melanoma b16fo cells in C57BL/6 mice by a nutrient mixture consisting of ascorbic Acid, lysine, proline, arginine, and green tea extract. *Exp Lung Res* 2006 November;32(10):517-30.

Abstract: The authors investigated the effect of a nutrient mixture (NM) on lung metastasis by B16F0 melanoma cells in C57BL/6 female mice. Mice were divided into equal groups (1 to 6) and injected via tail vein with B16F0 cells (groups 1 to 4), B16FO cells pretreated with NM (group 5), or saline (group 6). Groups 1, 3, 4, 5, and 6 were fed the control diet and group 2 the 0.5% NM supplemented diet. Groups 3 and 4 received NM intraperitoneally (IP) and intravenously (IV), respectively. Two weeks later, pulmonary metastatic colonies were counted. Pulmonary colonization was reduced by 63% in mice supplemented with NM diet, by 86% in mice receiving NM by IP and IV injections, and completely inhibited in mice injected with melanoma cells pretreated with NM. These results show that NM is effective in inhibiting the metastasis of B16FO melanoma cells

Kang TH, Lee JH, Song CK et al. Epigallocatechin-3-gallate enhances CD8+ T cell-mediated antitumor immunity induced by DNA vaccination. *Cancer Res* 2007 January 15;67(2):802-11.

Abstract: Immunotherapy and chemotherapy are generally effective against small tumors in animal models of cancer. However, these treatment regimens are generally ineffective against large, bulky tumors. We have found that a multimodality treatment regimen using DNA vaccination in combination with chemotherapeutic agent epigallocatechin-3-gallate (EGCG), a compound found in green tea, is effective in inhibiting large tumor growth. EGCG was found to induce tumor cellular apoptosis in a dose-dependent manner. The combination of EGCG and DNA vaccination led to an enhanced tumor-specific T-cell immune response and enhanced antitumor effects, resulting in a higher cure rate than either immunotherapy or EGCG alone. In addition, combined DNA vaccination and oral EGCG treatment provided long-term antitumor protection in cured mice. Cured animals rejected a challenge of E7-expressing tumors, such as TC-1 and B16E7, but not a challenge of B16 7 weeks after the combined treatment, showing antigen-specific immune responses. These results suggest that multimodality treatment strategies, such as combining immunotherapy with a tumor-killing cancer drug, may be a more effective anticancer strategy than single-modality treatments

Nihal M, Ahmad N, Mukhtar H, Wood GS. Anti-proliferative and proapoptotic effects of (-)-epigallocatechin-3-gallate on human melanoma: possible implications for the chemoprevention of melanoma. *Int J Cancer* 2005 April 20;114(4):513-21.

Abstract: Melanoma accounts for only about 4% of all skin cancer cases but most of skin cancer-related deaths. Standard systemic therapies such as interferon (IFN) have not been adequately effective in the management of melanoma. Therefore, novel approaches are needed for prevention and treatment of this disease. Chemoprevention by naturally occurring agents present in food and beverages has shown benefits in certain cancers including nonmelanoma skin cancers. Here, employing 2 human melanoma cell lines (A-375 amelanotic malignant melanoma and Hs-294T metastatic melanoma) and normal human epidermal melanocytes (NHEM), we studied the antiproliferative effects of epigallocatechin-3-gallate (EGCG), the major polyphenolic antioxidant present in green tea. EGCG treatment was found to result in a dose-dependent decrease in the viability and growth of both melanoma cell lines. Interestingly, at similar EGCG concentrations, the normal melanocytes were not affected. EGCG treatment of the melanoma cell lines resulted in decreased cell proliferation (as assessed by Ki-67 and PCNA protein

levels) and induction of apoptosis (as assessed cleavage of PARP, TUNEL assay and JC-1 assay). EGCG also significantly inhibited the colony formation ability of the melanoma cells studied. EGCG treatment of melanoma cells resulted in a downmodulation of anti-apoptotic protein Bcl2, upregulation of proapoptotic Bax and activation of caspases -3, -7 and -9. Furthermore, our data demonstrated that EGCG treatment resulted in a significant, dose-dependent decrease in cyclin D1 and cdk2 protein levels and induction of cyclin kinase inhibitors (ckis) p16INK4a, p21WAF1/CIP1 and p27KIP1. Our data suggest that EGCG causes significant induction of cell cycle arrest and apoptosis of melanoma cells that is mediated via modulations in the cki-cyclin-cdk network and Bcl2 family proteins. Thus, EGCG, alone or in conjunction with current therapies, could be useful for the management of melanoma

Liu JD, Chen SH, Lin CL, Tsai SH, Liang YC. Inhibition of melanoma growth and metastasis by combination with (-)-epigallocatechin-3-gallate and dacarbazine in mice. *J Cell Biochem* 2001;83(4):631-42.

Abstract: (-)-Epigallocatechin-3-gallate (EGCG), a major polyphenol in green tea, was shown to have cancer chemopreventive activity. In this study, we examined the antimetastatic effects of EGCG or the combination of EGCG and dacarbazine on B16-F3m melanoma cells in vitro and in vivo. First, the antimetastatic potentials of five green tea catechins were examined by soft agar colony formation assay, and the results show that EGCG was more effective than the other catechins in inhibiting soft agar colony formation. Second, EGCG dose-dependently inhibited B16-F3m cell migration and invasion by in vitro Transwell assay. Third, EGCG significantly inhibited the spread of B16-F3m cells on fibronectin, laminin, collagen, and Matrigel in a dose-dependent manner. In addition, EGCG significantly inhibited the tyrosine phosphorylation of focal adhesion kinase (FAK) and the activity of matrix metalloproteinase-9 (MMP-9). In animal experiments, EGCG alone reduced lung metastases in mice bearing B16-F3m melanomas. However, a combination of EGCG and dacarbazine was more effective than EGCG alone in reducing the number of pulmonary metastases and primary tumor growths, and increased the survival rate of melanoma-bearing mice. These results demonstrate that combination treatment with EGCG and dacarbazine strongly inhibits melanoma growth and metastasis, and the action mechanisms of EGCG are associated with the inhibition of cell spreading, cell-extracellular matrix and cell-cell interactions, MMP-9 and FAK activities

Taniguchi S, Fujiki H, Kobayashi H et al. Effect of (-)-epigallocatechin gallate, the main constituent of green tea, on lung metastasis with mouse B16 melanoma cell lines. *Cancer Lett* 1992 July 31;65(1):51-4.

Abstract: (-)-Epigallocatechin gallate (EGCG), the main polyphenolic constituent of green tea, inhibits tumor promotion and chemical carcinogenesis in animal experimental systems. Here we report that the peroral administration of EGCG inhibited metastasis of B16 melanoma cell lines, such as B16-F10 and BL6, in both experimental and spontaneous systems

Suzuki Y, Isemura M. Inhibitory effect of epigallocatechin gallate on adhesion of murine melanoma cells to laminin. *Cancer Lett* 2001 November 8;173(1):15-20.

Abstract: We examined the effects of five kinds of green tea catechin on the adhesion of mouse melanoma B16 cells to laminin. (-)-Epigallocatechin gallate (EGCG) and (-)-epicatechin gallate in the culture medium were found to inhibit the cell adhesion. The adhesion to laminin pre-treated with EGCG was also impaired. Affinity chromatography revealed the binding affinity between laminin and EGCG. These data suggest that the inhibitory effect of EGCG on adhesion of melanoma cells to laminin is included in the mechanism(s) of previously reported metastasis inhibition elicited by EGCG and green tea infusion

## Melatonin

Cabrera J, Negrin G, Estevez F, Loro J, Reiter RJ, Quintana J. Melatonin decreases cell proliferation and induces melanogenesis in human melanoma SK-MEL-1 cells. *J Pineal Res* 2010 August;49(1):45-54.

Abstract: Melatonin is an indoleamine synthesized in the pineal gland, and after its release into the blood, it has an extensive repertoire of biological activities, including antitumoral properties. In this study, we found that melatonin reduced the growth of the human melanoma cells SK-MEL-1. The antiproliferative effect was associated with an alteration in the progression of the phases of the cell cycle and also with an increase in tyrosinase activity, the key regulatory enzyme of melanogenesis. Antagonists for melatonin membrane receptors (luzindole and 4-P-PDOT) and the general G-coupled receptor inhibitor, pertussis toxin, did not prevent the melatonin-induced cell growth arrest; this suggests a mechanism independent of G-coupled membrane receptors. In contrast, p38 mitogen-activated protein kinase (p38 MAPK) signaling pathway seems to play a significant role in cell growth inhibition by melatonin. The indoleamine-induced phosphorylation of p38 MAPK and the effect on cell proliferation were abrogated by the specific inhibitor SB203580. Furthermore, comparative studies with known antioxidants such as N-acetyl-l-cysteine and trolox indicate that the growth of SK-MEL-1 cells is highly sensitive to antioxidants

Danielczyk K, Dziegiel P. The expression of MT1 melatonin receptor and Ki-67 antigen in melanoma malignum. *Anticancer Res* 2009 October;29(10):3887-95.

Abstract: BACKGROUND: Melatonin, the principal hormone produced by the pineal gland, manifests strong potency of inhibiting growth of dermal melanoma cells both under in vitro and in vivo conditions. Although the mechanism of the phenomenon has not been fully clarified yet, melatonin receptors seem to play a key role in the inhibition. In humans, two main types of high affinity membrane melatonin receptors have been identified, including MT1 (Mel1a) and MT2 (Mel1b) receptors, and their expression increases efficacy of the oncostatic melatonin activity. The principal aim of this study involved determination of location and intensity of expression of MT1 melatonin receptors and of Ki-67 proliferation-associated antigen in dermal melanoma using an immunohistochemical technique and an examination of their reciprocal correlation and their relationship with clinical advancement of the tumour, i.e. with its depth of infiltration. PATIENTS AND METHODS: Immunohistochemical studies were conducted on the material of 48 cases of dermal melanoma, including 38 primary tumours and 10 metastatic lymph nodes, fixed in formalin and embedded in paraffin. RESULTS: In all the examined cases, positive immunohistochemical reactions were obtained with antibodies to MT1 and Ki-67. Expression of MT1 receptor was more pronounced in primary tumours than in lymph nodes ( $p < 0.05$ ). Depth of tumour infiltration demonstrated a moderate positive correlation with the intensity of MT1 expression ( $r = 0.45$ ;  $p < 0.05$ ) and a strongly positive correlation with the expression of Ki-67 antigen ( $r = 0.79$ ;  $p < 0.05$ ). Moreover, both in primary tumours and in metastatic lymph nodes, a weak correlation was detected between the expression of MT1 receptor and expression of Ki-67 antigen. CONCLUSION: Confirmation of positive correlation between the expression of MT1 receptor and depth of melanoma infiltration may point to future use of MT1 as a prognostic index for such tumours

Otalora BB, Madrid JA, Alvarez N, Vicente V, Rol MA. Effects of exogenous melatonin and circadian synchronization on tumor progression in melanoma-bearing C57BL6 mice. *J Pineal Res* 2008 April;44(3):307-15.

Abstract: Circadian rhythmicity impairment reportedly becomes significant as a tumor progresses, while the incidence of cancer can be affected by disruption of the circadian system. Melatonin has oncostatic effects on several types of cancer (breast, prostate, and colorectal cancers), while it can be self-defeating in others, such as lymphoma. Melanoma is one of the most aggressive cancers in humans; however, it seems to respond positively to melatonin in vitro. The present work tested whether body temperature (BT)

rhythms are impaired by tumor progression, and whether exogenous melatonin restricts tumor growth and restores circadian rhythmicity; therefore, enhancing survival. To this end, C57 mice were intraperitoneal implanted with a temperature data logger and subcutaneously inoculated with melanoma cells. Animals were then submitted to light-dark (LD) 12:12 cycles or continuous light (LL), with or without melatonin administration. Under LD light conditions, the BT rhythm exhibited a marked reduction in the first circadian harmonic amplitude, and increased phase instability (Rayleigh vector) as the tumor progressed. Melatonin administration (2 mg/kg BW/day), on the other hand, increased the BT rhythm amplitude and phase stability, reduced tumor weight and prevented intraperitoneal dissemination. Exposure to LL induced a free-running rhythm (1500 min), significantly increasing tumor malignancy, and therefore reducing survival. Surprisingly, the highest tumor weights and morbidity by metastasis were seen in the LL group treated with melatonin probably because this indoleamine was being administered at different subjective hours to free-running animals. Circadian rhythmicity can thus be used as a marker rhythm for tumor progression, as rhythm impairment increases along with tumor malignancy. While melatonin administration improves rhythmicity and enhances survival under LD conditions, the results are self-defeating when they coexist with circadian disruption as it occurs under LL. This emphasizes the importance of taking into account endogenous rhythmicity and limiting melatonin administration to the subjective night in order to restrict melanoma progression

Kadekaro AL, Andrade LN, Floeter-Winter LM et al. MT-1 melatonin receptor expression increases the antiproliferative effect of melatonin on S-91 murine melanoma cells. *J Pineal Res* 2004 April;36(3):204-11.

Abstract: Melatonin, a derivative of tryptophan that is present in all vertebrates, was first described in bovine pineal gland. It is known that melatonin is a highly conserved molecule, present also in unicellular organisms and plants. Several effects of melatonin have been described, including receptor- and non-receptor-mediated actions. Herein, we studied the effects of melatonin on in vitro and in vivo cell proliferation of Cloudman S-91 murine melanoma cells. We demonstrated that melatonin treatment significantly inhibits S-91 melanoma cell proliferation in vitro ( $EC_{50} = 10^{-7}$  M) as well as reduces tumor growth in vivo. We also demonstrated that melatonin directly increases the activity of the antioxidant enzymes catalase and glutathione peroxidase. These effects are most likely triggered through the direct intracellular action of melatonin, since the presence of receptors could not be demonstrated in this cell line. Expression of MT-1 melatonin receptor by stable transfection, mediated a dramatic antiproliferative melatonin effect ( $EC_{50} = 10^{-10}$  M) in S-91 cells. The expressed receptor is negatively coupled to the adenylyl cyclase/cyclic AMP signaling pathway via Gi protein. These results suggest that expression of the MT-1 melatonin receptor in melanoma cells is a potential alternative approach to specifically target cells in cancer therapeutic treatment

Yerneni LK, Jayaraman S. Pharmacological action of high doses of melatonin on B16 murine melanoma cells depends on cell number at time of exposure. *Melanoma Res* 2003 April;13(2):113-7.

Abstract: Melatonin has been reported to possess growth inhibitory action at certain physiological doses in cancer cell lines in vitro and oncostatic action under in vivo conditions. In an attempt to achieve a pharmacologically effective anticancer action of melatonin, dose-response studies with high concentrations of melatonin ( $10^{-6}$  M,  $10^{-5}$  M,  $10^{-4}$  M and  $10^{-3}$  M) were conducted in the B16 murine melanoma cell line using three different numbers of exposed cells. A range of effects, including stimulatory, oncostatic and oncocidal action, were studied 3 days after exposure to melatonin. In order to standardize the results, the concentration of melatonin per cell was calculated from the amount of melatonin added to the culture, and compared with the growth patterns of the cells. Melatonin had a mild stimulatory effect on cell proliferation at the lower end of the dose spectrum and an oncostatic influence at intermediate concentrations, while the higher concentrations per cell demonstrated clear lethal (oncocidal) action. It is suggested that by using a pharmacologically appropriate dosage regimen,

melatonin could be useful in the treatment of responsive cancers. Furthermore, calculation of the concentration of melatonin per cell is important in understanding the true pharmacological potential of melatonin

Souza AV, Visconti MA, Castrucci AM. Melatonin biological activity and binding sites in human melanoma cells. *J Pineal Res* 2003 May;34(4):242-8.

Abstract: The effects of melatonin, N-acetylserotonin and serotonin on the growth and tyrosinase activity of SK-Mel 23 and SK-Mel 28 human melanoma cell lines were investigated. Binding assays were also performed to establish the nature of the binding site. SK-Mel 28 cells were responsive to melatonin and its precursors, exhibiting a decrease in growth and an increase in tyrosinase activity after a 72 hr treatment. N-acetylserotonin was as potent as melatonin, the minimal effective concentration (MEC, which is defined as the smallest concentration that elicits a measurable biological response, significantly different from control) being  $10^{-8}$  M. Serotonin was the least potent (MEC =  $10^{-6}$  M). Both melatonin antagonists, prazosin and luzindole, exhibited no effect per se and reversed both responses to melatonin. SK-Mel 23 cells, however, showed no significant responses to the indoleamines. Competition binding assays in SK-Mel 28 cells demonstrated the presence of binding sites to 2-[ $^{125}$ I]-iodomelatonin, which was displaced by the unlabelled hormone, by both antagonists, and by N-acetylserotonin. The curve adjustment of the displacement values with melatonin suggests the existence of two binding sites, with the following  $K_i$  values:  $1.0 \times 10^{-10}$  M and  $6.5 \times 10^{-6}$  M.  $K_i$  values for acetylserotonin, prazosin and luzindole were, respectively,  $3.8 \times 10^{-8}$  M,  $1.2 \times 10^{-8}$  M, and  $8.3 \times 10^{-6}$  M. Surprisingly, in SK-Mel 23 cells, melatonin and luzindole were able to compete with the radioligand, with  $K_i$  values of  $3.1 \times 10^{-8}$  M and  $2.4 \times 10^{-8}$  M, respectively. Our data suggest that SK-Mel 28 cells probably possess high affinity binding sites to melatonin and, in addition, MT3 low affinity binding sites, because N-acetylserotonin was as effective as the native hormone, and prazosin effectively blocked the actions of melatonin. Both sites are functional as demonstrated by the blockade promoted by both luzindole and prazosin on the proliferative and melanogenic responses. Although growth and tyrosinase activity of SK-Mel 23 cells were not affected by melatonin or its precursors, this cell line possesses high affinity binding sites, which may be non-functional, or trigger responses other than the ones herein investigated

Lissoni P, Vaghi M, Ardizzioia A et al. A phase II study of chemoneuroimmunotherapy with platinum, subcutaneous low-dose interleukin-2 and the pineal neurohormone melatonin (P.I.M.) as a second-line therapy in metastatic melanoma patients progressing on dacarbazine plus interferon-alpha. *In Vivo* 2002 March;16(2):93-6.

Abstract: Immunochemotherapeutic combinations containing IL-2 theoretically represent the most effective therapies for metastatic melanoma, particularly in association with cisplatin (CDDP); however, both IL-2 and CDDP have been generally utilized at high doses, with the consequence of considerable toxicity. According to psychoneuroimmunological knowledge, the antitumor activity of IL-2 has been proven to be enhanced by the immunomodulating pineal neurohormone melatonin (MLT), which has also been shown to increase the cytotoxicity of cancer chemotherapy and reduce its toxicity. On this basis, a study was planned with low-dose IL-2 and CDDP in association with MLT as a second-line therapy for metastatic melanoma patients progressing on dacarbazine plus interferon-alpha. The study included 13 evaluable patients. CDDP was injected i.v. at 30 mg/m<sup>2</sup>/day for 3 days every 21 days. IL-2 was administered s.c. at 3 million IU/day from days 4 to 9 and from days 11 to 16 of the cycle. Finally, MLT was given orally at 20 mg/day in the evening, every day without interruption. One patient obtained a complete response (CR), while partial response (PR) was achieved in 3 other patients. Therefore, the objective tumor response-rate (CR + PR) was 4 out of 13 (31%). A stable disease occurred in 5 patients, whereas the remaining 4 patients had a progressive disease. The treatment was extremely well-tolerated in all patients and, in particular, no CDDP-related neurotoxicity was observed. The results of this preliminary study would suggest that low-dose CDDP and IL-2 in association with the pineal hormone

MLT (P.I.M. schedule), given as a second line therapy, is an effective and well-tolerated treatment for metastatic melanoma, with a clinical efficacy at least comparable to that obtained with a first-line therapy of dacarbazine plus interferon-alpha

Cos S, Garcia-Bolado A, Sanchez-Barcelo EJ. Direct antiproliferative effects of melatonin on two metastatic cell sublines of mouse melanoma (B16BL6 and PG19). *Melanoma Res* 2001 April;11(2):197-201.

Abstract: The effects of melatonin on the growth of two highly tumorigenic rodent melanoma cells were studied in vitro. PG19, an amelanotic mouse melanoma cell line, and B16BL6, a melanotic melanoma cell line selected for its invasive potential in vitro, were cultured in the presence of different concentrations of melatonin (10 microM to 0.1 pM). Five days later, viable cells were determined in a haemocytometer by the trypan blue exclusion test. Melatonin at concentrations of 1 nM and 10 pM (within the range of concentrations that correspond to physiological night-time and daytime levels in human blood) significantly inhibited proliferation in both melanoma cell lines. Subphysiological (0.1 pM) or supraphysiological (10 microM to 100 nM) concentrations of melatonin lacked this effect. These results support the hypothesis that, at physiological concentrations, melatonin exerts a direct inhibitory effect on PG19 and B16BL6 cells proliferation

Hu DN, McCormick SA, Roberts JE. Effects of melatonin, its precursors and derivatives on the growth of cultured human uveal melanoma cells. *Melanoma Res* 1998 June;8(3):205-10.

Abstract: The effects of melatonin, its precursors and derivatives on the growth of cultured human uveal melanoma cells were studied. The melanoma cells were plated into 24-well plates. Melatonin, its 6-hydroxy or 6-chloro derivative, serotonin, tryptophan or kynurenine was added to the medium in concentrations of 0.001 to 1000 nM. After 5 days the cells were detached, counted, and compared with the controls. Melatonin inhibited the growth of uveal melanoma cell lines in a dose-dependent manner (0.1-10 nM). This growth inhibition occurred at concentrations of melatonin (2 nM) found in human aqueous humour. The melatonin derivatives also inhibited the growth of uveal melanoma cells; 6-chloromelatonin was more potent than melatonin and 6-hydroxymelatonin was the least active (6-chloromelatonin > melatonin > 6-hydroxymelatonin). The precursors of melatonin (tryptophan and serotonin) and the abnormal metabolite of tryptophan (kynurenine) did not inhibit the growth of the melanoma cells, indicating that changes to the metabolic processes of melatonin may play a role in the pathogenesis of uveal melanoma

Lissoni P, Brivio O, Brivio F et al. Adjuvant therapy with the pineal hormone melatonin in patients with lymph node relapse due to malignant melanoma. *J Pineal Res* 1996 November;21(4):239-42.

Abstract: Several experimental studies have shown that melatonin has an oncostatic action, either by stimulating host antitumor immune defenses or by directly inhibiting the growth of some cancer histotypes, including melanoma. Our previous clinical studies demonstrated that melatonin may induce stabilization of the disease in untreatable metastatic solid tumor patients, and these results have been confirmed by others, at least in patients with metastatic melanoma. On the contrary, at present there are no data related to the possible efficacy of melatonin as an adjuvant endocrine therapy. This study was performed to investigate the impact of melatonin therapy on the disease-free survival (DFS) in melanoma patients surgically treated for regional node recurrence. The study included 30 node-relapsed melanoma patients, who were randomized to receive no treatment or adjuvant therapy of melatonin (20 mg/day orally in the evening) every day until disease progression. After a median follow up of 31 months, the percent of DFS was significantly higher in melatonin-treated individuals than in controls. The DFS curve was also significantly longer in melatonin group than in controls. No melatonin-related toxicity was

observed. This preliminary study suggests that an adjuvant endocrine therapy with melatonin may be effective in preventing disease progression in node-relapsed melanoma patients

Lissoni P, Paolorossi F, Tancini G et al. A phase II study of tamoxifen plus melatonin in metastatic solid tumour patients. *Br J Cancer* 1996 November;74(9):1466-8.

Abstract: Preliminary data would suggest that the pineal hormone, melatonin (MLT), may enhance tamoxifen (TMX) anti-tumour efficacy. Both MLT and TMX have been used as single agents in the palliative treatment of metastatic neoplasms, other than the classical hormone-dependent tumours, without, however, any clear efficacy. On this basis, a phase II study with TMX plus MLT has been performed in untreatable metastatic solid tumour patients. The study included 25 metastatic solid tumour patients other than breast cancer and prostate cancer (six unknown primary tumour; four melanoma; four uterine cervix carcinoma; five pancreatic cancer; three hepatocarcinoma; two ovarian cancer; one non-small-cell lung cancer), for whom no other effective standard therapy was available, because of poor clinical conditions, no response to previous chemotherapies and/or chemotherapy-resistant tumours. Both drugs were given orally every day until disease progression (TMX, 20 mg day<sup>-1</sup> at noon; MLT, 20 mg day<sup>-1</sup> in the evening). Three patients had a partial response (PR) (12%; 95% confidence limits 2-24%) (one cervix carcinoma; one melanoma; one unknown primary tumour). A stable disease (SD) was achieved in 13 other patients, whereas the remaining nine patients progressed. Performance status (PS) improved in 9/25 patients, whose median score increased from 50% to 70%. Finally, a survival longer than 1 year was observed in 7/25 (28%) patients. This phase II study would suggest that the neuroendocrine combination with TMX plus MLT may have some benefit in untreatable metastatic solid tumour patients, either in controlling cancer cell proliferation or improving the PS

Ying SW, Niles LP, Crocker C. Human malignant melanoma cells express high-affinity receptors for melatonin: antiproliferative effects of melatonin and 6-chloromelatonin. *Eur J Pharmacol* 1993 July 15;246(2):89-96.

Abstract: In order to explore the potential oncostatic properties of the pineal hormone, melatonin, we have investigated its binding characteristics and functional effects in a human malignant melanoma (M-6) cell line. Binding studies in M-6 membranes showed the coexistence of 2-[125I]iodomelatonin binding sites with picomolar and nanomolar affinities. Guanine nucleotides caused conversion of all high-affinity sites to a low-affinity state without a change in binding capacity. Melatonin induced a marked concentration-dependent reduction in forskolin-stimulated cAMP accumulation in intact M-6 cells, indicating that it binds to a functional receptor in this cell line. The in vitro proliferation of M-6 cells was significantly inhibited by melatonin and its analogues 6-chloromelatonin, and 2-iodomelatonin, at concentrations ranging from 10<sup>-9</sup> to 10<sup>-4</sup> M, as demonstrated by cell counts and measurements of DNA content. These findings indicate that M-6 cells express functional receptors for melatonin which may be involved in mediating the antiproliferative effects of this hormone

Slominski A, Pruski D. Melatonin inhibits proliferation and melanogenesis in rodent melanoma cells. *Exp Cell Res* 1993 June;206(2):189-94.

Abstract: The effects of melatonin on proliferation and on the induction of melanogenesis in rodent melanoma cells were investigated. It was found that melatonin at low concentrations (0.1-10 nM) inhibited cell growth but had no effect on melanogenesis, while at high concentrations (> or = 0.1 microM) it inhibited the induction of melanogenesis but not cell growth. These effects were specific since corresponding concentrations of the direct precursor and product of melatonin degradation N-acetylserotonin (N-Ac-5HT) and 5-methoxytryptamine (5MT), respectively, did not have any effect on cell proliferation or melanogenesis. At very high concentration (100 microM) both N-Ac-5HT and melatonin could stimulate melanoma proliferation while 5MT inhibited it. The demonstration of

differential and unparalleled effects of melatonin on cell proliferation and melanogenesis suggests that melatonin can regulate or modify both processes via different mechanisms

Gonzalez R, Sanchez A, Ferguson JA et al. Melatonin therapy of advanced human malignant melanoma. *Melanoma Res* 1991 November;1(4):237-43.

Abstract: We undertook a study to investigate the therapeutic potential of orally administered melatonin in patients with advanced melanoma. Forty-two patients received melatonin in doses ranging from 5 mg/m<sup>2</sup>/day to 700 mg/m<sup>2</sup>/day in four divided doses. Two were excluded from analysis. After a median follow-up of 5 weeks, six patients had partial responses, six additional patients had stable disease. Sites of response included the central nervous system, subcutaneous tissue and lung. The median response duration was 33 weeks for the partial responders. There was a suggestion of a dose-response relationship. The toxicity encountered was minimal and consisted primarily of fatigue in 17 of 40 patients. Melatonin also appeared to reduce basal levels of follicle-stimulating hormone (FSH). No significant changes were encountered in serum levels of luteinizing hormone (LH) or thyroid stimulating hormone (TSH). We conclude that further study of melatonin as a potentially useful agent in metastatic melanoma is warranted

Narita T, Kudo H. Effect of melatonin on B16 melanoma growth in athymic mice. *Cancer Res* 1985 September;45(9):4175-7.

Abstract: The effect of melatonin on the growth of B16 mice melanoma was examined. Male and female BALB/c athymic mice, inoculated with 7 X 10<sup>4</sup> melanoma cells, were given drinking water containing melatonin (5 micrograms/g body weight/day) and 0.5% ethanol. Compared to control animals the melatonin treated male and female athymic mice had significantly smaller tumors on Day 40. The weights of the testes, the ovaries, and the adrenal glands of melatonin treated mice were significantly reduced compared to control animals. These data indicate that melatonin p.o. significantly inhibited the growth of B16 mouse melanoma and that the antitumor effect of melatonin was associated with a significant decrease in gonadal and adrenal weights

El-Domeiri AA, Das Gupta TK. The influence of pineal ablation and administration of melatonin on growth and spread of hamster melanoma. *J Surg Oncol* 1976;8(3):197-205.

Abstract: Removal of the pineal organ resulted in increase in the growth of transplanted melanoma in hamsters. Administration of melatonin, which is the principle indole found in pineal tissue, to pinealectomized animals abolished this effect. Therefore it was concluded that the effect of pinealectomy on tumor growth is due to a lack of endogenous melatonin. Administration of large doses of melatonin (4 mg/day) to intact animals, however, did not influence the rate of tumour growth, indicating that the drug has no direct effect on tumor growth and that the changes produced by melatonin deficiency is perhaps due to complex reaction involving centers in the hypothalamus and brain stem. Certain morphologic changes were noted in the pineal organs of animals treated with exogenous melatonin. Although the biologic significance of these changes is not clear, study of the electron micrographs indicates that the pinealocytes of the melatonin-treated animals are in a state of increased activity

## **Vitamin D**

Brozyna AA, Jozwicki W, Janjetovic Z, Slominski AT. Expression of the vitamin D-activating enzyme 1alpha-hydroxylase (CYP27B1) decreases during melanoma progression. *Hum Pathol* 2012 September 17.

Abstract: 1 $\alpha$ -Hydroxylase (CYP27B1), the enzyme responsible for the synthesis of the biologically active form of vitamin D (1,25(OH)(2)D(3)), is expressed in the skin. To assess the correlation between progression of melanocytic tumors and CYP27B1, we analyzed its expression in 29 benign nevi, 75 primary cutaneous melanomas, 40 metastases, and 4 re-excision and 6 normal skin biopsies. Immunoreactivity for CYP27B1 was significantly lower in the vertical growth phase and metastatic melanomas (0.6 and 0.5 arbitrary units, respectively) in comparison with nevi and radial growth phase tumors (1.2 and 1.1 arbitrary units, respectively); and expression was reduced in more advanced lesions (Clark levels III-V, Breslow thickness  $\geq$ 2.1 mm; 0.8 and 0.7 arbitrary units, respectively). There was an inverse correlation between CYP27B1 and Ki-67 expression. Furthermore, CYP27B1 expression was reduced in primary melanomas that created metastases in comparison with non-metastasizing melanomas. Reduced CYP27B1 expression in radial growth phase was related to shorter overall survival (810 versus 982 versus 1151 days in melanomas with absent, low, and high CYP27B1 immunoreactivity), and low CYP27B1 expression in radial growth phase and vertical growth phase was related to shorter disease-free survival (114 versus 339 versus 737 days and 129 versus 307 versus 737 days, respectively, in melanomas with absent, low, and high CYP27B1). Also, CYP27B1 expression was inversely related to melanin in melanoma cells in vivo and melanoma cells cultured in vitro. Thus, reduction of CYP27B1 correlates with melanoma phenotype and behavior, and its lack affects the survival of melanoma patients, indicating a role in the pathogenesis and progression of this cancer

Janjetovic Z, Brozyna AA, Tuckey RC et al. High basal NF-kappaB activity in nonpigmented melanoma cells is associated with an enhanced sensitivity to vitamin D3 derivatives. *Br J Cancer* 2011 December 6;105(12):1874-84.

Abstract: BACKGROUND: Melanoma is highly resistant to current modalities of therapy, with the extent of pigmentation playing an important role in therapeutic resistance. Nuclear factor-kappaB (NF-kappaB) is constitutively activated in melanoma and can serve as a molecular target for cancer therapy and steroid/secosteroid action. METHODS: Cultured melanoma cells were used for mechanistic studies on NF-kappaB activity, utilising immunofluorescence, western blotting, EMSA, ELISA, gene reporter, and estimated DNA synthesis assays. Formalin-fixed, paraffin-embedded specimens from melanoma patients were used for immunocytochemical analysis of NF-kappaB activity in situ. RESULTS: Novel 20-hydroxyvitamin (20(OH)D(3)) and classical 1 $\alpha$ ,25-dihydroxyvitamin D(3) (1,25(OH)(2)D(3)) secosteroids inhibited melanoma cell proliferation. Active forms of vitamin D were found to inhibit NF-kappaB activity in nonpigmented cells, while having no effect on pigmented cells. Treatment of nonpigmented cells with vitamin D3 derivatives inhibited NF-kappaB DNA binding and NF-kappaB-dependent reporter assays, as well as inhibited the nuclear translocation of the p65 NF-kappaB subunit and its accumulation in the cytoplasm. Moreover, analysis of biopsies of melanoma patients showed that nonpigmented and slightly pigmented melanomas displayed higher nuclear NF-kappaB p65 expression than highly pigmented melanomas. CONCLUSION: Classical 1,25(OH)(2)D(3) and novel 20(OH)D(3) hydroxyderivatives of vitamin D3 can target NF-kappaB and regulate melanoma progression in nonpigmented melanoma cells. Melanin pigmentation is associated with the resistance of melanomas to 20(OH)D(3) and 1,25(OH)(2)D(3) treatment

Lee JH, Park S, Cheon S et al. 1,25-Dihydroxyvitamin D(3) enhances NK susceptibility of human melanoma cells via Hsp60-mediated FAS expression. *Eur J Immunol* 2011 October;41(10):2937-46.

Abstract: The active metabolite of vitamin D(3), 1 $\alpha$ ,25(OH)(2)D(3), displays anticancer effects by regulating cell cycle and apoptosis in many cancer cells. However, it has not been determined whether 1 $\alpha$ ,25(OH)(2)D(3) increases the susceptibility of cancer cells to NK cells. Here, we investigated the anticancer effect of 1 $\alpha$ ,25(OH)(2)D(3) in human melanoma cell lines by investigating enhancement of NK susceptibility and elucidating the mediator of NK cytotoxicity. 1 $\alpha$ ,25(OH)(2)D(3)-resistant melanoma cells (G-361 and SK-MEL-5) treated with 1 $\alpha$ ,25(OH)(2)D(3) showed higher susceptibility

to NK cells with up-regulation of Fas expression. Furthermore, G-361 cells treated with 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> showed significantly increased caspase activity. In addition to Fas up-regulation, expression of heat shock protein 60 (Hsp60) was elevated by 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>. Increased expression of Hsp60 by 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> was related to not only up-regulation of Fas expression but also to NK susceptibility of G-361 cells. Taken together, our data suggest that 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> acts as an anticancer agent by increasing expression of Fas on the surface of melanoma cells through Hsp60 induction and strengthens caspase sensitivity to Fas-mediated apoptotic pathway by NK cells. 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> treatment may therefore have a preventive role in melanoma occurrence or potentiate the anticancer effects of NK-cell immune therapy

Field S, Newton-Bishop JA. Melanoma and vitamin D. *Mol Oncol* 2011 April;5(2):197-214.

Abstract: Vitamin D is a fat-soluble steroid hormone, which is essential to health and for which epidemiological studies suggest a role in autoimmune disease, infections, cardiovascular disease and cancer. It is ingested in foods such as oily fish and supplements, so that average levels vary between countries, but most individuals worldwide make most of their vitamin D as a result of the effects of sun exposure on the skin. Many studies in different populations around the world have in recent years shown that sub-optimal levels of vitamin D (<70 nmol/L) are common. A series of epidemiological studies have suggested that low vitamin D levels increase the risk of cancers, particularly of the breast and gastrointestinal tracts, so that there has been much interest in understanding the effects of vitamin D on cancer cells. Vitamin D binds to the vitamin D receptor (VDR) resulting in transcription of a number of genes playing a role in inhibition of MAPK signalling, induction of apoptosis and cell-cycle inhibition, and therefore vitamin D has anti-proliferative and pro-apoptotic effects in cells of many lineages. It also has suppressive effects on adaptive immunity and is reported to promote innate immunity. Here we review data on vitamin D and melanoma. There are in vitro data, which suggest that vitamin D has the same anti-proliferative effects on melanoma cells as have been demonstrated in other cells. We have reported data to suggest that vitamin D levels at diagnosis have a role in determining outcome for melanoma patients. There is a curious relationship between melanoma risk and sun exposure where sunburn is causal but occupational sun exposure is not (at least in temperate climes). Seeking to understand this, we discuss data, which suggest (but by no means prove) that vitamin D might also have a role in susceptibility to melanoma. In conclusion, much remains unknown about vitamin D in general and certainly about vitamin D and melanoma. However, the effects of avoidance of suboptimal vitamin D levels on cancer cell proliferation are likely to be beneficial to the melanoma patient. The possible results of high vitamin D levels on the immune system remain unclear however and a source of some concern, but the data support the view that serum levels in the range 70-100 nmol/L might be a reasonable target for melanoma patients as much as for other members of the population

Brozyna AA, Jozwicki W, Janjetovic Z, Slominski AT. Expression of vitamin D receptor decreases during progression of pigmented skin lesions. *Hum Pathol* 2011 May;42(5):618-31.

Abstract: 1,25-dihydroxyvitamin D<sub>3</sub> affects proliferation, differentiation, and apoptosis and protects DNA against oxidative damage with a net tumorostatic and anticarcinogenic effect. It acts through a specific nuclear receptor that is widely distributed through the body. Although a beneficial role of vitamin D in melanoma patients has been suggested, there is lack of information on the changes in the expression pattern of vitamin D receptor during progression of pigmented lesions. Using immunohistochemistry, we analyzed the expression of vitamin D receptor in 140 samples obtained from 82 patients, including 25 benign nevi, 70 primary cutaneous melanomas, 35 metastases, 5 re-excisions, and 5 normal skin biopsies. The strongest expression was observed in normal skin that significantly decreased in melanocytic proliferations with the following order of expression: normal skin > melanocytic nevi > melanomas = metastases. The vitamin D receptor expression in skin surrounding nevi and melanoma was also significantly reduced as compared to normal skin. Tumor-infiltrating and lymph node lymphocytes

retained high levels of vitamin D receptor. There was negative correlation between tumor progression and vitamin D receptor expression with a remarkable decrease of the immunoreactivity in nuclei of melanoma cells at vertical versus radial growth phases and with metastatic melanomas showing the lowest cytoplasmic receptor staining. Furthermore, lack of the receptor expression in primary melanomas and metastases was related to shorter overall patients' survival. In addition, the receptor expression decreased in melanized melanoma cells in comparison to amelanotic or poorly pigmented cells. Therefore, we propose that reduction or absence of vitamin D receptor is linked to progression of melanocytic lesions, that its lack affects survival of melanoma patients, and that melanogenesis can attenuate receptor expression. In conclusion, changes in vitamin D receptor expression pattern can serve as important variables for diagnosis, predicting clinical outcome of the disease, and/or as a guidance for novel therapy of melanomas based on use of vitamin D or its derivatives

Pinczewski J, Slominski A. The potential role of vitamin D in the progression of benign and malignant melanocytic neoplasms. *Exp Dermatol* 2010 October;19(10):860-4.

Abstract: Hormonally active vitamin D<sub>3</sub>, 1,25-(OH)<sub>2</sub>D<sub>3</sub>, is believed to have a role in the prevention of cancer formation and in limiting the aggressiveness of cancers that do arise. Therefore, much interest is presently being focused on 1,25-(OH)<sub>2</sub>D<sub>3</sub> and its analogues as potential treatments for various cancers including melanoma. This article discusses the evidence in favour of a role for 1,25-(OH)<sub>2</sub>D<sub>3</sub> in protection against the progression of melanocytic lesions and also summarizes the mechanisms by which 1,25-(OH)<sub>2</sub>D<sub>3</sub> may act to protect against melanoma development and progression

Hutchinson PE, Osborne JE, Pringle JH. Higher serum 25-hydroxy vitamin D<sub>3</sub> levels at presentation are associated with improved survival from melanoma, but there is no evidence that later prevailing levels are protective. *J Clin Oncol* 2010 September 20;28(27):e492-e493.

Sertznig P, Seifert M, Tilgen W, Reichrath J. Activation of vitamin D receptor (VDR)- and peroxisome proliferator-activated receptor (PPAR)-signaling pathways through 1,25(OH)<sub>2</sub>D<sub>3</sub> in melanoma cell lines and other skin-derived cell lines. *Dermatoendocrinol* 2009 July;1(4):232-8.

Abstract: We have investigated expression of vitamin D receptor (VDR) and peroxisome proliferator-activated receptors (PPAR)α, δ, γ in primary cultured normal melanocytes (NHM), melanoma cell lines (MeWo, SK-Mel-5, SK-Mel-25, SK-Mel-28), a cutaneous squamous cell carcinoma cell line (SCL-1) and an immortalized sebocyte cell line (SZ95). LNCaP prostate cancer cells, MCF-7 breast cancer cells and embryonic kidney cells (HEK-293) were used as controls. VDR and PPAR mRNA were detected, quantitated and compared in these cell lines using real-time quantitative polymerase chain reaction (RTqPCR). The expression patterns of these nuclear receptors (NRs) varied strongly between the different cell lines according to their origin. PPARδ and PPARγ were less strongly expressed in the melanoma cell lines and in the other skin-derived cell lines as compared to the control cell lines. PPARα and VDR were stronger expressed in the 1,25(OH)<sub>2</sub>D<sub>3</sub>-sensitive melanoma cells (MeWo and in SK-Mel-28) than in the 1,25(OH)<sub>2</sub>D<sub>3</sub>-resistant melanoma cell lines (SK-Mel-5 and SK-Mel-25) or in NHM. Interestingly, VDR expression was increased by the treatment with 1,25(OH)<sub>2</sub>D<sub>3</sub> in 1,25(OH)<sub>2</sub>D<sub>3</sub>-sensitive melanoma cells but not in 1,25(OH)<sub>2</sub>D<sub>3</sub>-resistant melanoma cell lines. 1,25(OH)<sub>2</sub>D<sub>3</sub> increased the expression of PPARα in almost all cell lines analyzed. Our results indicate a cross-talk between VDR- and PPAR-signaling pathways in various cell types including melanoma cells. Further investigations are required to investigate the physiological and pathophysiological relevance of this cross-talk. Because VDR and PPAR-signaling pathways regulate a multitude of genes that are of importance for a multitude of cellular functions including cell proliferation, cell differentiation, immune responses and apoptosis, the provided link between VDR and PPAR may open important new perspectives for treatment and prevention of melanoma and other diseases

Sertznig P, Seifert M, Tilgen W, Reichrath J. Peroxisome proliferator-activated receptor (PPAR) and vitamin D receptor (VDR) signaling pathways in melanoma cells: promising new therapeutic targets? *J Steroid Biochem Mol Biol* 2010 July;121(1-2):383-6.

Abstract: Peroxisome proliferator-activated receptor (PPAR) and vitamin D receptor (VDR) signaling pathways regulate a multitude of genes that are of importance for a multitude of cellular functions including cell proliferation, cell differentiation, immune responses and apoptosis. Ligands and other agents influencing the PPAR and VDR signaling pathways have been shown to reveal chemopreventive potential by mediating tumor suppressive activities in a variety of human cancers. Use of these compounds may represent a potential novel strategy to prevent melanoma pathogenesis and to inhibit melanoma progression. We recently showed that 1,25-dihydroxyvitamin D3 and some of the investigated PPAR ligands inhibited proliferation of the human melanoma cell line MeWo. In addition to this, our results gave an indication of an interconnection of the PPAR and VDR signaling pathways at the level of cross-regulation of their respective transcription factor mRNA levels. The provided link between VDR and PPAR may play an important role in treatment and prevention of melanoma. This review summarizes the currently available data on the roles of the PPARs and the VDR in pathogenesis and progression of melanoma as well as their role as promising future therapeutic targets

Essa S, Denzer N, Mahlknecht U et al. VDR microRNA expression and epigenetic silencing of vitamin D signaling in melanoma cells. *J Steroid Biochem Mol Biol* 2010 July;121(1-2):110-3.

Abstract: Malignant melanoma cells express the vitamin D receptor (VDR). However, some melanoma cell lines fail to respond to the antiproliferative effects of 1,25-dihydroxyvitamin D3 (1,25(OH)2D3). We reported previously that out of seven melanoma cell lines analyzed, three cell lines (MeWo, SK-Mel28, SM) respond to the antiproliferative effects of 1,25(OH)2D3, while the others (SK-Mel5, SK-Mel25, IGR, Meljuso) are resistant. It was the aim of this study to investigate whether epigenetic mechanisms are of importance for the abrogation of vitamin D signaling in vitamin D resistant melanoma cells. We used the histone deacetylase inhibitor (HDACI) trichostatin A (TSA) and the DNA methyltransferase inhibitor (DNMTI) 5-azacytidine (5-Aza) to elucidate the effects of protein acetylation and of DNA hypermethylation on 1,25(OH)2D3-induced effects on cell proliferation, respectively. Additionally we analyzed the expression of VDR microRNA in 1,25(OH)2D3-responding and resistant melanoma cells. TSA and 5-Aza exerted dose- and time-dependent antiproliferative effects on melanoma cell lines. Interestingly, combination therapy with 1,25(OH)2D3 and TSA exerted synergistic antiproliferative effects in a 1,25(OH)2D3-resistant melanoma cell line (IGR) ( $p < 0.05$ ). Combination therapy with 1,25(OH)2D3 and 5-Aza resulted in synergistic (MeWo after 72 h;  $p < 0.05$ ) or additive (other melanoma cell lines analyzed) antiproliferative effects. Additionally, we could show that VDR mRNA expression is relatively high in two of three 1,25(OH)2D3-responsive melanoma cells as compared to resistant cells, moreover this relatively high VDR expression is associated with low expression of miRNA125b in MeWo and SK-Mel28 cells. Our results suggest that the endogenous VDR mRNA level is inversely associated with expression of miRNA125b in melanoma cell lines analyzed. Moreover, miRNA125b may be involved in the regulation of VDR expression and in the resistance against 1,25(OH)2D3 in melanoma cells. It can be speculated whether miRNA125b may be of prognostic importance and/or may represent a therapeutic target for malignant melanoma. Drugs that influence epigenetic mechanisms might be promising therapeutics for the treatment of metastasized malignant melanoma, alone or in combination with antiproliferative or cytotoxic agents such as 1,25(OH)2D3

Newton-Bishop JA, Beswick S, Randerson-Moor J et al. Serum 25-hydroxyvitamin D3 levels are associated with breslow thickness at presentation and survival from melanoma. *J Clin Oncol* 2009 November 10;27(32):5439-44.

Abstract: PURPOSE: A cohort study was carried out to test the hypothesis that higher vitamin D levels

reduce the risk of relapse from melanoma. **METHODS:** A pilot retrospective study of 271 patients with melanoma suggested that vitamin D may protect against recurrence of melanoma. We tested these findings in a survival analysis in a cohort of 872 patients recruited to the Leeds Melanoma Cohort (median follow-up, 4.7 years). **RESULTS:** In the retrospective study, self-reports of taking vitamin D supplements were nonsignificantly correlated with a reduced risk of melanoma relapse (odds ratio = 0.6; 95% CI, 0.4 to 1.1; P = .09). Nonrelapsers had higher mean 25-hydroxyvitamin D(3) levels than relapsers (49 v 46 nmol/L; P = .3; not statistically significant). In the cohort (prospective) study, higher 25-hydroxyvitamin D(3) levels were associated with lower Breslow thickness at diagnosis (P = .002) and were independently protective of relapse and death: the hazard ratio for relapse-free survival (RFS) was 0.79 (95% CI, 0.64 to 0.96; P = .01) for a 20 nmol/L increase in serum level. There was evidence of interaction between the vitamin D receptor (VDR) BsmI genotype and serum 25-hydroxyvitamin D(3) levels on RFS. **CONCLUSION:** Results from the retrospective study were consistent with a role for vitamin D in melanoma outcome. The cohort study tests this hypothesis, providing evidence that higher 25-hydroxyvitamin D(3) levels, at diagnosis, are associated with both thinner tumors and better survival from melanoma, independent of Breslow thickness. Patients with melanoma, and those at high risk of melanoma, should seek to ensure vitamin D sufficiency. Additional studies are needed to establish optimal serum levels for patients with melanoma

Nurnberg B, Graber S, Gartner B et al. Reduced serum 25-hydroxyvitamin D levels in stage IV melanoma patients. *Anticancer Res* 2009 September;29(9):3669-74.

**Abstract: BACKGROUND:** Reduced serum 25-hydroxyvitamin D3 (25(OH)D) levels are associated with an increased incidence and an unfavorable outcome of various types of cancer. However, the influence of serum 25(OH)D on the incidence and outcome of patients with malignant melanoma is unknown. **PATIENTS AND METHODS:** The association between serum 25(OH)D levels and clinical and histopathological data among 205 patients with malignant melanoma was examined. Additionally, 141 healthy controls were investigated. All the blood samples were taken between October and April to minimize seasonal variations; basal serum 25(OH)D levels were analyzed using the LIAISON 25-OH Vitamin D-Assay (DiaSorin, Dietzenbach, Germany). The study started in 1997. The patients were observed until death or March 2007, whichever came first. **RESULTS:** Serum 25(OH)D levels were significantly reduced in stage IV melanoma patients as compared to stage I melanoma patients (p=0.006). A trend toward a greater tumor thickness of the primary cutaneous melanomas was seen in the patients with low (<10 ng/ml) serum 25(OH)D levels (median: 2.55 mm) as compared to those with 25(OH)D serum levels >20 ng/ml (median: 1.5 mm), although this difference was not statistically significant (p=0.078). The patients with low 25(OH)D serum levels (<10 ng/ml) had earlier distant metastatic disease (median: 24.37 months) as compared to those with 25(OH)D serum levels >20 ng/ml (median: 29.47 months), although this difference was also not statistically significant (p=0.641). **CONCLUSION:** Among the patients with malignant melanoma, significantly reduced serum 25(OH)D levels were found in the stage IV patients as compared to stage I patients, and those with low 25(OH)D serum levels (<10 ng/ml) may develop earlier distant metastatic disease compared to those with higher 25(OH)D serum levels (>20 ng/ml). Further study of the vitamin D pathway and its influence on pathogenesis and progression of malignant melanoma is warranted

Seifert M, Rech M, Meineke V, Tilgen W, Reichrath J. Differential biological effects of 1,25-dihydroxyVitamin D3 on melanoma cell lines in vitro. *J Steroid Biochem Mol Biol* 2004 May;89-90(1-5):375-9.

**Abstract:** 1,25-DihydroxyVitamin D(3) and analogs have been shown to inhibit proliferation and to induce differentiation in different cell types, including human melanocytes. However, various tumor cell lines that fail to respond to the antiproliferative effects of Vitamin D analogs have also been reported. Using real-time PCR (LightCycler), we have compared mRNA expression of Vitamin D receptor (VDR),

Vitamin D-25-hydroxylase (25-OHase), 25-hydroxyvitamin D-1alpha-hydroxylase (1alpha-OHase), and 1,25-dihydroxyvitamin D-24-hydroxylase (24-OHase) in a melanoma cell line that responds to antiproliferative effects of Vitamin D (MeWo) with a non-responsive melanoma cell line (SkMel5). Additionally, modulation of cell proliferation by calpain inhibitors, as well as regulation of mRNA expression of VDR, 1alpha-OHase, and 24-OHase genes by Vitamin D analogs were assessed in melanoma cell lines in vitro using a WST-1 based colorimetric assay and real-time PCR, respectively. RNA for VDR, 25-OHase, 1alpha-OHase, and 24-OHase was detected in melanoma cell lines. In contrast to SkMel5 cells, treatment of MeWo cells with calcitriol resulted in a dose-dependent increase in mRNA for VDR and 24-OHase as well as in a suppression of cell proliferation (up to approximately 50%). Our findings demonstrate that local synthesis or metabolism of Vitamin D metabolites may be of importance for growth regulation of MM and melanoma cell lines. Additionally, metastasizing MM represents a promising target for palliative treatment with new Vitamin D analogs that exert little calcemic side effects or for pharmacological modulation of calcitriol synthesis/metabolism in these tumors

Reichrath J, Rafi L, Rech M, Meineke V, Tilgen W, Seifert M. No evidence for amplification of 25-hydroxyvitamin D-1alpha-OHase (1alpha-OHase) or 1,25-dihydroxyvitamin D-24-OHase (24-OHase) genes in malignant melanoma (MM). *J Steroid Biochem Mol Biol* 2004 May;89-90(1-5):163-6.

Abstract: Increasing evidence points at an important function of Vitamin D metabolites for growth regulation in various tissues, including MM. Using array CGH, amplification of 24-OHase was recently detected as a likely target oncogene of the amplification unit 20q13.2 in breast cancer cell lines and tumors. Additionally, amplification of 1alpha-OHase has been reported in human malignant glioma. Using immunohistochemistry, we have now detected nuclear Vitamin D receptor (VDR) immunoreactivity in primary cutaneous malignant melanoma (MM), indicating that Vitamin D metabolites may be of importance for the growth regulation in these tumors. Using Southern analysis, we have analyzed MM and metastases for evidence of amplification of 1alpha-OHase or 24-OHase genes. Our results do not support the hypothesis that amplification of 1alpha-OHase or 24-OHase genes may be of importance for pathogenesis or progression of MM

Hutchinson PE, Osborne JE, Lear JT et al. Vitamin D receptor polymorphisms are associated with altered prognosis in patients with malignant melanoma. *Clin Cancer Res* 2000 February;6(2):498-504.

Abstract: Calcitriol [1,25(OH)2D3], the hormonal derivative of vitamin D3, is an antiproliferative and prodifferentiation factor for several cell types, including cultured melanocytes and malignant melanoma (MM) cells. Several polymorphisms of the vitamin D receptor (VDR) gene have been described including a FokI RFLP in exon 2, BsmI, and ApaI polymorphisms in intron 8 and an adjacent TaqI RFLP in exon 9. Alterations in vitamin D/1,25(OH)2D3 levels and polymorphisms of the VDR have been shown to be associated with several systemic malignancies. We hypothesize that polymorphism in this gene may be associated with altered susceptibility and outcome in patients with MM. A hospital-based case-control study, using 316 MM cases and 108 controls, was used to assess associations with MM susceptibility. Breslow thickness, the most important single prognostic factor in MM, was used as the outcome measure. Polymorphisms at the FokI and TaqI restriction sites were determined using PCR-based methods. Polymorphism at the FokI, but not TaqI, RFLP was associated with an altered risk of MM (P = 0.014). More importantly, variant alleles were associated with increased Breslow thickness. Thus, homozygosity for variant alleles at both RFLP (ttff genotype combination) was significantly associated with thicker tumors. (> or = 3.5 mm; P = 0.001; odds ratio = 31.5). Thus, polymorphisms of the VDR gene, which would be expected to result in impaired function, are associated with susceptibility and prognosis in MM. These data suggest that 1,25(OH)2D3, the ligand of the VDR, may have a protective influence in MM, as has been proposed for other malignancies

Yudoh K, Matsuno H, Kimura T. 1alpha,25-dihydroxyvitamin D3 inhibits in vitro invasiveness through the extracellular matrix and in vivo pulmonary metastasis of B16 mouse melanoma. *J Lab Clin Med* 1999 February;133(2):120-8.

Abstract: We investigated the role of 1alpha,25-dihydroxyvitamin D3 (1alpha,25(OH)2D3) in modulating tumor cell invasiveness through the extracellular matrix (ECM) and pulmonary metastasis in B16 mouse melanoma. The pretreatment of B16 cells for 48 hours with 1alpha,25(OH)2D3 significantly inhibited in vitro invasiveness through the ECM by a mechanism that is not directly correlated with the inhibition of cell proliferation. When cells were treated with 1alpha,25(OH)2D3 for only 8 hours during the assay, no inhibitory effect was observed, suggesting that pretreatment with the hormone for more than 8 hours is necessary to inhibit the invasive potential of B16 cells. The activity of B16 cells to adhere to reconstituted basement membrane (Matrigel) and type IV collagenolysis was inhibited by pretreatment of the cells with 1alpha,25(OH)2D3 for 48 hours. Cell motility was not influenced by the hormone. Mice were inoculated subcutaneously with  $3 \times 10^6$  B16 cells and were given 1alpha,25(OH)2D3 (0.5 microg/kg) or vehicle daily for 28 days, beginning 1 day after tumor inoculation. In the 1alpha,25(OH)2D3-treated group, no significant inhibition in exponential tumor growth, body weight, and serum level of calcium was observed until the twenty-eighth day. The mean serum concentration of the hormone was about 50 ng/mL, and there were no significant changes in its concentration during the treatment period. In both spontaneous and experimental metastasis models of tumor-bearing mice, treatment with 1alpha,25(OH)2D3 inhibited pulmonary metastasis. These findings suggest that 1alpha,25(OH)2D3 acts on B16 cells, inhibiting invasiveness through the ECM that is caused by the inhibition of cell adhesion to the ECM and the degradation of the ECM by the cells. 1alpha,25(OH)2D3 may have the potential to inhibit metastasis by a mechanism that is not exclusively based on its anti-cell proliferative effect

Evans SR, Houghton AM, Schumaker L et al. Vitamin D receptor and growth inhibition by 1,25-dihydroxyvitamin D3 in human malignant melanoma cell lines. *J Surg Res* 1996 February 15;61(1):127-33.

Abstract: The expression of vitamin D receptors (VDR) and growth inhibition induced by 1,25-dihydroxyvitamin D3 have been noted in certain human malignant melanoma cell lines. In this study, widely disparate levels of VDR mRNA expression were demonstrated in a panel of eight human malignant melanoma cell lines. Quantitation of receptor level by ligand binding assay showed a similar pattern. Proliferation and growth curve analysis was performed in two cell lines: RPMI 7951 (high VDR) and SK-MEL-28 (low VDR). Significant growth inhibition was noted in RPMI 7951 cells at  $10^{-9}$  M 1,25-dihydroxyvitamin D3. SK-MEL-28 cells, which express much lower levels of VDR, did not show any growth inhibition except at extremely high concentrations of 1,25-dihydroxyvitamin D3, namely  $10^{-5}$  M. These findings suggest a receptor-mediated mechanism of growth inhibition for 1,25-dihydroxyvitamin D3 and a role for this hormone in the growth of malignant melanoma cells

Brenner R, Shabahang M, Houghton A et al. Growth-inhibition of human-melanoma cells by vitamin-D analogs. *Oncol Rep* 1995 November;2(6):1157-62.

Abstract: The antiproliferative activity of 1,25(OH)(2)-vitamin D-3, and four vitamin D analogs was assessed in RPMI-7951, a human melanoma cell line which expresses the vitamin D receptor. Proliferation assays consisted of a [<sup>3</sup>H]-thymidine incorporation assay, and a 6-day growth study. The affinity of vitamin D analogs for vitamin D receptor relative to 125(OH)(2)-vitamin D-3 was determined with a hydroxyapatite-based competitive binding assay. For the proliferation assays, cells were treated with  $10^{-8}$  M 1,25(OH)(2)-vitamin D-3, 1,25(OH)(2)-16-ene-23-yne-vitamin D-3 (Ro 23-7553), 1,25(OH)(2)-16-ene-23-yne-26,27-hexafluoro-vitamin D-3 (Ro 24-5531), 1,25(OH)-,16,23Z-diene-26,27-hexafluoro-vitamin D-3 (Ro 25-5317), and 1 alpha-fluoro-25(OH)- 16-ene-23-yne-hexafluoro-vitamin D-3 (Ro 24-5583). 1,25(OH)(2)-vitamin D-3 and the four analogs all significantly inhibited melanoma cell

growth ( $P < 0.05$ ). Competitive binding of the vitamin D analogs to vitamin D receptor ranged from 51% to 72% that of 1,25(OH)<sub>2</sub>-vitamin D-3, suggesting a receptor-mediated response. These results demonstrate that analogs of 1,25(OH)<sub>2</sub>-vitamin D-3 are potent antiproliferative agents in human melanoma cells in vitro

Frankel TL, Mason RS, Hersey P, Murray E, Posen S. The synthesis of vitamin D metabolites by human melanoma cells. *J Clin Endocrinol Metab* 1983 September;57(3):627-31.

Abstract: Two melanin-producing human melanoma cell lines originally established from fresh surgical specimens were incubated with 25 hydroxyvitamin D<sub>3</sub> (25 OHD<sub>3</sub>). Both cell lines produced material comigrating with 1,25 dihydroxy-vitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>) and 24,25 dihydroxyvitamin D<sub>3</sub> (24,25(OH)<sub>2</sub>D<sub>3</sub>) in straight and reverse phase high performance liquid chromatography systems and displacing the relevant labeled ligands in competitive binding assays. The material designated 1,25(OH)<sub>2</sub>D<sub>3</sub> was found almost entirely within the cells, whereas 24,25(OH)<sub>2</sub>D<sub>3</sub> was evenly distributed between cells and medium. The synthesis of dihydroxylated materials was time dependent and was not observed if the cells were boiled before incubation with 25 OHD<sub>3</sub>. Preincubation with 1,25(OH)<sub>2</sub>D<sub>3</sub> caused an increase in the synthesis of 24,25(OH)<sub>2</sub>D<sub>3</sub> and a decrease in the synthesis of 1,25(OH)<sub>2</sub>D<sub>3</sub>. Michaelis-Menten constant (K<sub>m</sub>) values were  $1.4 \times 10^{-9}$  mol/liter 25 OHD<sub>3</sub> for the 1-alpha-hydroxylase enzyme and  $72 \times 10^{-9}$  mol/liter for 24-hydroxylase. These studies constitute further evidence for the extrarenal synthesis of 1,25(OH)<sub>2</sub>D<sub>3</sub>. The suppressibility of 1 alpha-hydroxylase by preincubation with 1,25(OH)<sub>2</sub>D<sub>3</sub> suggests a regulatory function for this system in the skin

Colston K, Colston MJ, Feldman D. 1,25-dihydroxyvitamin D<sub>3</sub> and malignant melanoma: the presence of receptors and inhibition of cell growth in culture. *Endocrinology* 1981 March;108(3):1083-6.

Abstract: In this study we demonstrate the presence of specific, high-affinity receptors for 1,25-dihydroxyvitamin D<sub>3</sub> in malignant melanoma. Receptors are present both in cultured melanoma cells and in melanoma tumor tissue produced by inoculation of cells into athymic rats. The receptor sediments at 3.25 on sucrose density gradients, possesses a preferential affinity for 1,25-(OH)<sub>2</sub>D<sub>3</sub> and has an apparent K<sub>d</sub> of 0.18 nM by Scatchard analysis. We also demonstrate that human melanoma cells are responsive to 1,25-(OH)<sub>2</sub>D<sub>3</sub> in vitro. Inclusion of 1,25-(OH)<sub>2</sub>D<sub>3</sub> in the culture medium produced a marked increase in cell doubling time. This inhibitory effect of the hormone on melanoma cell proliferation was dose-related and represents the first demonstration of a 1,25-(OH)<sub>2</sub>D<sub>3</sub> mediated action on tumor cells

### **Spirulina (inhibitor of NADPH oxidase)**

Calvani M, Comito G, Giannoni E, Chiarugi P. Time-dependent stabilization of hypoxia inducible factor-1alpha by different intracellular sources of reactive oxygen species. *PLoS ONE* 2012;7(10):e38388.

Abstract: Intratumoral hypoxia is a major obstacle in the development of effective cancer chemotherapy, decreasing the efficacy of anti-neoplastic drugs in several solid tumours. The hypoxic environment, through its master regulator hypoxia inducible factor-1 (HIF-1), is able to maintain an anti-apoptotic potential through activation of critical genes associated with drug resistance. Besides affecting metabolism and motility of tumour cells, hypoxia also paradoxically increases production of reactive oxygen species (ROS), which contribute to stabilize HIF-1 through a redox-mediated inhibition of its proteolysis. Here we reported that 1% O<sub>2</sub> hypoxia increases the resistance of human metastatic melanoma cells to conventional chemotherapy with etoposide, and that the increase in chemoresistance strongly depends on ROS delivery due to hypoxia. We reported a biphasic redox-dependent role of HIF-1,

involving mitochondrial complex III and NADPH oxidase as oxidants sources, synergising in enhancing survival to chemotherapy. The feed-forward loop engaged by hypoxia involves first an HIF-1-dependent vascular endothelial growth factor-A (VEGF-A) autocrine production and, in the later phase, activation of NADPH oxidase from VEGF/VEGFR2 interaction, finally leading to a further redox-dependent long lasting stabilization of HIF-1. We therefore identified a redox-dependent circuitry linking hypoxia-driven ROS to VEGF-A secretion and to enhanced melanoma cell survival to etoposide chemotherapy

Liu F, Gomez Garcia AM, Meyskens FL, Jr. NADPH oxidase 1 overexpression enhances invasion via matrix metalloproteinase-2 and epithelial-mesenchymal transition in melanoma cells. *J Invest Dermatol* 2012 August;132(8):2033-41.

Abstract: NADPH oxidase 1 (Nox1) is a member of the NADPH oxidase family that has not been well characterized in the melanocytic cell lineage. Here we demonstrated that Nox1 and Nox4 were detected in melanocytic lineage, with only Nox1 detected in normal human melanocytes and Nox4 in a subset of metastatic melanoma cell lines. The protein level and enzymatic activity of Nox1 was elevated in all melanoma cells as compared with normal melanocytes. Overexpression of GFP-Nox1 protein in Wm3211 primary melanoma cells increased invasion rate by 4- to 6-fold as measured by Matrigel invasion assay, whereas knocking down or inhibiting Nox1 decreased invasion by approximately 40-60% in Wm3211 and SK-Mel-28 cells. Matrix metalloproteinase-2 (MMP-2) was increased by Nox1 overexpression at the mRNA, protein, and activity levels, and decreased by Nox1 knockdown. MMP-2 promoter activity was also regulated by Nox1 knockdown. In addition, stable clones overexpressing Nox1 exhibited an epithelial-mesenchymal transition (EMT) as examined by cell morphology and EMT markers; knockdown or inhibiting Nox1 led to a reversal of EMT. Supplementing MMP-2 to culture media did not induce EMT, suggesting that EMT induction by Nox1 was not through MMP-2 upregulation. In summary, Nox1 was overexpressed in all melanoma cell lines examined, and enhanced cell invasion by MMP-2 upregulation and EMT induction

Park SJ, Kim YT, Jeon YJ. Antioxidant dieckol downregulates the Rac1/ROS signaling pathway and inhibits Wiskott-Aldrich syndrome protein (WASP)-family verprolin-homologous protein 2 (WAVE2)-mediated invasive migration of B16 mouse melanoma cells. *Mol Cells* 2012 April;33(4):363-9.

Abstract: Reactive oxygen species (ROS) generation is linked to dynamic actin cytoskeleton reorganization, which is involved in tumor cell motility and metastasis. Thus, inhibition of ROS generation and actin polymerization in tumor cells may represent an effective anticancer strategy. However, the molecular basis of this signaling pathway is currently unknown. Here, we show that the Ecklonia cava-derived antioxidant dieckol downregulates the Rac1/ROS signaling pathway and inhibits Wiskott-Aldrich syndrome protein (WASP)-family verprolin-homologous protein 2 (WAVE2)-mediated invasive migration of B16 mouse melanoma cells. Steady-state intracellular ROS levels were higher in malignant B16F10 cells than in parental, nonmetastatic B16F0 cells. Elevation of ROS by H<sub>2</sub>O<sub>2</sub> treatment increased migration and invasion ability of B16F0 cells to level similar to that of B16F10 cells, suggesting that intracellular ROS signaling mediates the prometastatic properties of B16 mouse melanoma cells. ROS levels and the cell migration and invasion ability of B16 melanoma cells correlated with Rac1 activation and WAVE2 expression. Overexpression of dominant negative Rac1 and depletion of WAVE2 by siRNA suppressed H<sub>2</sub>O<sub>2</sub>-induced cell invasion of B16F0 and B16F10 cells. Similarly, dieckol attenuates the ROS-mediated Rac1 activation and WAVE2 expression, resulting in decreased migration and invasion of B16 melanoma cells. In addition, we found that dieckol decreases association between WAVE2 and NADPH oxidase subunit p47(phox). Therefore, this finding suggests that WAVE2 acts to couple intracellular Rac1/ROS signaling to the invasive migration of B16 melanoma cells, which is inhibited by dieckol

Jajoo S, Mukherjea D, Watabe K, Ramkumar V. Adenosine A(3) receptor suppresses prostate cancer metastasis by inhibiting NADPH oxidase activity. *Neoplasia* 2009 November;11(11):1132-45.

Abstract: Prostate cancer is the most commonly diagnosed and second most lethal malignancy in men, due mainly to a lack of effective treatment for the metastatic disease. A number of recent studies have shown that activation of the purine nucleoside receptor, adenosine A(3) receptor (A(3)AR), attenuates proliferation of melanoma, colon, and prostate cancer cells. In the present study, we determined whether activation of the A(3)AR reduces the ability of prostate cancer cells to migrate in vitro and metastasize in vivo. Using severe combined immunodeficient mice, we show that proliferation and metastasis of AT6.1 rat prostate cancer cells were decreased by the administration of A(3)AR agonist N(6)-(3-iodobenzyl) adenosine-5'-N-methyluronamide. In vitro studies show that activation of A(3)AR decreased high basal nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity present in these cells, along with the expression of Rac1 and p47(phox) subunits of this enzyme. Inhibition of NADPH oxidase activity by the dominant-negative RacN17 or short interfering (si)RNA against p47(phox) reduced both the generation of reactive oxygen species and the invasion of these cells on Matrigel. In addition, we show that membrane association of p47(phox) and activation of NADPH oxidase is dependent on the activity of the extracellular signal-regulated kinase (ERK)1/2 mitogen-activated protein kinase pathway. We also provide evidence that A(3)AR inhibits ERK1/2 activity in prostate cancer cells through inhibition of adenylyl cyclase and protein kinase A. We conclude that activation of the A(3)AR in prostate cancer cells reduces protein kinase A-mediated stimulation of ERK1/2, leading to reduced NADPH oxidase activity and cancer cell invasiveness

Yamaura M, Mitsushita J, Furuta S et al. NADPH oxidase 4 contributes to transformation phenotype of melanoma cells by regulating G2-M cell cycle progression. *Cancer Res* 2009 March 15;69(6):2647-54.

Abstract: Generation of reactive oxygen species (ROS) has been implicated in carcinogenic development of melanoma, but the underlying molecular mechanism has not been fully elucidated. We studied the expression and function of the superoxide-generating NADPH oxidase (Nox)4 in human melanoma cells. Nox4 was up-regulated in 13 of 20 melanoma cell lines tested. Silencing of Nox4 expression in melanoma MM-BP cells by small interfering RNAs decreased ROS production and thereby inhibited anchorage-independent cell growth and tumorigenicity in nude mice. Consistently, a general Nox inhibitor, diphenylene iodonium, and antioxidants vitamin E and pyrrolidine dithiocarbamate blocked cell proliferation of MM-BP cells. Flow cytometric analysis indicated that Nox4 small interfering RNAs and diphenylene iodonium induced G(2)-M cell cycle arrest, which was also observed with another melanoma cell line, 928mel. This was accompanied by induction of the Tyr-15 phosphorylated, inactive form of cyclin-dependent kinase 1 (a hallmark of G(2)-M checkpoint) and hyperphosphorylation of cdc25c leading to its increased binding to 14-3-3 proteins. Ectopic expression of catalase, a scavenger of ROS, also caused accumulation of cells in G(2)-M phase. Immunohistochemistry revealed that expression of Nox4 was detected in 31.0% of 13 melanoma patients samples, suggesting the association of Nox4 expression with some steps of melanoma development. The findings suggest that Nox4-generated ROS are required for transformation phenotype of melanoma cells and contribute to melanoma growth through regulation of G(2)-M cell cycle progression

Zhao Y, Liu J, McMartin KE. Inhibition of NADPH oxidase activity promotes differentiation of B16 melanoma cells. *Oncol Rep* 2008 May;19(5):1225-30.

Abstract: The activity of NADPH oxidase is increased in malignant skin keratinocytes. We demonstrated that inhibition of NADPH oxidase activity by diphenyleneiodonium (DPI) suppressed free radical production, inhibited cell growth and promoted cell differentiation of B16 melanoma cells, as indicated by cell morphology, increased production of melanin, and increased expression of microphthalmia-associated transcription factor (MITF). siRNA to NADPH oxidase subunit Rac1 or p47 induced the

expression of MITF, verifying that the pro-differentiation effects are due to the inhibition of NADPH oxidase. Biochemical studies suggest that ERK plays a positive role whereas PKC $\alpha$  plays a negative role during this differentiation event. In addition, the protein levels of the tumor suppressor p53 were suppressed by DPI, suggesting that p53 is activated by oxidative stress and may negatively regulate differentiation in melanoma cells. Taken together, these results suggest that inhibiting NADPH oxidase activity promotes cell differentiation of B16 melanoma cells

Brar SS, Kennedy TP, Sturrock AB et al. An NAD(P)H oxidase regulates growth and transcription in melanoma cells. *Am J Physiol Cell Physiol* 2002 June;282(6):C1212-C1224.

Abstract: Malignant melanoma cells spontaneously generate reactive oxygen species (ROS) that promote constitutive activation of the transcription factor nuclear factor-kappaB (NF-kappaB). Although antioxidants and inhibitors of NAD(P)H oxidases significantly reduce constitutive NF-kappaB activation and suppress cell proliferation (11), the nature of the enzyme responsible for ROS production in melanoma cells has not been determined. To address this issue, we now have characterized the source of ROS production in melanoma cells. We report that ROS are generated by isolated, cytosol-free melanoma plasma membranes, with inhibition by NAD(P)H oxidase inhibitors. The p22(phox), gp91(phox), and p67(phox) components of the human phagocyte NAD(P)H oxidase and the gp91(phox) homolog NOX4 were demonstrated in melanomas by RT-PCR and sequencing, and protein product for both p22(phox) and gp91(phox) was detected in cell membranes by immunoassay. Normal human epidermal melanocytes expressed only p22(phox) and NOX4. Melanoma proliferation was reduced by NAD(P)H oxidase inhibitors and by transfection of antisense but not sense oligonucleotides for p22(phox) and NOX4. Also, the flavoprotein inhibitor diphenylene iodonium inhibited constitutive DNA binding of nuclear protein to the NF-kappaB and cAMP-response element consensus oligonucleotides, without affecting DNA binding activity to activator protein-1 or OCT-1. This suggests that ROS generated in autocrine fashion by an NAD(P)H oxidase may play a role in signaling malignant melanoma growth

McCarty MF. Clinical potential of Spirulina as a source of phycocyanobilin. *J Med Food* 2007 December;10(4):566-70.

Abstract: Recent research reveals that free bilirubin functions physiologically as a potent inhibitor of NADPH oxidase activity. The chromophore phycocyanobilin (PCB), found in blue-green algae and cyanobacteria such as Spirulina, also has been found to be a potent inhibitor of this enzyme complex, likely because in mammalian cells it is rapidly reduced to phycocyanorubin, a close homolog of bilirubin. In light of the protean roles of NADPH oxidase activation in pathology, it thus appears likely that PCB supplementation may have versatile potential in prevention and therapy -- particularly in light of rodent studies demonstrating that orally administered Spirulina or phycocyanin (the Spirulina holoprotein that contains PCB) can exert a wide range of anti-inflammatory effects. Until PCB-enriched Spirulina extracts or synthetically produced PCB are commercially available, the most feasible and least expensive way to administer PCB is by ingestion of whole Spirulina. A heaping tablespoon (about 15 g) of Spirulina can be expected to provide about 100 mg of PCB. By extrapolating from rodent studies, it can be concluded that an intake of 2 heaping tablespoons daily would be likely to have important antioxidant activity in humans -- assuming that humans and rodents digest and absorb Spirulina-bound PCB in a comparable manner. An intake of this magnitude can be clinically feasible if Spirulina is incorporated into "smoothies" featuring such ingredients as soy milk, fruit juices, and whole fruits. Such a regimen should be evaluated in clinical syndromes characterized and in part mediated by NADPH oxidase overactivity in affected tissues

McCarty MF, Barroso-Aranda J, Contreras F. A two-phase strategy for treatment of oxidant-dependent cancers. *Med Hypotheses* 2007;69(3):489-96.

Abstract: In many cancers, a chronic increase in oxidant stress - associated with elevated levels of hydrogen peroxide - contributes to the increased proliferative rate, diminished apoptosis, increased

angiogenic and metastatic capacity, and chemoresistance that often characterize advanced malignancies. This oxidant stress often reflects up-regulation of expression and activity of NADPH oxidase, and/or decreased activity of catalase, which functions as suppressor gene in oxidant-dependent cancers. These characteristics of oxidant-dependent cancers suggest a dual strategy for treatment of these cancers. Since ascorbate can react spontaneously with molecular oxygen to generate hydrogen peroxide, high-dose intravenous ascorbate should be selectively toxic to tumors that are low in catalase activity - as suggested by numerous cell culture studies. Measures which concurrently improve the oxygenation of hypoxic tumor regions would be expected to boost the efficacy of such therapy; calcitriol and high-dose selenium might also be useful in this regard. Secondly, during the intervals between sessions of ascorbate therapy, administration of agents which can safely inhibit NADPH oxidase would be expected to slow the proliferation and spread of surviving tumor cells - while providing selection pressure for a further decline in catalase activity. In effect, cancers treated in this way would be whipsawed between lethally excessive and inadequately low oxidant stress. An additional possibility is that ascorbate-induced oxidant stress in tumors might potentiate the cell kill achieved with concurrently administered cytotoxic drugs, inasmuch as oxidant mechanisms appear to play a mediating role in the apoptosis induced by many such drugs, largely via activation of c-Jun NH(2)-terminal kinase; cell culture studies would be useful for evaluating this possibility

### **NF-kappaB Inhibitors - Salsalate and Anti-inflammatory Phytochemicals**

Katerinaki E, Haycock JW, Lalla R et al. Sodium salicylate inhibits TNF-alpha-induced NF-kappaB activation, cell migration, invasion and ICAM-1 expression in human melanoma cells. *Melanoma Res* 2006 February;16(1):11-22.

Abstract: We have previously shown that tumour necrosis factor-alpha (TNF-alpha) upregulates human melanoma cell integrin expression, migration and invasion in vitro. The aim of this study was to investigate the effect of the non-steroidal anti-inflammatory agent sodium salicylate on TNF-alpha-induced activation of the transcription factor nuclear factor-kappaB (NF-kappaB) and upregulation of intercellular adhesion molecule-1 (ICAM-1), and TNF-alpha-stimulated cell migration and invasion through fibronectin. HBL human melanoma cells were pre-incubated with sodium salicylate prior to stimulation with TNF-alpha for 24 h. NF-kappaB activation was measured using an assay that detects changes in the expression of a luciferase reporter gene under the direct control of NF-kappaB transcriptional activity. The effect of sodium salicylate and TNF-alpha on HBL cell invasion over 20 h and migration over 24 h was studied using fibronectin invasion and 'scratch wound' migration models in vitro, as described previously. Sodium salicylate inhibited TNF-alpha-stimulated NF-kappaB activation in melanoma cells in a concentration-dependent manner, and this was achieved with pre-incubation times as short as 15 min. TNF-alpha-stimulated ICAM-1 expression in HBL cells was also downregulated by sodium salicylate, although in a manner inversely related to the concentration of this agent. In functional assays, TNF-alpha stimulated migration and invasion, and sodium salicylate significantly reduced the extent of melanoma invasion and migration in both the presence and absence of TNF-alpha. In conclusion, sodium salicylate effectively inhibited TNF-alpha-induced upregulation of NF-kappaB, ICAM-1 expression, in-vitro migration and invasion in human melanoma cells, indicating that non-steroidal anti-inflammatory drugs may be a useful therapeutic approach to oppose inflammation-induced melanoma invasion and metastasis in vivo

SHERMAN JD, FRIEDEL GH. The effect of salicylate treatment of hamsters on the growth of a transplantable human melanoma. *Transplant Bull* 1958 October;5(4):406-7.

D'Anneo A, Carlisi D, Lauricella M et al. Parthenolide induces caspase-independent and AIF-mediated cell death in human osteosarcoma and melanoma cells. *J Cell Physiol* 2012 June 11.

Abstract: The mechanism of the cytotoxic effect exerted by parthenolide on tumor cells is not clearly defined today. This paper shows that parthenolide stimulates in human osteosarcoma MG63 and melanoma SK-MEL-28 cells a mechanism of cell death, which is not prevented by z-VAD-fmk and other caspase inhibitors. In particular treatment with parthenolide rapidly stimulated (1-2 h) ROS generation by inducing activation of extracellular signal-regulated kinase1/2 (ERK1/2) and NADPH oxidase. This event caused depletion of thiol groups and glutathione, NF-kappaB inhibition, JNK activation, cell detachment from the matrix and cellular shrinkage. The increase of ROS generation together with the mitochondrial accumulation of Ca(2+) also favoured dissipation of triangle uppsim, which seemed primarily determined by PTP opening, since triangle uppsim loss was partially prevented by the inhibitor cyclosporin A. Staining with Hoechst 33342 revealed in most cells, at 3-5h of treatment, chromatin condensation and fragmentation, while only few cells were PI-positive. In addition, at this stage AIF translocated to the nucleus and co-localized with areas of condensed chromatin. Prolonging the treatment (5-15h) ATP content declined while PI-positive cells strongly augmented, denouncing the increase of necrotic effects. All these effects were prevented by NAC, while caspase inhibitors were ineffective. We suggest that AIF exerts a crucial role in parthenolide action. In accordance, down-regulation of AIF markedly inhibited parthenolide effect on the production of cells with apoptotic or necrotic signs. Taken together our results demonstrate that parthenolide causes in the two cell lines a caspase-independent cell death, which is mediated by AIF. *J. Cell. Physiol.* (c) 2012 Wiley Periodicals, Inc

Madonna G, Ullman CD, Gentilcore G, Palmieri G, Ascierto PA. NF-kappaB as potential target in the treatment of melanoma. *J Transl Med* 2012;10:53.

Abstract: The RAS/MAP kinase pathway has attracted attention because activating mutations of the BRAF serine/threonine kinase was described in over 50% of melanomas. Very recently, selective and potent BRAF inhibitors have been developed. Several other signal transduction pathways have been found to be constitutively active or mutated in other subsets of melanoma tumors that are potentially targetable with new agents. Among these, NFkappaB is another pathway that melanoma tumors use to achieve survival, proliferation and resistance to apoptosis. Inhibition of NF-kappaB activation appears to be a very promising option for anti-cancer therapies

Enzler T, Sano Y, Choo MK et al. Cell-selective inhibition of NF-kappaB signaling improves therapeutic index in a melanoma chemotherapy model. *Cancer Discov* 2011 November;1(6):496-507.

Abstract: The transcription factor NF-kappaB promotes survival of cancer cells exposed to doxorubicin and other chemotherapeutic agents. IkappaB kinase is essential for chemotherapy-induced NF-kappaB activation and considered a prime target for anticancer treatment. An IkappaB kinase inhibitor sensitized human melanoma xenografts in mice to killing by doxorubicin, yet also exacerbated treatment toxicity in the host animals. Using mouse models that simulate cell-selective targeting, we found that impaired NF-kappaB activation in melanoma and host myeloid cells accounts for the therapeutic and the adverse effects, respectively. Ablation of tumor-intrinsic NF-kappaB activity resulted in apoptosis-driven tumor regression following doxorubicin treatment. By contrast, chemotherapy in mice with myeloid-specific loss of NF-kappaB activation led to a massive intratumoral recruitment of interleukin-1beta-producing neutrophils and necrotic tumor lesions, a condition associated with increased host mortality but not accompanied by tumor regression. Therefore, a molecular target-based therapy may be steered toward different clinical outcomes depending on the drug's cell-specific effects. SIGNIFICANCE: Our findings show that the IkappaB kinase-NF-kappaB signaling pathway is important for both promoting treatment resistance and preventing host toxicity in cancer chemotherapy; however, the two functions are exerted by

distinct cell type-specific mechanisms and can therefore be selectively targeted to achieve an improved therapeutic outcome

Pletz N, Schon M, Ziegelbauer K et al. Doxorubicin-induced activation of NF-kappaB in melanoma cells is abrogated by inhibition of IKKbeta, but not by a novel IKKalpha inhibitor. *Exp Dermatol* 2012 April;21(4):301-4.

Abstract: Drug resistance is arguably the most important challenge in cancer therapy. Here, doxorubicin induced profound of NF-kappaB activation in melanoma cells with a maximum (3.5-fold) at concentrations relevant in vivo. This was followed by transcriptional induction of several gene products involved in tumor progression. A novel IKKalpha inhibitor (BAY32-5915) was identified and characterized, and doxorubicin-induced NF-kappaB activation was assessed following inhibition of IKKalpha or IKKbeta by small-molecular compounds. While the IKKalpha inhibitor did not affect doxorubicin-induced NF-kappaB activation, this process was completely abrogated when the IKKbeta inhibitor, KINK-1, was used. Moreover, inhibition of IKKbeta, but not IKKalpha, led to significantly increased apoptosis in response to doxorubicin. Our results indicate that the net outcome of chemotherapy is difficult to predict and may even involve mechanisms conferring chemoresistance. In case of doxorubicin-induced NF-kappaB activation, blocking IKKbeta, but not IKKalpha, by small molecules can antagonize this activity and, thus, increase chemosensitivity

Thu YM, Su Y, Yang J et al. NF-kappaB inducing kinase (NIK) modulates melanoma tumorigenesis by regulating expression of pro-survival factors through the beta-catenin pathway. *Oncogene* 2012 May 17;31(20):2580-92.

Abstract: Nuclear factor-kappaB (NF-kappaB) inducing kinase (NIK) is a MAP3K that regulates the activation of NF-kappaB. NIK is often highly expressed in tumor cells, including melanoma, but the significance of this in melanoma progression has been unclear. Tissue microarray analysis of NIK expression reveals that dysplastic nevi (n=22), primary (n=15) and metastatic melanoma (n=13) lesions showed a statistically significant elevation in NIK expression when compared with benign nevi (n=30). Moreover, when short hairpin RNA techniques were used to knock-down NIK, the resultant NIK-depleted melanoma cell lines exhibited decreased proliferation, increased apoptosis, delayed cell cycle progression and reduced tumor growth in a mouse xenograft model. As expected, when NIK was depleted there was decreased activation of the non-canonical NF-kappaB pathway, whereas canonical NF-kappaB activation remained intact. NIK depletion also resulted in reduced expression of genes that contribute to tumor growth, including CXCR4, c-MYC and c-MET, and pro-survival factors such as BCL2 and survivin. These changes in gene expression are not fully explained by the attenuation of the non-canonical NF-kappaB pathway. Shown here for the first time is the demonstration that NIK modulates beta-catenin-mediated transcription to promote expression of survivin. NIK-depleted melanoma cells exhibited downregulation of survivin as well as other beta-catenin regulated genes including c-MYC, c-MET and CCND2. These data indicate that NIK mediates both beta-catenin and NF-kappaB regulated transcription to modulate melanoma survival and growth. Thus, NIK may be a promising therapeutic target for melanoma

Levati L, Ruffini F, Muzi A et al. Placenta growth factor induces melanoma resistance to temozolomide through a mechanism that involves the activation of the transcription factor NF-kappaB. *Int J Oncol* 2011 January;38(1):241-7.

Abstract: Placenta growth factor (PIGF) and its receptor vascular endothelial growth factor receptor-1 (VEGFR-1) are co-expressed in a large number of human melanoma cell lines. Moreover, a correlation between in vivo PIGF production and melanoma progression has been suggested. To investigate whether PIGF might have a role in protecting melanoma cells from the cytotoxic effects of the anticancer agent

temozolomide (TMZ), which is used for the treatment of this malignancy, we stably transfected a doxycycline-inducible PIGF antisense mRNA into a human melanoma cell clone that secretes VEGF-A and PIGF and expresses receptors for both growth factors. Induction of PIGF antisense mRNA in the transfected cells (13443/ASP3 subclone) halved TMZ IC(50), and exogenous addition of PIGF to the culture medium 24 h before TMZ treatment, partially restored IC(50) values to that of control cells. The increased sensitivity of 13443/ASP3 cells upon PIGF antisense mRNA expression was not due to down-regulation of O6-methylguanine-DNA methyltransferase, a DNA repair protein that represents the main mechanism of resistance to TMZ. Since the activity of the transcription factor nuclear factor-kappaB (NF-kappaB) has been correlated to melanoma chemoresistance, we investigated whether NF-kappaB was involved in PIGF-induced melanoma cell resistance to TMZ. Induction of PIGF antisense mRNA in 13443/ASP3 cells halved the levels of active NF-kappaB and the specific inhibition of this transcription factor increased sensitivity of 13443/ASP3 cells to TMZ. In conclusion, our data strongly suggest that PIGF plays a role in melanoma cell resistance to TMZ through a pathway that involves NF-kappaB activation

Lin K, Baritaki S, Militello L, Malaponte G, Bevelacqua Y, Bonavida B. The Role of B-RAF Mutations in Melanoma and the Induction of EMT via Dysregulation of the NF-kappaB/Snail/RKIP/PTEN Circuit. *Genes Cancer* 2010 May;1(5):409-20.

Abstract: Melanoma is a highly metastatic cancer, and there are no current therapeutic modalities to treat this deadly malignant disease once it has metastasized. Melanoma cancers exhibit B-RAF mutations in up to 70% of cases. B-RAF mutations are responsible, in large part, for the constitutive hyperactivation of survival/antiapoptotic pathways such as the MAPK, NF-kappaB, and PI3K/AKT. These hyperactivated pathways regulate the expression of genes targeting the initiation of the metastatic cascade, namely, the epithelial to mesenchymal transition (EMT). EMT is the result of the expression of mesenchymal gene products such as fibronectin, vimentin, and metalloproteinases and the invasion and inhibition of E-cadherin. The above pathways cross-talk and regulate each other's activities and functions. For instance, the NF-kappaB pathway directly regulates EMT through the transcription of gene products involved in EMT and indirectly through the transcriptional up-regulation of the metastasis inducer Snail. Snail, in turn, suppresses the expression of the metastasis suppressor gene product Raf kinase inhibitor protein RKIP (inhibits the MAPK and the NF-kappaB pathways) as well as PTEN (inhibits the PI3K/AKT pathway). The role of B-RAF mutations in melanoma and their direct role in the induction of EMT are not clear. This review discusses the hypothesis that B-RAF mutations are involved in the dysregulation of the NF-kappaB/Snail/RKIP/PTEN circuit and in both the induction of EMT and metastasis. The therapeutic implications of the dysregulation of the above circuit by B-RAF mutations are such that they offer novel targets for therapeutic interventions in the treatment of EMT and metastasis

Wu FH, Yuan Y, Li D et al. Endothelial cell-expressed Tim-3 facilitates metastasis of melanoma cells by activating the NF-kappaB pathway. *Oncol Rep* 2010 September;24(3):693-9.

Abstract: T cell immunoglobulin and mucin domain-3 (Tim-3) is originally recognized as a receptor of Th1 cells. We found that Tim-3 could be expressed in endothelial cells after stimulation with tumor cell-released TLR4 ligand. Tim-3 expressed by endothelial cells does not function as the receptor of galectin-9, but mediates the interaction of endothelial cells with tumor cells. The engagement of endothelial cell-expressed Tim-3 with a non-galectin 9 putative receptor on B16 melanoma cells could trigger the NF-kappaB signaling pathway in B16 cells. The activated NF-kappaB not only promoted the proliferation of B16 cells, but also enhanced apoptosis resistance of B16 cells by up-regulating Bcl-2 and Bcl-xL and down-regulating Bax. Consistently, Tim-3 facilitated the survival of B16 cells in the blood stream, arrested in the lung and following invasion, resulted in more metastatic nodules in the lung. These findings suggest that endothelial cell-expressed Tim-3 increases tumor cell metastatic potential by

facilitating tumor cell intravasation, survival in blood stream and extravasation. Thus, anti-inflammation or blockade of Tim-3 may contribute to the prevention of metastasis

Biswas SK, Lewis CE. NF-kappaB as a central regulator of macrophage function in tumors. *J Leukoc Biol* 2010 November;88(5):877-84.

Abstract: TAMs are usually abundant in the tumor microenvironment and are now known to play an essential role in tumor progression. For example, TAMs influence many aspects of tumorigenesis, such as the growth, survival, invasion, and metastasis of tumor cells, tumor angiogenesis, and the suppression of other tumor-infiltrating immune effector cells. The molecular pathways that regulate these tumor-promoting functions of TAMs are currently under intense investigation. Several recent studies about transgenic murine tumor models have shown that the transcription factor NF-kappaB is a key player in tumor progression with distinct roles in regulating the functions of macrophages and tumor cells in malignant tumors. Here, we outline the evidence for classical and noncanonical NF-kappaB signaling pathways driving the tumor-promoting repertoire of TAMs

Cheng Y, Li J, Martinka M, Li G. The expression of NAD(P)H:quinone oxidoreductase 1 is increased along with NF-kappaB p105/p50 in human cutaneous melanomas. *Oncol Rep* 2010 April;23(4):973-9.

Abstract: NAD(P)H:quinone oxidoreductase 1 (NQO1) is a key enzyme involved in metabolism of quinones and may perform multiple functions within the cell. Recent studies demonstrated that NQO1 is overexpressed in many types of tumors, including the lung, ovary, adrenal gland, thyroid, liver, colon, breast, and pancreas. To investigate whether NQO1 plays a role in melanoma pathogenesis, we used tissue microarray technology and immunohistochemistry to examine NQO1 expression in 56 dysplastic nevi and 93 primary melanoma biopsies. Our data showed that NQO1 expression is significantly increased in primary melanomas compared with dysplastic nevi ( $P=0.015$ , chi2 test). Our results also revealed that the increase of NQO1 was not associated with patient age, tumor thickness, ulceration, tumor site, American Joint Committee on Cancer (AJCC) stage, and 5-year patient survival. Interestingly, we found that female patients had more NQO1 expression than male patients ( $P=0.022$ , chi2 test). Furthermore, NQO1 expression level was significantly higher in superficial spreading melanomas compared with other tumor subtypes ( $P=0.020$ , chi2 test). Moreover, we found that NQO1 expression is significantly correlated with the expression of NF-kappaB subunit p50 ( $P=0.032$ , chi2 test). Our findings suggest that NQO1 may play an important role in the initiation stage of melanoma development

Kuphal S, Winklmeier A, Warnecke C, Bosserhoff AK. Constitutive HIF-1 activity in malignant melanoma. *Eur J Cancer* 2010 April;46(6):1159-69.

Abstract: The hypoxia-inducible factor-1 (HIF-1), which consists of the constitutive HIF-1beta and the oxygen-responsive HIF-1alpha subunit, is the master activator of the cellular transcriptional response to hypoxia coordinating gene expression during reduced oxygen tension. Overexpression of HIF-1 and increased transcriptional activity induced by hypoxia are linked to progression of many tumour types such as head and neck cancer, cervical carcinoma, leukaemia and renal cell carcinoma. In this study, we demonstrate that HIF activity is increased in malignant melanoma cells already under normoxic conditions in contrast to other tumour types. HIF-1alpha and -2alpha knockdown by siRNA transfection revealed that this effect is due to constitutive HIF-1alpha expression. Furthermore, the inhibition or activation of reactive oxygen species (ROS) decreased or activated, respectively, HIF-1 activity and HIF-1alpha protein expression. Interestingly, the inhibition of the NFkappaB pathway also reduced the accumulation of HIF-1alpha assuming a context between ROS and NFkappaB, and suggesting that ROS and NFkappaB activity contribute to HIF-1alpha accumulation. In summary, we identified an increased HIF-1alpha protein expression and activity in melanoma under normoxia mediated by ROS and the NFkappaB pathway

Amschler K, Schon MP, Pletz N, Wallbrecht K, Erpenbeck L, Schon M. NF-kappaB inhibition through proteasome inhibition or IKKbeta blockade increases the susceptibility of melanoma cells to cytostatic treatment through distinct pathways. *J Invest Dermatol* 2010 April;130(4):1073-86.

Abstract: Metastasized melanoma is almost universally resistant to chemotherapy. Given that constitutive or drug-induced upregulation of NF-kappaB activity is associated with this chemoresistance, NF-kappaB inhibition may increase the susceptibility to antitumoral therapy. On the cellular level, two principles of NF-kappaB inhibition, proteasome inhibition by bortezomib and IkappaB kinase-beta (IKKbeta) inhibition by the kinase inhibitor of NF-kappaB-1 (KINK-1), significantly increased the antitumoral efficacy of camptothecin. When combined with camptothecin, either of the two NF-kappaB-inhibiting principles synergistically influenced progression-related in vitro functions, including cell growth, apoptosis, and invasion through an artificial basement membrane. In addition, when C57BL/6 mice were intravenously injected with B16F10 melanoma cells, the combination of cytostatic treatment with either of the NF-kappaB-inhibiting compounds revealed significantly reduced pulmonary metastasis compared to either treatment alone. However, on the molecular level, nuclear translocation of p65, cell cycle analysis, and expression of NF-kappaB-dependent gene products disclosed distinctly different molecular mechanisms, resulting in the same functional effect. That proteasome inhibition and IKKbeta inhibition affect distinct molecular pathways downstream of NF-kappaB, both leading to increased chemosensitivity, is previously unreported. Thus, it is conceivable that switching the two principles of NF-kappaB inhibition, once resistance to one of the agents occurs, will improve future treatment regimens

Dror R, Lederman M, Umezawa K, Barak V, Pe'er J, Chowers I. Characterizing the involvement of the nuclear factor-kappa B (NF kappa B) transcription factor in uveal melanoma. *Invest Ophthalmol Vis Sci* 2010 April;51(4):1811-6.

Abstract: Purpose. To examine the involvement of nuclear factor-kappa B (NFkappaB) pathways in uveal melanoma (UM) and to assess their potential as a therapeutic target for metastatic UM. Methods. Samples from primary (n = 7) and metastatic (n = 7) UM were evaluated for NFkappaB transcription factor family expression by quantitative PCR (QPCR), immunofluorescent staining, and Western blot analysis. The effect of two NFkappaB inhibitors, DHMEQ and BMS-345541, on two cell lines derived from UM liver metastases was assessed. Cell proliferation was examined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, methylene blue assay, and immunostaining for Ki-67. Apoptosis was assessed by immunostaining for activated caspase 3. Results. NFkappaB1, NFkappaB2, RelA, RelB, and NIK were expressed in primary UM and in its liver metastases. NFkappaB2, RelB, and NIK showed significantly higher mRNA levels in metastases from UM compared with primary tumors (3.4-fold, P = 0.03; 3.6-fold, P = 0.05; 3.5-fold, P = 0.03; respectively). NFkappaB2 protein activation was 3.9-fold higher in metastases (P = 0.03). NFkappaB inhibition reduced metastatic cell proliferation by 9.2-fold and 1.9-fold according to Ki67 staining (P = 0.04) and methylene blue assay (P = 6 x 10<sup>-7</sup>), respectively. Both NFkappaB inhibitors achieved dose-dependent reductions of UM cell proliferation in both cell lines (P < 0.001). NFkappaB inhibition resulted in a 6.3-fold increase of apoptosis (P = 7 x 10<sup>-7</sup>). Conclusions. These data indicate that the NFkappaB1 and NFkappaB2 pathways are active in both primary and metastatic UM and that these pathways regulate metastatic cell proliferation and apoptosis. The role of NFkappaB as a therapeutic target for UM should be further evaluated

Hao H, Zhou HS, McMasters KM. Chemosensitization of tumor cells: inactivation of nuclear factor-kappa B associated with chemosensitivity in melanoma cells after combination treatment with E2F-1 and doxorubicin. *Methods Mol Biol* 2009;542:301-13.

Abstract: Combination chemotherapy has been shown to be more effective than single-agent therapy for many types of cancer, but both are known to induce drug resistance in cancer cells. Two major culprits in the development of this drug resistance are nuclear factor-kappaB (NF-kappaB) and the multidrug

resistance (MDR) gene. For this reason, chemogene therapy is emerging as a viable alternative to conventional chemotherapy combinations. We have shown that transduction of the E2F-1 gene in melanoma cells markedly increases cell sensitivity to doxorubicin, thereby producing a synergistic effect on melanoma cell apoptosis. Our microarray results show that the NF-kappaB pathway and related genes undergo significant changes after the combined treatment of E2F-1 and doxorubicin. In fact, inactivation of NF-kappaB is associated with melanoma cell apoptosis induced by E2F-1 and doxorubicin, providing a link between the NF-kappaB signaling pathway and the chemosensitivity of melanoma cells after this treatment

Gavriil M, Tsao CC, Mandiyan S, Arndt K, Abraham R, Zhang Y. Specific IKKbeta inhibitor IV blocks Streptonigrin-induced NF-kappaB activity and potentiates its cytotoxic effect on cancer cells. *Mol Carcinog* 2009 August;48(8):678-84.

Abstract: Many anticancer agents activate NF-kappaB, which plays an important role in the survival of cancer cells. Inhibition of NF-kappaB activity may therefore potentiate the efficacy of anticancer agents. We found that a previously used anticancer agent Streptonigrin (SN) was also a potent NF-kappaB inducer. Using a specific IKKbeta inhibitor IV (Podolin et al., *J Pharmacol Exp Ther* 2005; 312: 373-381), we revealed that the activation of NF-kappaB was mediated through DNA damage-induced activation of IKK complex. Furthermore, we demonstrated that SN-induced DNA damage was unrelated to reactive oxygen species but to the hydroquinone form of SN converted by the NAD(P)H:quinine oxidoreductase (NQO1). The study suggests that the combination of SN with IKK inhibitor may improve efficacy over the use of single agent

Ianaro A, Tersigni M, Belardo G et al. NEMO-binding domain peptide inhibits proliferation of human melanoma cells. *Cancer Lett* 2009 February 18;274(2):331-6.

Abstract: Melanoma is the most aggressive form of skin cancer, it originates from melanocytes and its incidence has increased in the last decade. Recent advances in the understanding of the underlying biology of the progression of melanoma have identified key signalling pathways that are important in promoting melanoma tumourigenesis, thus providing dynamic targets for therapy. One such important target identified in melanoma tumour progression is the Nuclear Factor-kappaB (NF-kappaB) pathway. In vitro studies have shown that NF-kappaB binding is constitutively elevated in human melanoma cultures compared to normal melanocytes. It has been found that a short cell-permeable peptide spanning the IKK-beta NBD, named NBD peptide, disrupted the association of NEMO with IKKs in vitro and blocked TNFalpha-induced NF-kappaB activation in vivo. In the present study we investigated the effect of the NBD peptide on NF-kappaB activity and survival of A375 human melanoma cells. We found that NBD peptide is able to inhibit the proliferation of A375 cells, which present constitutively elevated NF-kappaB levels. Inhibition of cell proliferation by NBD peptide was associated with direct inhibition of constitutive NF-kappaB DNA-binding activity and induction of apoptosis by activation of caspase-3 as confirmed by the cleavage and consequently inactivation of poly (ADP ribose) polymerase (PARP-1) known as the best marker of this process

Karst AM, Gao K, Nelson CC, Li G. Nuclear factor kappa B subunit p50 promotes melanoma angiogenesis by upregulating interleukin-6 expression. *Int J Cancer* 2009 January 15;124(2):494-501.

Abstract: Nuclear factor kappa B (NF-kappaB) signaling is deregulated in many tumor types, resulting in aberrant expression and/or activation of NF-kappaB transcriptional complexes. We have previously reported that nuclear expression of the NF-kappaB subunit p50 is strongly correlated with melanoma progression and poor 5-year patient survival. In this study, we used cDNA microarray to analyze the gene expression profiles of melanoma cells overexpressing NF-kappaB p50. We found that NF-kappaB p50 expression strongly induced interleukin-6 (IL-6) upregulation in melanoma cells at both the

transcriptional and translational levels and that IL-6 production by melanoma cells enhanced the growth of endothelial cells in vitro. Expression of activating transcription factor 3 (ATF3), a negative regulator of IL-6 gene transcription, inhibited p50-mediated IL-6 upregulation. Knockdown of p50 expression using lentiviral-based shRNA abrogated IL-6 induction in melanoma cells and inhibited its effects on endothelial cell growth. Finally, we used an in vivo matrigel plug assay to show that NF-kappaB p50 overexpression promotes angiogenesis, while silencing NF-kappaB p50 inhibits blood vessel formation. Our results demonstrate for the first time that the NF-kappaB p50 subunit mediates melanoma angiogenesis by specifically upregulating IL-6, highlighting a novel and important role for the NF-kappaB p50/IL-6 signaling axis in melanoma progression

Uffort DG, Grimm EA, Ellerhorst JA. NF-kappaB mediates mitogen-activated protein kinase pathway-dependent iNOS expression in human melanoma. *J Invest Dermatol* 2009 January;129(1):148-54.

Abstract: Tumor expression of inducible nitric oxide synthase (iNOS) predicts poor outcomes for melanoma patients. We have reported the regulation of melanoma iNOS by the mitogen-activated protein kinase (MAPK) pathway. In this study, we test the hypothesis that NF-kappaB mediates this regulation. Western blotting of melanoma cell lysates confirmed the constitutive expression of iNOS. Western blot detected baseline levels of activated nuclear extracellular signal-regulated kinase and NF-kappaB. Indirect immunofluorescence confirmed the presence of NF-kappaB p50 and p65 in melanoma cell nuclei, with p50 being more prevalent. Electrophoretic mobility shift assay demonstrated baseline NF-kappaB activity, the findings confirmed by supershift analysis. Treatment of melanoma cells with the MEK inhibitor U0126 decreased NF-kappaB binding to its DNA recognition sequence, implicating the MAPK pathway in NF-kappaB activation. Two specific NF-kappaB inhibitors suppressed iNOS expression, demonstrating regulation of iNOS by NF-kappaB. Several experiments indicated the presence of p50 homodimers, which lack a transactivation domain and rely on the transcriptional coactivator Bcl-3 to carry out this function. Bcl-3 was detected in melanoma cells and co-immunoprecipitated with p50. These data suggest that the constitutively activated melanoma MAPK pathway stimulates activation of NF-kappaB hetero- and homodimers, which, in turn, drive iNOS expression and support melanoma tumorigenesis

Yang J, Pan WH, Clawson GA, Richmond A. Systemic targeting inhibitor of kappaB kinase inhibits melanoma tumor growth. *Cancer Res* 2007 April 1;67(7):3127-34.

Abstract: Constitutive activation of nuclear factor-kappaB (NF-kappaB) has been directly implicated in tumorigenesis of various cancer types, including melanoma. Inhibitor of kappaB kinase (IKK) functions as a major mediator of NF-kappaB activation. Thus, development of an IKK-specific inhibitor has been a high priority, although it remains unclear whether systemic inhibition of IKK will provide therapeutic benefit. In this study, we show that inhibition of NF-kappaB activity in melanocytes that are persistently expressing an active H-Ras(V12) gene and are deficient in the tumor suppressors inhibitor A of cyclin-dependent kinase 4/alternative reading frame results in reduction of melanoma tumor growth in vivo. This effect is, at least in part, via regulation of NF-kappaB nuclear activation and RelA phosphorylation. Based on this result, we developed a double hammerhead ribozyme long-term expression system to silence either IKKalpha or IKKbeta. The ribozymes were placed in an EBV construct and delivered i.v. to nude mice bearing melanoma lesions, which developed after i.v. injection of H-Ras-transformed melanoma cells. Our in vivo data show that knockdown of endogenous IKKbeta significantly reduces the growth of the melanoma lesions and knockdown of either IKKalpha or IKKbeta prolongs the life span of immunocompetent mice

Yang J, Su Y, Richmond A. Antioxidants tiron and N-acetyl-L-cysteine differentially mediate apoptosis in melanoma cells via a reactive oxygen species-independent NF-kappaB pathway. *Free Radic Biol Med* 2007 May 1;42(9):1369-80.

Abstract: Tiron and N-acetyl-L-cysteine (NAC) have been recognized as potential antioxidants capable of inhibiting apoptosis induced by reactive oxygen species (ROS). Although the ROS-scavenging function of tiron and NAC is clear, the mechanism for their regulation of apoptosis is still elusive. Here we demonstrate that tiron increases nuclear factor-kappaB (NF-kappaB)/DNA binding and as a result enhances NF-kappaB transcriptional activity. In contrast, NAC inhibits NF-kappaB activation by reducing inhibitor of kappaB kinase (IKK) activity. Moreover, the expression of an NF-kappaB target gene, the chemokine CXCL1, is promoted by tiron and suppressed by NAC. Finally, tiron confers an antiapoptotic function, while NAC imparts a proapoptotic function in melanoma cells. These functions correlate with the alteration of mitochondrial membrane potential but not ROS production or induction of activating protein-1 (AP-1). This study underscores the potential benefits of regulating NF-kappaB activity in melanoma cells as a therapeutic approach

Liu J, Suresh Kumar KG, Yu D et al. Oncogenic BRAF regulates beta-Trcp expression and NF-kappaB activity in human melanoma cells. *Oncogene* 2007 March 22;26(13):1954-8.

Abstract: Mutational activation of BRAF is a frequent event in human malignant melanomas suggesting that BRAF-dependent signaling is conducive to melanoma cell growth and survival. Previously published work reported that melanoma cells exhibit constitutive anti-apoptotic nuclear factor kappaB (NF-kappaB) transcription factor activation triggered by proteolysis of its inhibitor IkappaB. IkappaB degradation is dependent upon its phosphorylation by the IkappaB kinase (IKK) complex and subsequent ubiquitination facilitated by beta-Trcp E3 ubiquitin ligase. Here, we report that melanocytes expressing a conditionally oncogenic form of BRAF(V600E) exhibit enhanced beta-Trcp expression, increased IKK activity and a concomitant increase in the rate of IkappaBalpha degradation. Conversely, inhibition of BRAF signaling using either a broad-spectrum Raf inhibitor (BAY 43-9006) or by selective knock-down of BRAF(V600E) expression by RNA interference in human melanoma cells leads to decreased IKK activity and beta-Trcp expression, stabilization of IkappaB, inhibition of NF-kappaB transcriptional activity and sensitization of these cells to apoptosis. Taken together, these data support a model in which mutational activation of BRAF in human melanomas contributes to constitutive induction of NF-kappaB activity and to increased survival of melanoma cells

Ueda Y, Richmond A. NF-kappaB activation in melanoma. *Pigment Cell Res* 2006 April;19(2):112-24.

Abstract: Metastatic melanoma is an aggressive skin cancer that is notoriously resistant to current cancer therapies. In human melanoma, nuclear factor-kappa B (NF-kappaB) is upregulated, leading to the deregulation of gene transcription. In this review, we discuss (i) the relationship between gene alteration in melanoma and upregulation of NF-kappaB, (ii) mechanisms by which activated NF-kappaB switch from pro-apoptotic to anti-apoptotic functions in melanoma and (iii) autocrine mechanisms that promote constitutive activation of NF-kappaB in metastatic melanoma

Amiri KI, Richmond A. Role of nuclear factor-kappa B in melanoma. *Cancer Metastasis Rev* 2005 June;24(2):301-13.

Abstract: Nuclear Factor-kappa B (NF-kappa B) is an inducible transcription factor that regulates the expression of many genes involved in the immune response. Recently, NF-kappa B activity has been shown to be upregulated in many cancers, including melanoma. Data indicate that the enhanced activation of NF-kappa B may be due to deregulations in upstream signaling pathways such as Ras/Raf, PI3K/Akt, and NIK. Multiple studies have shown that NF-kappa B is involved in the regulation of apoptosis, angiogenesis, and tumor cell invasion, all of which indicate the important role of NF-kappa B in tumorigenesis. Thus, understanding the molecular mechanism of melanoma progression will aid in designing new therapeutic approaches for melanoma. In this review, the association between NF-kappa B

and melanoma tumorigenesis are discussed. Additionally, the potential of emerging selective NF-kappa B inhibitors for the treatment of melanoma is reviewed

Kuphal S, Poser I, Jobin C, Hellerbrand C, Bosserhoff AK. Loss of E-cadherin leads to upregulation of NFkappaB activity in malignant melanoma. *Oncogene* 2004 November 4;23(52):8509-19.

Abstract: Malignant transformation of melanocytes frequently coincides with loss of E-cadherin expression. Here, we show that loss of E-cadherin leads to induction of nuclear factor kappa B (NFkappaB) activity in melanoma cell lines. Melanoma cells show constitutively active NFkappaB, whereas no activity is found in primary melanocytes. After re-expression of E-cadherin in melanoma cells, strong downregulation of NFkappaB activity was found. Consistently, NFkappaB activity was induced in primary human melanocytes after inhibition of E-cadherin activity by functionally blocking anti-E-cadherin antibodies. Interestingly, re-expression of E-cadherin-blocked p38 MAPK activity and the p38 MAPK inhibitors SB203580 and SB202190 almost completely prevented NFkappaB activation in melanoma cells. Furthermore, cytoplasmatic beta-catenin induced p38 and NFkappaB activation in malignant melanoma. To our knowledge, this is the first report suggesting a correlation between E-cadherin and NFkappaB activity in melanocytes and melanoma cells. In summary, we conclude that loss of E-cadherin and cytoplasmatic beta-catenin induces p38-mediated NFkappaB activation, potentially revealing an important mechanism of tumorigenesis in malignant melanomas

Munshi A, Kurland JF, Nishikawa T, Chiao PJ, Andreeff M, Meyn RE. Inhibition of constitutively activated nuclear factor-kappaB radiosensitizes human melanoma cells. *Mol Cancer Ther* 2004 August;3(8):985-92.

Abstract: Melanoma tumors and cultured cell lines are relatively resistant to the cytotoxic effects of ionizing radiation, thereby limiting the use of radiotherapy for the clinical treatment of melanoma. New strategies for sensitizing melanoma cells therefore deserve examination. In an attempt to identify and target signaling pathways that contribute to radioresistance, we investigated the role of nuclear factor-kappaB (NF-kappaB), a transcription factor known to inhibit apoptosis induced by a variety of stimuli and promote radioresistance. Two human metastatic melanoma cell lines, A375 and MeWo, were used to examine the radiosensitizing effects of inhibitors of the NF-kappaB pathway. Nuclear extracts from these cell lines were tested for active NF-kappaB using the electrophoretic mobility shift assay. Both melanoma cell lines had constitutively activated NF-kappaB as observed by electrophoretic mobility shift assay. In an attempt to reverse NF-kappaB activity, cells were treated either with vehicle alone (DMSO) or with a proteasome inhibitor Z-Leu-Leu-Leu-H (MG132; 10 micromol/L for 2 hours prior to irradiation) that inhibited both constitutive and radiation-induced NF-kappaB activity. The clonogenic cell survival assay showed that pretreatment with MG132 enhanced tumor cell radiosensitivity with the survival factor at 2 Gy being reduced from 48 +/- 0.8% and 48 +/- 1.6% in vehicle-treated cells to 27.7 +/- 0.32% and 34.3 +/- 0.7% in MG132-treated MeWo and A375 cells, respectively. To test the role of NF-kappaB in radioresistance more directly, MeWo cells were stably transfected with a dominant-negative mutant IkappaBalpha construct, which led to the inhibition of both constitutive and radiation-induced NF-kappaB activity. A modest restoration of radiosensitivity was also observed in the stably transfected MeWo cells with survival factor at 2 Gy values being reduced from 47 +/- 0.8% in parental MeWo cells to 32.9 +/- 0.7% in stable transfectants. Because constitutively activated mitogen-activated protein kinase kinase (MEK) pathway has been shown to lead to activated NF-kappaB, we wanted to determine the relative contribution of activated MEK in the human melanoma cells. To test this, MeWo and A375 melanoma cells were exposed to the MEK inhibitor PD184352. Treatment with PD184352 partially reversed NF-kappaB activity but did not impart radiation sensitivity to these cells. Our results indicate that activated NF-kappaB may be one of the pathways responsible for the radioresistance of melanoma cells and that strategies for inhibiting its influence may be useful in restoring the radioresponse of melanomas

Zheng M, Ekmekcioglu S, Walch ET, Tang CH, Grimm EA. Inhibition of nuclear factor-kappaB and nitric oxide by curcumin induces G2/M cell cycle arrest and apoptosis in human melanoma cells. *Melanoma Res* 2004 June;14(3):165-71.

**Abstract:** Curcumin (diferuloylmethane) inhibits tumour cell growth by inducing apoptosis in many tumour types, including melanoma, via complex and ill-defined pathways. Recent studies have shown that curcumin is both a nitric oxide scavenger and an inhibitor of inducible nitric oxide synthase (iNOS) expression, low levels of which correlate with antiapoptotic function and poor survival and which may be regulated by inhibition of nuclear factor-kappaB (NFkappaB) activation. To elucidate the mechanisms by which curcumin inhibits melanoma proliferation, we tested the in vitro effects of curcumin on specific cell cycle pathways and melanoma cell survival, including NFkappaB activation. Curcumin induced melanoma cell apoptosis and cell cycle arrest, which is associated with the downregulation of NFkappaB activation, iNOS and DNA-dependent protein kinase catalytic subunit expression, and upregulation of p53, p21(Cip1), p27(Kip1) and checkpoint kinase 2. Curcumin also downregulated constitutive iNOS activity in melanoma cells. Our results demonstrate that curcumin arrested cell growth at the G(2)/M phase and induced apoptosis in human melanoma cells by inhibiting NFkappaB activation and thus depletion of endogenous nitric oxide. Therefore, curcumin should be considered further as a potential therapy for patients with melanoma

Kashani-Sabet M, Shaikh L, Miller JR, III et al. NF-kappa B in the vascular progression of melanoma. *J Clin Oncol* 2004 February 15;22(4):617-23.

**Abstract:** **PURPOSE:** To examine a model of melanoma progression based on vascular factors and the role of NF-kappa B in the vascular progression of melanoma. **PATIENTS AND METHODS:** A data set of 526 patients from the University of California San Francisco Melanoma Center with 2 years of follow-up or first relapse was studied. The impact of the presence or absence of various prognostic factors on overall survival of melanoma patients was assessed using Cox regression and Kaplan-Meier analysis. A matched-pair analysis of NF-kappa B expression was performed in cases with vascular involvement and increased tumor vascularity versus matched controls lacking these factors. **RESULTS:** Cox regression analysis of factors evaluated by the American Joint Committee on Cancer Melanoma Staging Committee reproduced the powerful impact of tumor thickness and ulceration in this data set. With the inclusion of vascular factors such as tumor vascularity and vascular involvement, ulceration was no longer significant in predicting overall survival. By multivariate analysis, vascular involvement and tumor vascularity were the strongest predictors of melanoma outcome. Tumor vascularity seems to be a precursor of both vascular involvement and ulceration. A matched-pair tissue array analysis demonstrated the significant correlation between overexpression of NF-kappa B-p65 and the development of vascular factors. **CONCLUSION:** Vascular factors play an important role in the progression of malignant melanoma. Ulceration may be a surrogate marker for the interactions between melanoma and the tumor vasculature. NF-kappa B seems to play an important role in the development of these factors

Brar SS, Kennedy TP, Quinn M, Hoidal JR. Redox signaling of NF-kappaB by membrane NAD(P)H oxidases in normal and malignant cells. *Protoplasma* 2003 May;221(1-2):117-27.

**Abstract:** Evidence is rapidly accumulating that low-activity NAD(P)H oxidases homologous to that in phagocytic cells generate reactive oxygen species as signaling intermediates. In this review we discuss evidence that signaling NAD(P)H oxidases in part influence normal and malignant cell division by activating the redox-regulated transcription factor nuclear factor kappaB. The roles of growth-regulatory NAD(P)H oxidases in human airway smooth muscle and malignant melanoma are used as examples

Yang J, Richmond A. Constitutive IkappaB kinase activity correlates with nuclear factor-kappaB activation in human melanoma cells. *Cancer Res* 2001 June 15;61(12):4901-9.

Abstract: Constitutive IKK activity associated with increased IkappaBalpha phosphorylation and degradation contribute to the high level of endogenous nuclear factor-kappaB (NF-kappaB) activation in Hs294T melanoma cells as compared with RPE cells (R. L. Shattuck-Brandt and A. Richmond, *Cancer Res.*, 57: 3032-3039, 1997; M. N. Devalaraja et al., *Cancer Res.*, 59: 1372-1377, 1999). To determine whether this endogenous NF-kappaB activation was characteristic of melanoma, we examined the level of constitutive activation of NF-kappaB in a number of melanoma cell lines. We demonstrate here that eight melanoma cell lines exhibit increased IkappaB kinase (IKK) activity, enhanced phosphorylation of IkappaBalpha and p65, and enhanced nuclear localization of p65/p50 in comparison to normal human epidermal melanocytes. The chemokines, CXC ligand 1 (CXCL1) and CXCL8, but not CXCL5, are highly expressed in most of the melanoma cell lines, suggesting that the constitutive production of chemokines is highly correlated to endogenous NF-kappaB activity. Our failure to observe a direct relationship between the fold activation of IKK, CXCL1, or CXCL8 mRNA levels and secretion of these chemokines into the culture medium suggest that regulation of chemokine expression also occurs at the posttranscription level of mRNA stability and/or translational control. Moreover, recombinant CXCL1 can directly induce IKK activity in normal human epidermal melanocytes in a concentration-dependent manner after up-modulation of CXCL1 protein expression, whereas inhibition of IKKbeta activity results in down-modulation of CXCL1 protein expression. Finally, CXCL1 antibody blocks IKK activity and inhibits the proliferation of melanoma cells to further support the concept that the constitutive activation of NF-kappaB and autocrine effects of CXCL1 play an important role in the pathogenesis of melanoma

Brar SS, Kennedy TP, Whorton AR et al. Reactive oxygen species from NAD(P)H:quinone oxidoreductase constitutively activate NF-kappaB in malignant melanoma cells. *Am J Physiol Cell Physiol* 2001 March;280(3):C659-C676.

Abstract: The transcription factor nuclear factor-kappaB (NF-kappaB) is constitutively activated in malignancies from enhanced activity of inhibitor of NF-kappaB (IkappaB) kinase, with accelerated IkappaBalpha degradation. We studied whether redox signaling might stimulate these events. Cultured melanoma cells generated superoxide anions (O(2)(-)) without serum stimulation. O(2)(-) generation was reduced by the NAD(P)H:quinone oxidoreductase (NQO) inhibitor dicumarol and the quinone analog capsaicin, suggesting that electron transfer from NQO through a quinone-mediated pathway may be an important source of endogenous reactive oxygen species (ROS) in tumor cells. Treatment of malignant melanoma cells with the H(2)O(2) scavenger catalase, the sulfhydryl donor N-acetylcysteine, the glutathione peroxidase mimetic ebselen, or dicumarol decreased NF-kappaB activation. Catalase, N-acetylcysteine, ebselen, dicumarol, and capsaicin also inhibited growth of melanoma and other malignant cell lines. These results raise the possibility that ROS produced endogenously by mechanisms involving NQO can constitutively activate NF-kappaB in an autocrine fashion and suggest the potential for new antioxidant strategies for interruption of oxidant signaling of melanoma cell growth

Huang S, Deguzman A, Bucana CD, Fidler IJ. Nuclear factor-kappaB activity correlates with growth, angiogenesis, and metastasis of human melanoma cells in nude mice. *Clin Cancer Res* 2000 June;6(6):2573-81.

Abstract: The purpose of this study was to determine the role of nuclear factor (NF)-kappaB/relA activity in the induction of angiogenesis and production of metastasis by human melanoma cells. Highly metastatic melanoma variant cells expressed high levels of constitutive NF-kappaB activity. Transfection of highly metastatic human melanoma variant cells with a dominant-negative mutant inhibitor of nuclear factor-kappaB alpha (Ikappabeta alpha) expression vector (Ikappabeta alphaM) decreased the level of constitutive NF-kappaB activity, inhibited s.c. tumor growth, and prevented lung metastasis in nude mice. Furthermore, the slow-growing s.c. tumors formed by the IkappaB alphaM-transfected cells exhibited a decrease in microvessel density (angiogenesis), which correlated with a decrease in the level of

interleukin-8 expression. Collectively, these results demonstrate that NF-kappaB/reLA activity significantly contributes to tumorigenicity, angiogenesis, and metastasis of human melanoma cells implanted in nude mice

## **Tocotrienols**

Fu JY, Zhang W, Blatchford DR, Tetley L, McConnell G, Dufes C. Novel tocotrienol-entrapping vesicles can eradicate solid tumors after intravenous administration. *J Control Release* 2011 August 25;154(1):20-6.

Abstract: The therapeutic potential of tocotrienol, a vitamin E extract with anti-cancer properties, is hampered by its failure to specifically reach tumors after intravenous administration. In this work, we demonstrated that novel transferrin-bearing, tocopheryl-based multilamellar vesicles entrapping tocotrienol significantly improved tocotrienol uptake by cancer cells overexpressing transferrin receptors. This led to a dramatically improved therapeutic efficacy in vitro, ranging from 17-fold to 72-fold improvement depending on the cell lines, compared to the free drug. In vivo, the intravenous administration of this novel tocotrienol formulation led to complete tumor eradication for 40% of B16-F10 murine melanoma tumors and 20% of A431 human epidermoid carcinoma tumors. Animal survival was improved by more than 20 days compared to controls, for the two tumor models tested. These therapeutic effects, together with the lack of toxicity, potentially make transferrin-bearing vesicles entrapping tocotrienol a highly promising therapeutic system as part as an anti-cancer therapeutic strategy

de Mesquita ML, Araujo RM, Bezerra DP et al. Cytotoxicity of delta-tocotrienols from *Kielmeyera coriacea* against cancer cell lines. *Bioorg Med Chem* 2011 January 1;19(1):623-30.

Abstract: In the search for new anti-cancer compounds, Brazilian Cerrado plant species have been investigated. The hexane root bark extract of *Kielmeyera coriacea* lead to a mixture of delta-tocotrienol (1) and its dimer (2). The structures of both compounds 1 and 2 were established based on detailed 1D and 2D NMR and EI-MS analyses. The cytotoxicity of the mixture was tested against four human tumor cell lines in the following cultures: MDA-MB-435 (melanoma), HCT-8 (colon), HL-60 (leukemia), and SF-295 (glioblastoma), and displayed IC(50) values ranging from 8.08 to 23.58µg/mL. Additional assays were performed in order to investigate the mechanism of action of the mixture (1+2) against the human leukemia cell line HL-60. The results suggested that the mixture suppressed leukemia growth and reduced cell survival, triggering both apoptosis and necrosis, depending on the concentration

McAnally JA, Gupta J, Sodhani S, Bravo L, Mo H. Tocotrienols potentiate lovastatin-mediated growth suppression in vitro and in vivo. *Exp Biol Med (Maywood)* 2007 April;232(4):523-31.

Abstract: 3-Hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase is the rate-limiting enzyme in the mevalonate pathway that provides essential intermediates for the membrane anchorage and biologic functions of growth-related proteins. Contrary to preclinical studies showing the growth-suppressive activity of statins, competitive inhibitors of HMG CoA reductase, clinical application of statins in cancer is precluded by their lack of activity at levels prescribed for the prevention of cardiovascular disease and by their dose-limiting toxicities at high doses. The dysregulated and elevated HMG CoA reductase activity in tumors retains sensitivity to the isoprenoid-mediated posttranscriptional down-regulation, an action that complements the statin-mediated inhibition and may lead to synergistic impact of blends of isoprenoids and lovastatin on tumor HMG CoA reductase activity and consequently tumor growth. d-gamma- and d-delta-tocotrienols, vitamin E isomers containing an isoprenoid moiety, and lovastatin-

induced concentration-dependent inhibition of the 48-hr proliferation of murine B16 melanoma cells with IC<sub>50</sub> values of 20 +/- 3, 14 +/- 3, and 1.5 +/- 0.4 microM respectively. A blend of lovastatin (1 microM) and d-gamma-tocotrienol (5 microM) totally blocked cell growth, an impact far exceeding the sum of inhibitions induced by lovastatin (12%) and d-gamma-tocotrienol (8%) individually. Synergistic impact of these two agents was also shown in human DU145 prostate carcinoma and human A549 lung carcinoma cells. C57BL6 mice were fed diets supplemented with 12.5 mg lovastatin/kg body weight, 62.5 mg d-delta-tocotrienol/kg body weight, or a blend of both agents for 22 days following B16 cell implantation; only the latter had significantly lower tumor weight than those with no supplementation. Co-administration of isoprenoids that posttranscriptionally down-regulate tumor reductase may lower the effective dose of statins and offer a novel approach to cancer chemo-prevention and/or therapy

He L, Mo H, Hadisusilo S, Qureshi AA, Elson CE. Isoprenoids suppress the growth of murine B16 melanomas in vitro and in vivo. *J Nutr* 1997 May;127(5):668-74.

Abstract: Sundry mevalonate-derived constituents (isoprenoids) of fruits, vegetables and cereal grains suppress the growth of tumors. This study estimated the concentrations of structurally diverse isoprenoids required to inhibit the increase in a population of murine B16(F10) melanoma cells during a 48-h incubation by 50% (IC<sub>50</sub> value). The IC<sub>50</sub> values for d-limonene and perillyl alcohol, the monoterpenes in Phase I trials, were 450 and 250 micromol/L, respectively; related cyclic monoterpenes (perillaldehyde, carvacrol and thymol), an acyclic monoterpene (geraniol) and the end ring analog of beta-carotene (beta-ionone) had IC<sub>50</sub> values in the range of 120-150 micromol/L. The IC<sub>50</sub> value estimated for farnesol, the side-chain analog of the tocotrienols (50 micromol/L) fell midway between that of alpha-tocotrienol (110 micromol/L) and those estimated for gamma- (20 micromol/L) and delta- (10 micromol/L) tocotrienol. A novel tocotrienol lacking methyl groups on the tocol ring proved to be extremely potent (IC<sub>50</sub>, 0.9 micromol/L). In the first of two diet studies, experimental diets were fed to weanling C57BL female mice for 10 d prior to and 28 d following the implantation of the aggressively growing and highly metastatic B16(F10) melanoma. The isomolar (116 micromol/kg diet) and the Vitamin E-equivalent (928 micromol/kg diet) substitution of d-gamma-tocotrienol for dl-alpha-tocopherol in the AIN-76A diet produced 36 and 50% retardations, respectively, in tumor growth (P < 0.05). In the second study, melanomas were established before mice were fed experimental diets formulated with 2 mmol/kg d-gamma-tocotrienol, beta-ionone individually and in combination. Each treatment increased (P < 0.03) the duration of host survival. Our finding that the effects of individual isoprenoids were additive suggests the possibility that one component of the anticarcinogenic action of plant-based diets is the tumor growth-suppressive action of the diverse isoprenoid constituents of fruits, vegetables and cereal grains

## Low-Dose Naltrexone

Donahue RN, McLaughlin PJ, Zagon IS. Low-dose naltrexone targets the opioid growth factor-opioid growth factor receptor pathway to inhibit cell proliferation: mechanistic evidence from a tissue culture model. *Exp Biol Med (Maywood)* 2011 September 1;236(9):1036-50.

Abstract: Naltrexone (NTX) is an opioid antagonist that inhibits or accelerates cell proliferation in vivo when utilized in a low (LDN) or high (HDN) dose, respectively. The mechanism of opioid antagonist action on growth is not well understood. We established a tissue culture model of LDN and HDN using short-term and continuous opioid receptor blockade, respectively, in human ovarian cancer cells, and found that the duration of opioid receptor blockade determines cell proliferative response. The alteration of growth by NTX also was detected in cells representative of pancreatic, colorectal and squamous cell carcinomas. The opioid growth factor (OGF; [Met(5)]-enkephalin) and its receptor (OGFr) were responsible for mediating the action of NTX on cell proliferation. NTX upregulated OGF and OGFr at the

translational but not at the transcriptional level. The mechanism of inhibition by short-term NTX required p16 and/or p21 cyclin-dependent inhibitory kinases, but was not dependent on cell survival (necrosis, apoptosis). Sequential administration of short-term NTX and OGF had a greater inhibitory effect on cell proliferation than either agent alone. Given the parallels between short-term NTX in vitro and LDN in vivo, we now demonstrate at the molecular level that the OGF-OGFr axis is a common pathway that is essential for the regulation of cell proliferation by NTX

Zagon IS, Donahue RN, McLaughlin PJ. Opioid growth factor-opioid growth factor receptor axis is a physiological determinant of cell proliferation in diverse human cancers. *Am J Physiol Regul Integr Comp Physiol* 2009 October;297(4):R1154-R1161.

Abstract: The opioid growth factor (OGF) regulates cell proliferation of human cancer cells through the cyclin-dependent kinase inhibitory pathway, with mediation of this action by the OGF receptor (OGFr). The ubiquity of the OGF-OGFr axis in human cancer is unknown. We used 31 human cancer cell lines, representative of more than 90% of neoplasias occurring in humans, and found that OGF and OGFr were detected in the cytoplasm and nucleus by immunohistochemistry. The addition of OGF to cultures depressed cell number up to 41%, whereas naltrexone (NTX) increased cell proliferation by up to 44%, a total of 85% in the modulating capacity for the OGF-OGFr axis. Neutralization of OGF by specific antibodies led to a marked increase in cell number. Knockdown of OGFr by OGFr-siRNA resulted in a significant increase in the number of cells, even in the face of the addition of exogenous OGF. The cultures to which NTX was added and subjected to OGFr-siRNA were similar to those with OGF-siRNA alone. The OGF-OGFr axis, a physiological determinant of cell-proliferative activity, is a ubiquitous feature of human cancer cells. The identification of this native biological system in neoplasia may be important in understanding the pathophysiology of neoplasia, and in designing treatment modalities that utilize the body's own chemistry

## **Macrophage Activating Factor - GcMAF**

Kisker O, Onizuka S, Becker CM et al. Vitamin D binding protein-macrophage activating factor (DBP-maf) inhibits angiogenesis and tumor growth in mice. *Neoplasia* 2003 January;5(1):32-40.

Abstract: We have isolated a selectively deglycosylated form of vitamin D binding protein (DBP-maf) generated from systemically available DBP by a human pancreatic cancer cell line. DBP-maf is antiproliferative for endothelial cells and antiangiogenic in the chorioallantoic membrane assay. DBP-maf administered daily was able to potently inhibit the growth of human pancreatic cancer in immune compromised mice (T/C=0.09). At higher doses, DBP-maf caused tumor regression. Histological examination revealed that treated tumors had a higher number of infiltrating macrophages as well as reduced microvessel density, and increased levels of apoptosis relative to untreated tumors. Taken together, these data suggest that DBP-maf is an antiangiogenic molecule that can act directly on endothelium as well as stimulate macrophages to attack both the endothelial and tumor cell compartment of a growing malignancy

## **Intravenous Ascorbate Therapy**

Chen Q, Espey MG, Sun AY et al. Pharmacologic doses of ascorbate act as a prooxidant and decrease growth of aggressive tumor xenografts in mice. *Proc Natl Acad Sci U S A* 2008 August 12;105(32):11105-9.

Abstract: Ascorbic acid is an essential nutrient commonly regarded as an antioxidant. In this study, we

showed that ascorbate at pharmacologic concentrations was a prooxidant, generating hydrogen-peroxide-dependent cytotoxicity toward a variety of cancer cells in vitro without adversely affecting normal cells. To test this action in vivo, normal oral tight control was bypassed by parenteral ascorbate administration. Real-time microdialysis sampling in mice bearing glioblastoma xenografts showed that a single pharmacologic dose of ascorbate produced sustained ascorbate radical and hydrogen peroxide formation selectively within interstitial fluids of tumors but not in blood. Moreover, a regimen of daily pharmacologic ascorbate treatment significantly decreased growth rates of ovarian ( $P < 0.005$ ), pancreatic ( $P < 0.05$ ), and glioblastoma ( $P < 0.001$ ) tumors established in mice. Similar pharmacologic concentrations were readily achieved in humans given ascorbate intravenously. These data suggest that ascorbate as a prodrug may have benefits in cancers with poor prognosis and limited therapeutic options

Chen Q, Espey MG, Sun AY et al. Ascorbate in pharmacologic concentrations selectively generates ascorbate radical and hydrogen peroxide in extracellular fluid in vivo. *Proc Natl Acad Sci U S A* 2007 May 22;104(21):8749-54.

Abstract: Ascorbate (ascorbic acid, vitamin C), in pharmacologic concentrations easily achieved in humans by i.v. administration, selectively kills some cancer cells but not normal cells. We proposed that pharmacologic ascorbate is a prodrug for preferential steady-state formation of ascorbate radical ( $\text{Asc}^{*-}$ ) and  $\text{H}(2)\text{O}(2)$  in the extracellular space compared with blood. Here we test this hypothesis in vivo. Rats were administered parenteral (i.v. or i.p.) or oral ascorbate in typical human pharmacologic doses (approximately 0.25-0.5 mg per gram of body weight). After i.v. injection, ascorbate baseline concentrations of 50-100  $\mu\text{M}$  in blood and extracellular fluid increased to peaks of  $>8$  mM. After i.p. injection, peaks approached 3 mM in both fluids. By gavage, the same doses produced ascorbate concentrations of  $<150$   $\mu\text{M}$  in both fluids. In blood,  $\text{Asc}^{*-}$  concentrations measured by EPR were undetectable with oral administration and always  $<50$  nM with parenteral administration, even when corresponding ascorbate concentrations were  $>8$  mM. After parenteral dosing,  $\text{Asc}^{*-}$  concentrations in extracellular fluid were 4- to 12-fold higher than those in blood, were as high as 250 nM, and were a function of ascorbate concentrations. By using the synthesized probe peroxyxanthone,  $\text{H}(2)\text{O}(2)$  in extracellular fluid was detected only after parenteral administration of ascorbate and when  $\text{Asc}^{*-}$  concentrations in extracellular fluid exceeded 100 nM. The data show that pharmacologic ascorbate is a prodrug for preferential steady-state formation of  $\text{Asc}^{*-}$  and  $\text{H}(2)\text{O}(2)$  in the extracellular space but not blood. These data provide a foundation for pursuing pharmacologic ascorbate as a prooxidant therapeutic agent in cancer and infections

Chen Q, Espey MG, Krishna MC et al. Pharmacologic ascorbic acid concentrations selectively kill cancer cells: action as a pro-drug to deliver hydrogen peroxide to tissues. *Proc Natl Acad Sci U S A* 2005 September 20;102(38):13604-9.

Abstract: Human pharmacokinetics data indicate that i.v. ascorbic acid (ascorbate) in pharmacologic concentrations could have an unanticipated role in cancer treatment. Our goals here were to test whether ascorbate killed cancer cells selectively, and if so, to determine mechanisms, using clinically relevant conditions. Cell death in 10 cancer and 4 normal cell types was measured by using 1-h exposures. Normal cells were unaffected by 20 mM ascorbate, whereas 5 cancer lines had  $\text{EC}(50)$  values of  $<4$  mM, a concentration easily achievable i.v. Human lymphoma cells were studied in detail because of their sensitivity to ascorbate ( $\text{EC}(50)$  of 0.5 mM) and suitability for addressing mechanisms. Extracellular but not intracellular ascorbate mediated cell death, which occurred by apoptosis and pyknosis/necrosis. Cell death was independent of metal chelators and absolutely dependent on  $\text{H}(2)\text{O}(2)$  formation. Cell death from  $\text{H}(2)\text{O}(2)$  added to cells was identical to that found when  $\text{H}(2)\text{O}(2)$  was generated by ascorbate treatment.  $\text{H}(2)\text{O}(2)$  generation was dependent on ascorbate concentration, incubation time, and the presence of 0.5-10% serum, and displayed a linear relationship with ascorbate radical formation. Although ascorbate addition to medium generated  $\text{H}(2)\text{O}(2)$ , ascorbate addition to blood generated no detectable  $\text{H}(2)\text{O}(2)$  and only trace detectable ascorbate radical. Taken together, these data indicate that ascorbate at concentrations achieved only by i.v.

administration may be a pro-drug for formation of H<sub>2</sub>O<sub>2</sub>, and that blood can be a delivery system of the pro-drug to tissues. These findings give plausibility to i.v. ascorbic acid in cancer treatment, and have unexpected implications for treatment of infections where H<sub>2</sub>O<sub>2</sub> may be beneficial

## Valproate

Landreville S, Agapova OA, Matatall KA et al. Histone deacetylase inhibitors induce growth arrest and differentiation in uveal melanoma. *Clin Cancer Res* 2012 January 15;18(2):408-16.

Abstract: PURPOSE: Metastasis is responsible for the death of most cancer patients, yet few therapeutic agents are available which specifically target the molecular events that lead to metastasis. We recently showed that inactivating mutations in the tumor suppressor gene BAP1 are closely associated with loss of melanocytic differentiation in uveal melanoma (UM) and metastasis. The purpose of this study was to identify therapeutic agents that reverse the phenotypic effects of BAP1 loss in UM. EXPERIMENTAL DESIGN: In silico screens were done to identify therapeutic compounds predicted to differentiate UM cells using Gene Set Enrichment Analysis and Connectivity Map databases. Valproic acid (VPA), trichostatin A, LBH-589, and suberoylanilide hydroxamic acid were evaluated for their effects on UM cells using morphologic evaluation, MTS viability assays, bromodeoxyuridine incorporation, flow cytometry, clonogenic assays, gene expression profiling, histone acetylation and ubiquitination assays, and a murine xenograft tumorigenicity model. RESULTS: Histone deacetylase (HDAC) inhibitors induced morphologic differentiation, cell-cycle exit, and a shift to a differentiated, melanocytic gene expression profile in cultured UM cells. VPA inhibited the growth of UM tumors in vivo. CONCLUSIONS: These findings suggest that HDAC inhibitors may have therapeutic potential for inducing differentiation and prolonged dormancy of micrometastatic disease in UM

Ouyang D, Zhang Y, Xu L, Li J, Zha Q, He X. Histone deacetylase inhibitor valproic acid sensitizes B16F10 melanoma cells to cucurbitacin B treatment. *Acta Biochim Biophys Sin (Shanghai)* 2011 June;43(6):487-95.

Abstract: Cucurbitacin B (CuB) is reported to have anti-proliferation effects on a variety of tumors including melanoma, and more effective regimens by combination of this agent with others are under investigation. In this study, the anti-melanoma effect of CuB as a single agent and in combination with valproic acid (VPA), an inhibitor of histone deacetylase (HDAC), was evaluated in B16F10, a mouse melanoma cell line. The results demonstrated that CuB inhibited the proliferation of the cell line in a dose-dependent manner. However, it was likely that a pro-survival compensatory response, involving the induction of autophagy and upregulation of anti-apoptotic Bcl-2 protein, was induced by CuB treatment, which might greatly decrease the cytotoxicity of this agent. Supporting this, the melanoma cells were found to be more sensitive to the combination of CuB with chloroquine, a well-known autophagy inhibitor. And CuB-induced autophagy was associated with c-Jun N-terminal kinase (JNK) activation, at least partly, since inhibition of JNK activity by SP600125 could alleviate the autophagy. When CuB was combined with VPA, the two drugs showed synergistic cytotoxicity by induction of cell apoptosis. Moreover, the multiploidization effect of CuB was also suppressed in the presence of VPA. In contrast to the transient activation of JNKs by CuB, the combination of CuB and VPA resulted in prolonged JNK activation, although at low level after 4 h. Our results demonstrated that HDAC inhibitor VPA can sensitize B16F10 cells to CuB treatment through induction of apoptotic pathway

Roos WP, Jost E, Belohlavek C, Nagel G, Fritz G, Kaina B. Intrinsic anticancer drug resistance of malignant melanoma cells is abrogated by IFN-beta and valproic acid. *Cancer Res* 2011 June 15;71(12):4150-60.

Abstract: Malignant melanoma, once metastasized, has a dismal prognosis because of intrinsic resistance

to anticancer drugs. First-line therapy includes the methylating agents dacarbazine and temozolomide. Although DNA mismatch repair and O(6)-methylguanine (O(6)MeG)-DNA methyltransferase (MGMT) are key determinants of cellular resistance to these drugs, there is no correlation between these markers and the therapeutic response in melanoma, indicating as yet unknown mechanisms of drug resistance. We show that in malignant melanoma cells with wild-type p53, the temozolomide-induced DNA damage O(6)MeG triggers upregulation of the Fas/CD95/Apo-1 receptor without activating the apoptosis cascade. This is due to silencing of procaspase-8. A single treatment with IFN-beta reactivated procaspase-8 and sensitized melanoma cells to temozolomide. The key role of procaspase-8 in melanoma cell sensitization was verified by experiments in which the death receptor pathway was blocked by expression of dominant-negative FADD, siRNA knockdown of procaspase-8, or stimulation with Fas/CD95/Apo-1 activating antibody. The expression of procaspase-8 could further be enhanced by additional pretreatment with the histone deacetylase inhibitor valproic acid (VPA), which together with IFN-beta caused significant sensitization of melanoma cells in vitro. Sensitization of melanoma cells to temozolomide by IFN-beta and VPA was also shown in a xenograft mouse model. The data provide a plausible explanation why therapy of malignant melanomas with alkylating anticancer drugs failed even in trials where the repair of the critical toxic lesion O(6)MeG was blocked by MGMT inhibitors and suggest approaches to abrogate intrinsic drug resistance by IFN and VPA-mediated reactivation of the death receptor pathway

Daud AI, Dawson J, DeConti RC et al. Potentiation of a topoisomerase I inhibitor, karenitecin, by the histone deacetylase inhibitor valproic acid in melanoma: translational and phase I/II clinical trial. *Clin Cancer Res* 2009 April 1;15(7):2479-87.

Abstract: **PURPOSE:** The novel topoisomerase I inhibitor karenitecin (KTN) shows activity against melanoma. We examined whether histone deacetylase inhibition could potentiate the DNA strand cleavage, cytotoxicity as well as the clinical toxicity, and efficacy of KTN in melanoma. **EXPERIMENTAL DESIGN:** Apoptosis, COMET, and xenograft experiments were carried out as described previously. A phase I/II trial of valproic acid (VPA) and KTN was conducted in patients with stage IV melanoma, with any number of prior therapies, Eastern Cooperative Oncology Group performance status 0-2, and adequate organ function. **RESULTS:** VPA pretreatment potentiated KTN-induced apoptosis in multiple melanoma cell lines and in mouse A375 xenografts. VPA increased KTN-induced DNA strand breaks. In the phase I/II trial, 39 patients were entered, with 37 evaluable for toxicity and 33 evaluable for response. Somnolence was the dose-limiting toxicity. The maximum tolerated dose for VPA was 75 mg/kg/d; at maximum tolerated dose, serum VPA was approximately 200 microg/mL (1.28 mmol/L). At the dose expansion cohort, 47% (7 of 15) of patients had stable disease; median overall survival and time to progression were 32.8 and 10.2 weeks, respectively. Histone hyperacetylation was observed in peripheral blood mononuclear cells at maximum tolerated dose. **CONCLUSION:** VPA potentiates KTN-induced DNA strand breaks and cytotoxicity. VPA can be combined at 75 mg/kg/d for 5 days with full-dose KTN without overlapping toxicities. In metastatic poor prognosis melanoma, this combination is associated with disease stabilization in 47% of patients. Further testing of this combination appears warranted

Rocca A, Minucci S, Tosti G et al. A phase I-II study of the histone deacetylase inhibitor valproic acid plus chemoimmunotherapy in patients with advanced melanoma. *Br J Cancer* 2009 January 13;100(1):28-36.

Abstract: We explored in a phase I/II clinical trial the combination of valproic acid (VPA), a clinically available histone deacetylase inhibitor, with standard chemoimmunotherapy in patients with advanced melanoma, to evaluate its clinical activity, to correlate the clinical response with the biological activity of VPA and to assess toxicity. Patients were treated initially with VPA alone for 6 weeks. The inhibition of the target in non-tumour peripheral blood cells (taken as a potential surrogate marker) was measured periodically, and valproate dosing adjusted with the attempt to reach a measurable inhibition. After the

treatment with valproate alone, dacarbazine plus interferon-alpha was started in combination with valproate. Twenty-nine eligible patients started taking valproate and 18 received chemoimmunotherapy and are assessable for response. We observed one complete response, two partial remissions and three disease stabilisations lasting longer than 24 weeks. With the higher valproate dosages needed to reach a measurable inhibition of the target, we observed an increase of side effects in those patients who received chemoimmunotherapy. The combination of VPA and chemoimmunotherapy did not produce results overtly superior to standard therapy in patients with advanced melanoma and toxicity was not negligible, casting some doubts on the clinical use of VPA in this setting (at least in the administration schedule adopted)

Khan AN, Gregorie CJ, Tomasi TB. Histone deacetylase inhibitors induce TAP, LMP, Tapasin genes and MHC class I antigen presentation by melanoma cells. *Cancer Immunol Immunother* 2008 May;57(5):647-54.

Abstract: Histone deacetylase inhibitors (HDACi), including trichostatin A (TSA) and valproic acid, can alter the acetylation of histones in chromatin and enhance gene transcription. Previously we demonstrated that HDACi-treated tumor cells are capable of presenting antigen via the MHC class II pathway. In this study, we show that treatment with HDACi enhances the expression of molecules (TAP1, TAP2, LMP2, LMP7, Tapasin and MHC class I) involved in antigen processing and presentation via the MHC class I pathway in melanoma cells. HDACi treatment of B16F10 cells also enhanced cell surface expression of class I and costimulatory molecules CD40 and CD86. Enhanced transcription of these genes is associated with a significant increase in direct presentation of whole protein antigen and MHC class I-restricted peptides by TSA-treated B16F10 cells. Our data indicate that epigenetic modification can convert a tumor cell to an antigen presenting cell capable of activating IFN-gamma secreting T cells via the class I pathway. These findings suggest that the abnormalities, observed in some tumors in the expression of MHC class I antigen processing and presentation molecules, may result from epigenetic repression

Valentini A, Gravina P, Federici G, Bernardini S. Valproic acid induces apoptosis, p16INK4A upregulation and sensitization to chemotherapy in human melanoma cells. *Cancer Biol Ther* 2007 February;6(2):185-91.

Abstract: It is known that melanoma develops as a consequence of multifactorial alterations. To date several studies indicate the effective implication of p16 as a tumor suppressor gene with a major role in either the development or progression of human melanoma. Deregulation of melanoma cell growth has been widely associated with mutations in the p16-cyclin D/cdk4-pRb pathway. Recently anticancer therapies are focused on restoration of p16 CDK inhibitory function and other proteins unregulated in melanoma cell cycle pathway (e.g., c-myc, p27). A combined strategy for restoration of normal homeostasis in the melanoma skin with targeted delivery of apoptosis-inducing agents does not seem to be far obtained. New class of antitumoral agents are emerging: histone deacetylase (HDAC) inhibitors have attracted much interest because of their ability to arrest cell growth, induce cell differentiation, and in some cases, induce apoptosis of cancer cells. Recently, attention has been focused on the ability of HDAC inhibitors to induce perturbation in cell cycle regulatory protein (e.g., p21(CIP1)) and down-regulation of survival signalling pathway. In the present study, we have examined the effect of valproic acid (VPA) on M14 human melanoma cell line. Here we observed that VPA induces cell cycle arrest and apoptosis sensitising melanoma cells to cis-platin and etoposide treatment. IC(50) dose (2.99 mM) of VPA was able to induce G(1) arrest (up to 75%) in association with upregulation of p16, p21 and cyclin-D1 related to Rb ipo-phosphorylation. In addition VPA activated apoptosis (50%) in M14 cells, when given alone or in combination with antitumoral agents. The ability of valproic acid to reestablished the G(1) pathway in melanoma cells suggests a potential application of VPA in melanoma therapeutic protocols

Facchetti F, Previdi S, Ballarini M, Minucci S, Perego P, La Porta CA. Modulation of pro- and anti-apoptotic factors in human melanoma cells exposed to histone deacetylase inhibitors. *Apoptosis* 2004 September;9(5):573-82.

Abstract: Valproic acid (VPA, 2-propylpentanoic acid) is an established drug in the long-term therapy of epilepsy. Recently, VPA was demonstrated to inhibit histone deacetylases (HDACs) class I enzyme at therapeutically relevant concentrations, thereby, mimicking the prototypical histone deacetylase inhibitors, trichostatin A (TSA) or suberoylanilide hydroxamic acid (SAHA). In the present study, we investigated the cellular effects of VPA, TSA and SAHA on four human melanoma cell lines (WM115, WM266, A375, SK-Mel28) with particular reference to the modulation of regulators of apoptosis, including Bcl-2, BclXL, Mcl-1, Apaf-1, BclXs, NOXA, TRAIL-R1, TRAIL-R2, caspase 8, and survivin). Firstly, we found that VPA induced apoptosis in two of the four human melanoma cell lines, while both TSA and SAHA exhibited an antiproliferative and apoptotic effects in all four cell lines, a different expression of Bcl-2 and BclX(L/S) occurred. On the other hand, SAHA and VPA modulated differently pro- and anti-apoptotic factors. In particular, the treatment with VPA enhanced the level of expression of survivin only in VPA-resistant cell lines, whereas down-regulation of survivin was induced by VPA and SAHA in VPA-sensitive cells. In the latter, since activation of caspase 8 was documented, a receptor-mediated apoptosis was suggested. Taken together, our results suggest that HDAC inhibitors may represent a promising therapeutic strategy to treat melanoma

## **Nelfinavir**

Jiang W, Mikochik PJ, Ra JH et al. HIV protease inhibitor nelfinavir inhibits growth of human melanoma cells by induction of cell cycle arrest. *Cancer Res* 2007 February 1;67(3):1221-7.

Abstract: HIV protease inhibitors (HIV PI) are a class of antiretroviral drugs that are designed to target the viral protease. Unexpectedly, this class of drugs is also reported to have antitumor activity. In this study, we have evaluated the in vitro activity of nelfinavir, a HIV PI, against human melanoma cells. Nelfinavir inhibits the growth of melanoma cell lines at low micromolar concentrations that are clinically attainable. Nelfinavir promotes apoptosis and arrests cell cycle at G(1) phase. Cell cycle arrest is attributed to inhibition of cyclin-dependent kinase 2 (CDK2) and concomitant dephosphorylation of retinoblastoma tumor suppressor. We further show that nelfinavir inhibits CDK2 through proteasome-dependent degradation of Cdc25A phosphatase. Our results suggest that nelfinavir is a promising candidate chemotherapeutic agent for advanced melanoma, for which novel and effective therapies are urgently needed

Bernstein WB, Dennis PA. Repositioning HIV protease inhibitors as cancer therapeutics. *Curr Opin HIV AIDS* 2008 November;3(6):666-75.

Abstract: PURPOSE OF REVIEW: Although designed to target only the HIV protease, HIV protease inhibitors induce toxicities in patients such as insulin resistance and lipodystrophy that suggest that protease inhibitors have other targets in mammalian cells. Akt controls insulin signaling and is an important target in cancer, but no Akt inhibitors are approved as cancer therapeutics. These observations have prompted the study of HIV protease inhibitors as inhibitors of Akt and possible cancer therapeutics. This review will highlight the latest advances in repositioning HIV protease inhibitors as cancer therapeutics. RECENT FINDINGS: Although protease inhibitors can inhibit Akt activation and the proliferation of over 60 cancer cell lines, as well as improve sensitivity to radiation or chemotherapy, these effects do not always correlate with Akt inhibition. Other important processes, such as the induction of endoplasmic reticulum stress, appear critical to the biological activity of

protease inhibitors. These impressive and surprising preclinical data have prompted clinical testing of nelfinavir as a lead HIV protease inhibitor in cancer patients. **SUMMARY:** Although mechanisms of action for the antitumor effects of HIV protease inhibitors are complex, their broad spectrum of activity, minimal toxicity, and wide availability make protease inhibitors ideal candidates for repositioning as cancer therapeutics

## **Chloroquine/Hydroxychloroquine**

Ma XH, Piao S, Wang D et al. Measurements of tumor cell autophagy predict invasiveness, resistance to chemotherapy, and survival in melanoma. *Clin Cancer Res* 2011 May 15;17(10):3478-89.

**Abstract:** **PURPOSE:** Autophagy consists of lysosome-dependent degradation of cytoplasmic contents sequestered by autophagic vesicles (AV). The role of autophagy in determining tumor aggressiveness and response to therapy in melanoma was investigated in this study. **EXPERIMENTAL DESIGN:** Autophagy was measured in tumor biopsies obtained from metastatic melanoma patients enrolled on a phase II trial of temozolomide and sorafenib and correlated to clinical outcome. These results were compared with autophagy measurements in aggressive and indolent melanoma cells grown in two- and three-dimensional (3D) culture and as xenograft tumors. The effects of autophagy inhibition with either hydroxychloroquine or inducible shRNA (short hairpin RNA) against the autophagy gene ATG5 were assessed in three-dimensional spheroids. **RESULTS:** Patients whose tumors had a high autophagic index were less likely to respond to treatment and had a shorter survival compared with those with a low autophagic index. Differences in autophagy were less evident in aggressive and indolent melanoma cells grown in monolayer culture. In contrast, autophagy was increased in aggressive compared with indolent melanoma xenograft tumors. This difference was recapitulated when aggressive and indolent melanoma cells were grown as spheroids. Autophagy inhibition with either hydroxychloroquine or inducible shRNA against ATG5 resulted in cell death in aggressive melanoma spheroids, and significantly augmented temozolomide-induced cell death. **CONCLUSIONS:** Autophagy is a potential prognostic factor and therapeutic target in melanoma. Three dimensional culture mimics the tumor microenvironment better than monolayer culture and is an appropriate model for studying therapeutic combinations involving autophagy modulators. Autophagy inhibition should be tested clinically in patients with melanoma

Noman MZ, Janji B, Kaminska B et al. Blocking hypoxia-induced autophagy in tumors restores cytotoxic T-cell activity and promotes regression. *Cancer Res* 2011 September 15;71(18):5976-86.

**Abstract:** The relationship between hypoxic stress, autophagy, and specific cell-mediated cytotoxicity remains unknown. This study shows that hypoxia-induced resistance of lung tumor to cytolytic T lymphocyte (CTL)-mediated lysis is associated with autophagy induction in target cells. In turn, this correlates with STAT3 phosphorylation on tyrosine 705 residue (pSTAT3) and HIF-1alpha accumulation. Inhibition of autophagy by siRNA targeting of either beclin1 or Atg5 resulted in impairment of pSTAT3 and restoration of hypoxic tumor cell susceptibility to CTL-mediated lysis. Furthermore, inhibition of pSTAT3 in hypoxic Atg5 or beclin1-targeted tumor cells was found to be associated with the inhibition Src kinase (pSrc). Autophagy-induced pSTAT3 and pSrc regulation seemed to involve the ubiquitin proteasome system and p62/SQSTM1. In vivo experiments using B16-F10 melanoma tumor cells indicated that depletion of beclin1 resulted in an inhibition of B16-F10 tumor growth and increased tumor apoptosis. Moreover, in vivo inhibition of autophagy by hydroxychloroquine in B16-F10 tumor-bearing mice and mice vaccinated with tyrosinase-related protein-2 peptide dramatically increased tumor growth inhibition. Collectively, this study establishes a novel functional link between hypoxia-induced autophagy and the regulation of antigen-specific T-cell lysis and points to a major role of autophagy in the control of in vivo tumor growth

Harhaji-Trajkovic L, Arsikin K, Kravic-Stevovic T et al. Chloroquine-mediated lysosomal dysfunction enhances the anticancer effect of nutrient deprivation. *Pharm Res* 2012 August;29(8):2249-63.

Abstract: PURPOSE: To investigate the ability of chloroquine, a lysosomotropic autophagy inhibitor, to enhance the anticancer effect of nutrient deprivation. METHODS: Serum-deprived U251 glioma, B16 melanoma and L929 fibrosarcoma cells were treated with chloroquine in vitro. Cell viability was measured by crystal violet and MTT assay. Oxidative stress, apoptosis/necrosis and intracellular acidification were analyzed by flow cytometry. Cell morphology was examined by light and electron microscopy. Activation of AMP-activated protein kinase (AMPK) and autophagy were monitored by immunoblotting. RNA interference was used for AMPK and LC3b knockdown. The anticancer efficiency of intraperitoneal chloroquine in calorie-restricted mice was assessed using a B16 mouse melanoma model. RESULTS: Chloroquine rapidly killed serum-starved cancer cells in vitro. This effect was not mimicked by autophagy inhibitors or LC3b shRNA, indicating autophagy-independent mechanism. Chloroquine-induced lysosomal accumulation and oxidative stress, leading to mitochondrial depolarization, caspase activation and mixed apoptotic/necrotic cell death, were prevented by lysosomal acidification inhibitor bafilomycin. AMPK downregulation participated in chloroquine action, as AMPK activation reduced, and AMPK shRNA mimicked chloroquine toxicity. Chloroquine inhibited melanoma growth in calorie-restricted mice, causing lysosomal accumulation, mitochondrial disintegration and selective necrosis of tumor cells. CONCLUSION: Combined treatment with chloroquine and calorie restriction might be useful in cancer therapy

Liang X, de Vera ME, Buchser WJ et al. Inhibiting systemic autophagy during interleukin 2 immunotherapy promotes long-term tumor regression. *Cancer Res* 2012 June 1;72(11):2791-801.

Abstract: Administration of high-dose interleukin-2 (HDIL-2) has durable antitumor effects in 5% to 10% of patients with melanoma and renal cell carcinoma. However, treatment is often limited by side effects, including reversible, multiorgan dysfunction characterized by a cytokine-induced systemic autophagic syndrome. Here, we hypothesized that the autophagy inhibitor chloroquine would enhance IL-2 immunotherapeutic efficacy and limit toxicity. In an advanced murine metastatic liver tumor model, IL-2 inhibited tumor growth in a dose-dependent fashion. These antitumor effects were significantly enhanced upon addition of chloroquine. The combination of IL-2 with chloroquine increased long-term survival, decreased toxicity associated with vascular leakage, and enhanced immune cell proliferation and infiltration in the liver and spleen. HDIL-2 alone increased serum levels of HMGB1, IFN-gamma, IL-6, and IL-18 and also induced autophagy within the liver and translocation of HMGB1 from the nucleus to the cytosol in hepatocytes, effects that were inhibited by combined administration with chloroquine. In tumor cells, chloroquine increased autophagic vacuoles and LC3-II levels inhibited oxidative phosphorylation and ATP production and promoted apoptosis, which was associated with increased Annexin-V(+)/propidium iodide (PI)(-) cells, cleaved PARP, cleaved caspase-3, and cytochrome c release from mitochondria. Taken together, our findings provide a novel clinical strategy to enhance the efficacy of HDIL-2 immunotherapy for patients with cancer

Inoue S, Hasegawa K, Ito S, Wakamatsu K, Fujita K. Antimelanoma activity of chloroquine, an antimalarial agent with high affinity for melanin. *Pigment Cell Res* 1993 October;6(5):354-8.

Abstract: The antimalarial agent chloroquine is known for high affinity for melanin. This 4-aminoquinoline derivative was examined for anti-melanoma activity and uptake into melanoma cells. Chloroquine inhibited growth of cultured melanoma cells; the effect was much greater to a moderately pigmented cell line HMV-II than to a nonpigmented HMV-I. Treatment with chloroquine at a dose of 62 mg/kg i.p. for 12 days prolonged by 71% the life span of mice bearing B16 melanoma, while 24-day treatment at 31 mg/kg resulted in a 81% increase in life span. HMV-II cells showed a two-fold increase in uptake of chloroquine as compared with HMV-I cells. Chloroquine, 24 hr after administration to mice

implanted s.c. with B16 melanoma, was selectively accumulated in the pigmented tissues, melanoma and eyes. Other nonpigmented tissues such as the liver, lung, and kidney showed rapid uptake (within 1 hr) and release. These results suggest that chloroquine is toxic to pigmented melanoma cells, the process being partly mediated by binding to melanin

Djordjevic B, Lange CS, Rotman M. Potentiation of radiation lethality in mouse melanoma cells by mild hyperthermia and chloroquine. *Melanoma Res* 1992 December;2(5-6):321-6.

Abstract: To test the hypothesis that radiosensitization by combined mild hyperthermia and chloroquine may be increased by the presence of melanin in treated cells, Cloudman melanotic mouse melanoma S91/6 cells, and the amelanotic S91/amel cells were incubated during a 3 h post-irradiation period with 0.03 mM chloroquine at 41 degrees C. A considerable increase in radiation lethality was observed (radiation potentiation factor > 1.6) in both cases. Addition of 0.1 mM isobutyl-methyl xanthine (IBMX), a promoter of melanin synthesis, to the growth medium of S91/6 cells 10 days before irradiation, did not further increase the lethality of radiation followed by combined heat and chloroquine treatment. Under these conditions, toxicity to unirradiated cells was slight. On the other hand, 10 microM chloroquine showed similar toxicity to unirradiated B-16 mouse melanoma cells, but did not increase radiation lethality. Factors other than melanin content therefore play a role in the potentiation of radiation lethality by mild hyperthermia and chloroquine

Zbytniewski Z, Drewa G. Inhibiting effect of chloroquine diphosphate on the growth of tumors and activity of some acid hydrolases of a melanotic melanoma in golden hamsters (*Mesocricetus auratus*, Waterhouse). *Arch Immunol Ther Exp (Warsz)* 1973;21(6):877-81.

### **Itraconazole (Inhibitor of Hedgehog Pathway)**

Kim J, Tang JY, Gong R et al. Itraconazole, a commonly used antifungal that inhibits Hedgehog pathway activity and cancer growth. *Cancer Cell* 2010 April 13;17(4):388-99.

Abstract: In a screen of drugs previously tested in humans we identified itraconazole, a systemic antifungal, as a potent antagonist of the Hedgehog (Hh) signaling pathway that acts by a mechanism distinct from its inhibitory effect on fungal sterol biosynthesis. Systemically administered itraconazole, like other Hh pathway antagonists, can suppress Hh pathway activity and the growth of medulloblastoma in a mouse allograft model and does so at serum levels comparable to those in patients undergoing antifungal therapy. Mechanistically, itraconazole appears to act on the essential Hh pathway component Smoothed (SMO) by a mechanism distinct from that of cyclopamine and other known SMO antagonists, and prevents the ciliary accumulation of SMO normally caused by Hh stimulation

Santini R, Vinci MC, Pandolfi S et al. Hedgehog-GLI signaling drives self-renewal and tumorigenicity of human melanoma-initiating cells. *Stem Cells* 2012 September;30(9):1808-18.

Abstract: The question of whether cancer stem/tumor-initiating cells (CSC/TIC) exist in human melanomas has arisen in the last few years. Here, we have used nonadherent spheres and the aldehyde dehydrogenase (ALDH) enzymatic activity to enrich for CSC/TIC in a collection of human melanomas obtained from a broad spectrum of sites and stages. We find that melanomaspheres display extensive in vitro self-renewal ability and sustain tumor growth in vivo, generating human melanoma xenografts that recapitulate the phenotypic composition of the parental tumor. Melanomaspheres express high levels of Hedgehog (HH) pathway components and of embryonic pluripotent stem cell factors SOX2, NANOG,

OCT4, and KLF4. We show that human melanomas contain a subset of cells expressing high ALDH activity (ALDH(high)), which is endowed with higher self-renewal and tumorigenic abilities than the ALDH(low) population. A good correlation between the number of ALDH(high) cells and sphere formation efficiency was observed. Notably, both pharmacological inhibition of HH signaling by the SMOOTHENED (SMO) antagonist cyclopamine and GLI antagonist GANT61 and stable expression of shRNA targeting either SMO or GLI1 result in a significant decrease in melanoma stem cell self-renewal in vitro and a reduction in the number of ALDH(high) melanoma stem cells. Finally, we show that interference with the HH-GLI pathway through lentiviral-mediated silencing of SMO and GLI1 drastically diminishes tumor initiation of ALDH(high) melanoma stem cells. In conclusion, our data indicate an essential role of the HH-GLI1 signaling in controlling self-renewal and tumor initiation of melanoma CSC/TIC. Targeting HH-GLI1 is thus predicted to reduce the melanoma stem cell compartment

Li C, Chi S, Xie J. Hedgehog signaling in skin cancers. *Cell Signal* 2011 August;23(8):1235-43.

Abstract: An increasing progress on the role of Hedgehog (Hh) signaling for carcinogenesis has been achieved since the link of Hh pathway to human cancer was firstly established. In particular, the critical role of Hh signaling in the development of Basal cell carcinoma (BCC) has been convincingly demonstrated by genetic mutation analyses, mouse models of BCCs, and successful clinical trials of BCCs using Hh signaling inhibitors. In addition, the Hh pathway activity is also reported to be involved in the pathogenesis of Squamous Cell Carcinoma (SCC), melanoma and Merkel Cell Carcinoma. These findings have significant new paradigm on Hh signaling transduction, its mechanisms in skin cancer and even therapeutic approaches for BCC. In this review, we will summarize the major advances in the understanding of Hh signaling transduction, the roles of Hh signaling in skin cancer development, and the current implications of "mechanism-based" therapeutic strategies

Alexaki VI, Javelaud D, Van Kempen LC et al. GLI2-mediated melanoma invasion and metastasis. *J Natl Cancer Inst* 2010 August 4;102(15):1148-59.

Abstract: BACKGROUND: The transforming growth factor-beta (TGF-beta) pathway, which has both tumor suppressor and pro-oncogenic activities, is often constitutively active in melanoma and is a marker of poor prognosis. Recently, we identified GLI2, a mediator of the hedgehog pathway, as a transcriptional target of TGF-beta signaling. METHODS: We used real-time reverse transcription-polymerase chain reaction (RT-PCR) and western blotting to determine GLI2 expression in human melanoma cell lines and subsequently classified them as GLI2high or as GLI2low according to their relative GLI2 mRNA and protein expression levels. GLI2 expression was reduced in a GLI2high cell line with lentiviral expression of short hairpin RNA targeting GLI2. We assessed the role of GLI2 in melanoma cell invasiveness in Matrigel assays. We measured secretion of matrix metalloproteinase (MMP)-2 and MMP-9 by gelatin zymography and expression of E-cadherin by western blotting and RT-PCR. The role of GLI2 in development of bone metastases was determined following intracardiac injection of melanoma cells in immunocompromised mice (n = 5-13). Human melanoma samples (n = 79) at various stages of disease progression were analyzed for GLI2 and E-cadherin expression by immunohistochemistry, in situ hybridization, or RT-PCR. All statistical tests were two-sided. RESULTS: Among melanoma cell lines, increased GLI2 expression was associated with loss of E-cadherin expression and with increased capacity to invade Matrigel and to form bone metastases in mice (mean osteolytic tumor area: GLI2high vs GLI2low, 2.81 vs 0.93 mm(2), difference = 1.88 mm(2), 95% confidence interval [CI] = 1.16 to 2.60, P < .001). Reduction of GLI2 expression in melanoma cells that had expressed high levels of GLI2 substantially inhibited both basal and TGF-beta-induced cell migration, invasion (mean number of Matrigel invading cells: shGLI2 vs shCtrl (control), 52.6 vs 100, difference = 47.4, 95% CI = 37.0 to 57.8, P = .024; for shGLI2 + TGF-beta vs shCtrl + TGF-beta, 31.0 vs 161.9, difference = -130.9, 95% CI = -96.2 to -165.5, P = .002), and MMP secretion in vitro and the development of experimental bone

metastases in mice. Within human melanoma lesions, GLI2 expression was heterogeneous, associated with tumor regions in which E-cadherin was lost and increased in the most aggressive tumors. CONCLUSION: GLI2 was directly involved in driving melanoma invasion and metastasis in this preclinical study

Das S, Harris LG, Metge BJ et al. The hedgehog pathway transcription factor GLI1 promotes malignant behavior of cancer cells by up-regulating osteopontin. *J Biol Chem* 2009 August 21;284(34):22888-97.

Abstract: The role of Hedgehog (Hh) signaling as a developmental pathway is well established. Several recent studies have implicated a role for this pathway in multiple cancers. In this study we report that expression of GLI1 and osteopontin (OPN) increase progressively with the progression of melanoma from primary cutaneous cancer to metastatic melanoma in clinically derived specimens. We have further determined that OPN is a direct transcriptional target of GLI1. We have observed that OPN expression is stimulated in the presence of Hh ligands and inhibited in the presence of the Smoothed (SMO) inhibitor, cyclopamine. Transcriptional silencing of GLI1 negatively impacts OPN expression and compromises the ability of cancer cells to proliferate, migrate, and invade in vitro and interferes with their ability to grow as xenografts and spontaneously metastasize in nude mice. These altered attributes could be rescued by re-expressing OPN in the GLI1-silenced cells, suggesting that OPN is a critical downstream effector of active GLI1 signaling. Our observations lead us to conclude that the GLI1-mediated up-regulation of OPN promotes malignant behavior of cancer cells

Bar-Eli M. The HOG-GLI face of melanoma. *Pigment Cell Res* 2007 October;20(5):341-2.

Geng L, Cuneo KC, Cooper MK et al. Hedgehog signaling in the murine melanoma microenvironment. *Angiogenesis* 2007;10(4):259-67.

Abstract: The Hedgehog intercellular signaling pathway regulates cell proliferation and differentiation. This pathway has been implicated to play a role in the pathogenesis of cancer and in embryonic blood vessel development. In the current study, Hedgehog signaling in tumor related vasculature and microenvironment was examined using human umbilical vein endothelial cells and B16F0 (murine melanoma) tumors models. Use of exogenous Sonic hedgehog (Shh) peptide significantly increased BrdU incorporation in endothelial cells in vitro by a factor of 2 ( $P < 0.001$ ). The Hedgehog pathway antagonist cyclopamine effectively reduced Shh-induced proliferation to control levels. To study Hedgehog signaling in vivo a hind limb tumor model with the B16F0 cell line was used. Treatment with 25 mg/kg cyclopamine significantly attenuated BrdU incorporation in tumor cells threefold ( $P < 0.001$ ), in tumor related endothelial cells threefold ( $P = 0.004$ ), and delayed tumor growth by 4 days. Immunohistochemistry revealed that the Hedgehog receptor Patched was localized to the tumor stroma and that B16F0 cells expressed Shh peptide. Furthermore, mouse embryonic fibroblasts required the presence of B16F0 cells to express Patched in a co-culture assay system. These studies indicate that Shh peptide produced by melanoma cells induces Patched expression in fibroblasts. To study tumor related angiogenesis a vascular window model was used to monitor tumor vascularity. Treatment with cyclopamine significantly attenuated vascular formation by a factor of 2.5 ( $P < 0.001$ ) and altered vascular morphology. Furthermore, cyclopamine reduced tumor blood vessel permeability to FITC labeled dextran while having no effect on normal blood vessels. These studies suggest that Hedgehog signaling regulates melanoma related vascular formation and function

Stecca B, Mas C, Clement V et al. Melanomas require HEDGEHOG-GLI signaling regulated by interactions between GLI1 and the RAS-MEK/AKT pathways. *Proc Natl Acad Sci U S A* 2007 April 3;104(14):5895-900.

Abstract: Melanoma is one of the most aggressive cancers, and its incidence is increasing. These tumors derive from the melanocyte lineage and remain incurable after metastasis. Here we report that SONIC HEDGEHOG (SHH)-GLI signaling is active in the matrix of human hair follicles, and that it is required for the normal proliferation of human melanocytes in culture. SHH-GLI signaling also regulates the proliferation and survival of human melanomas: the growth, recurrence, and metastasis of melanoma xenografts in mice are prevented by local or systemic interference of HH-GLI function. Moreover, we show that oncogenic RAS-induced melanomas in transgenic mice express Gli1 and require Hh-Gli signaling in vitro and in vivo. Finally, we provide evidence that endogenous RAS-MEK and AKT signaling regulate the nuclear localization and transcriptional activity of GLI1 in melanoma and other cancer cells. Our data uncover an unsuspected role of HH-GLI signaling in melanocytes and melanomas, demonstrate a role for this pathway in RAS-induced tumors, suggest a general integration of the RAS/AKT and HH-GLI pathways, and open a therapeutic approach for human melanomas

## Metronomic Cyclophosphamide

Cruz-Munoz W, Man S, Kerbel RS. Effective treatment of advanced human melanoma metastasis in immunodeficient mice using combination metronomic chemotherapy regimens. *Clin Cancer Res* 2009 August 1;15(15):4867-74.

Abstract: PURPOSE: The development of effective therapeutic approaches for treatment of metastatic melanoma remains an immense challenge. Present therapies offer minimal benefit. Although dacarbazine chemotherapy remains the standard therapy, it mediates only low response rates, usually of short duration, even when combined with other chemotherapeutic agents. Thus, new therapeutic strategies are urgently needed. EXPERIMENTAL DESIGN: Using a newly developed preclinical model, we evaluated the efficacy of various doublet metronomic combination chemotherapy against established advanced melanoma metastasis and compared these with the standard maximum tolerated dose dacarbazine (alone or in combination with chemotherapeutic agents or vascular endothelial growth factor receptor-blocking antibody). RESULTS: Whereas maximum tolerated dose dacarbazine therapy did not cause significant improvement in median survival, a doublet combination of low-dose metronomic vinblastine and low-dose metronomic cyclophosphamide induced a significant increase in survival with only minimal toxicity. Furthermore, we show that the incorporation of the low-dose metronomic vinblastine/low-dose metronomic cyclophosphamide combination with a low-dose metronomic dacarbazine regimen also results in a significant increase in survival, but not when combined with maximum tolerated dose dacarbazine therapy. We also show that a combination of metronomic vinblastine therapy and a vascular endothelial growth factor receptor 2-blocking antibody (DC101) results in significant control of metastatic disease and that the combination of low-dose metronomic vinblastine/DC101 and low-dose metronomic dacarbazine induced a significant improvement in median survival. CONCLUSIONS: The effective control of advanced metastatic melanoma achieved by these metronomic-based chemotherapeutic approaches warrants clinical consideration of this treatment concept, given the recent results of a number of metronomic-based chemotherapy clinical trials

Borne E, Desmedt E, Duhamel A et al. Oral metronomic cyclophosphamide in elderly with metastatic melanoma. *Invest New Drugs* 2010 October;28(5):684-9.

Abstract: INTRODUCTION: In Western countries, the number of frail elderly people with metastatic melanoma (MM) keeps increasing. Conventional chemotherapy frequently induces imbalance in frail physiological cases. We propose to treat these patients with oral metronomic cyclophosphamide. PATIENTS AND METHODS: This retrospective study analyses the data of patients with unresectable MM who received 50 to 100 mg of cyclophosphamide a day, 3 weeks out of 4. Main evaluation criterion

was safety. Secondary evaluation criteria were objective response rate and overall survival. RESULTS: Thirteen patients were included (median age: 80, 5 AJCC stage III and 8 AJCC stage IV). Clinical and biological safety were good, leading to long home staying and rare treatment discontinuations. Main toxicity observed was lymphopenia; no opportunist infection occurred. The control rate was 46%: one partial response and five stable diseases (median: 10 months). Survival after beginning of treatment ranged from 4 to 37 months (median: 8 months). DISCUSSION: Literature about MM in frail elderly is limited. Still, specific treatment is necessary. Cyclophosphamide in metronomic schema was well tolerated. The response rate was difficult to assess (small population) but several patients presented with long lasting stabilisation. The mechanisms of action of this treatment are original, associating antiangiogenic action and immunomodulation. Conclusion: Cyclophosphamide in metronomic schema showed good safety results for this frail population. Oral treatment enabled patients to stay at home longer and limited hospitalisation time. A larger controlled study will be necessary to confirm these encouraging results in elderly with MM, a classical chemoresistant tumor

Ellebaek E, Engell-Noerregaard L, Iversen TZ et al. Metastatic melanoma patients treated with dendritic cell vaccination, Interleukin-2 and metronomic cyclophosphamide: results from a phase II trial. *Cancer Immunol Immunother* 2012 October;61(10):1791-804.

Abstract: Dendritic cells (DC) are the most potent antigen presenting cells and have proven effective in stimulation of specific immune responses in vivo. Competing immune inhibition could limit the clinical efficacy of DC vaccination. In this phase II trial, metronomic Cyclophosphamide and a Cox-2 inhibitor have been added to a DC vaccine with the intend to dampen immunosuppressive mechanisms. Twenty-eight patients with progressive metastatic melanoma were treated with autologous DCs pulsed with survivin, hTERT, and p53-derived peptides (HLA-A2(+)) or tumor lysate (HLA-A2(-)). Concomitantly the patients were treated with IL-2, Cyclophosphamide, and Celecoxib. The treatment was safe and tolerable. Sixteen patients (57 %) achieved stable disease (SD) at 1st evaluation and 8 patients had prolonged SD (7-13.7 months). The median OS was 9.4 months. Patients with SD had an OS of 10.5 months while patients with progressive disease (PD) had an OS of 6.0 months ( $p = 0.048$ ) even though there were no differences in prognostic factors between the two groups. Despite the use of metronomic Cyclophosphamide, regulatory T cells did not decrease during treatment. Indirect IFN-gamma ELISPOT assays showed a general increase in immune responses from baseline to the time of 4th vaccination. Induction of antigen-specific immune responses was seen in 9 out of 15 screened HLA-A2(+) patients. In conclusion, the number of patients obtaining SD more than doubled and 6-month survival significantly increased compared to a previous trial without Cyclophosphamide and Celecoxib. A general increase in immune responses against the tested peptides was observed

Reichle A, Vogt T, Coras B et al. Targeted combined anti-inflammatory and angiostatic therapy in advanced melanoma: a randomized phase II trial. *Melanoma Res* 2007 December;17(6):360-4.

Abstract: An angiostatic approach was used to assess the impact of anti-inflammatory therapy in combination with metronomic low-dose chemotherapy. A randomized multi-institutional phase II trial was designed to select metronomic chemotherapy (arm A: trofosfamide 50 mg orally three times daily, day 1+) or combined anti-inflammatory/angiostatic treatment (arm B: trofosfamide as above mentioned plus rofecoxib 25 mg orally, day 1+, and pioglitazone 60 mg orally, day 1+) for further evaluation. A total of 76 patients, mostly (>60%) refractory to at least one previous chemotherapy with maximum tolerated doses, and progression of metastatic melanoma were included. The estimated progression-free survival (PFS) rates at one year were 0% for metronomic chemotherapy (A), but 9% for additional anti-inflammatory therapy (B). Vice versa the hazard ratio for the intent-to-treat analysis of A versus B was 1.9 ( $P=0.008$ ). By Cox analysis, the impact of anti-inflammatory therapy on PFS achieved significance ( $P=0.016$ ) as well as C-reactive protein response on overall survival ( $P=0.045$ ). WHO grade 3 (no grade 4) toxicities were reported in arm A/B in 19 and 28%, respectively. In conclusion, control of tumour-

associated inflammatory processes (C-reactive protein response) is associated with longer PFS than achieved with metronomic chemotherapy alone in metastatic melanoma

Jia LJ, Wei DP, Sun QM et al. Tumor-targeting Salmonella typhimurium improves cyclophosphamide chemotherapy at maximum tolerated dose and low-dose metronomic regimens in a murine melanoma model. *Int J Cancer* 2007 August 1;121(3):666-74.

Abstract: Chemotherapy for cancer is partly limited by the inability of drugs to act on poorly vascularized or avascularized areas of tumors. Tumor-targeting bacteria are capable of preferentially replicating in these poorly perfused regions. Some strains have been combined with chemotherapeutic agents and the results have been promising. However, no systematic work has been carried out to test the effect of bacteria on clinical modes of chemotherapy, such as standard maximum tolerated dose (MTD) and novel low-dose metronomic (LDM) chemotherapy. Here Salmonella typhimurium VNP20009 was combined with cyclophosphamide (CTX) at both MTD and LDM schedules in a murine melanoma model. The results showed that VNP20009 significantly improved the effects of all forms of CTX treatments. The combination of VNP20009 and CTX led to a more significant decrease in tumor microvessel density and serum vascular endothelial growth factor (VEGF) level, compared with either treatment alone. Furthermore, combination therapy remarkably increased the number of bacteria within tumors when compared with bacteria treatment alone. These findings suggest that tumor-targeting bacteria, in conjunction with CTX at standard MTD and LDM regimens, might be of clinical value for the treatment of melanoma

Hermans IF, Chong TW, Palmowski MJ, Harris AL, Cerundolo V. Synergistic effect of metronomic dosing of cyclophosphamide combined with specific antitumor immunotherapy in a murine melanoma model. *Cancer Res* 2003 December 1;63(23):8408-13.

Abstract: Immunotherapy could be combined with conventional chemotherapeutic modalities aimed at reducing tumor burden. Such combination therapy may be most useful when "metronomic" doses of antineoplastic drugs are used, thereby potentially avoiding some of the immunosuppressive effects of these drugs. Recent studies have shown that some conventional antineoplastic drugs can be exploited for antiangiogenic capacities, a strategy that requires drugs to be administered at regular intervals. We therefore investigated whether such metronomic therapy with the alkylating agent cyclophosphamide (CTX) could be effectively combined with immunotherapy eliciting tumor-reactive CTLs. An immunization protocol using injection of recombinant DNA followed by injection of recombinant modified vaccinia virus Ankara strain was used to initiate a specific CTL response in mice capable of providing resistance to challenge with the murine melanoma B16.F10. Combining this immunotherapeutic regime with metronomic delivery of CTX resulted in antitumor activity that was dramatically enhanced over either treatment administered alone and was also significantly greater than combining immunotherapy with CTX administered by a maximum tolerated dose regime. Whereas both metronomic and maximum tolerated dose delivery of CTX did cause deletion of proliferating tumor-specific CTLs in the blood, this deletion occurred with slower kinetics with the metronomic schedule. Further analysis showed that metronomic CTX treatment did not delete cells with low expression of CD43, a "memory" phenotype, and that these cells maintained potent restimulatory capacity. The combination of immunotherapy and metronomic CTX therapy may be well suited to clinical management of cancer