THE EFFECTS OF BEHAVIORAL STRESS AND ENDOTHELIN RECEPTOR ANTAGONISTS ON CANCER

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ABSTRACT

This work examines two potential mediators of cancer. Section one considers the putative link between behavioral stress and cancer. We developed a stress paradigm of alternating established stressors that dramatically increases serum corticosterone and causes thymus involution. When this stress paradigm was applied to mice implanted with either melanoma or lymphosarcoma tumors we observed no alteration in the growth of either tumor. In addition, we applied a protocol established in another laboratory that demonstrated a dramatic enhancement of lymphosarcoma tumors following rotational stress (Riley V. (1981). Psychoneuroendocrine influences on immunocompetence and neoplasia. *Science* **212,** 1100-1109). We find no significant effect on lymphosarcoma progression. Therefore, under the conditions used in our studies, strong behavioral stress does not influence tumor growth.

Section two considers the effects of endothelin receptor (ETR) antagonists on cancer progression. Their ability to inhibit growth of many cancers is well documented, but similar research on glioma has been limited. We find that two ETRB-specific antagonists, BQ788 and A-192621, reduce the number of viable cells in glioma and melanoma cell lines in a dose- and time-dependent manner. In glioma cells, A-192621 induces a G2/M arrest, decreases the mean number of cell divisions and enhances apoptosis. BQ123, an ETRA-specific antagonist, has no effect on cell viability. A-192621 also up-regulates several DNA damage-inducible genes. Interestingly, reducing ETRB expression with small interfering RNAs does not abrogate the effects of either A-192621 or BQ788 in glioma or melanoma cells. Thus, while ETRB antagonists are effective against glioma, it appears unlikely that their therapeutic effects are mediated by

ETRB. Further investigation is needed to define the mechanism by which these compounds decrease cell viability.

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Section I

Chapter I

Evidence for an Association Between Stress and Cancer

Stress

Stress activates two major neural pathways, the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS) (Chrousos, 1998; Moynihan, 2003; Antoni *et al.*, 2006) (Figure 1). Stressful stimuli are processed in the locus ceruleus and the hypothalamus by the paraventricular nucleus. In the HPA axis, the hypothalamus releases corticotropin-releasing hormone (CRH) and arginine vasopressin, which signal the pituitary to release peptides including cleavage produces of proopiomelanocortin, endorphins, enkaphalins and adrenocorticotropic hormone (ACTH). ACTH then signals the adrenal cortex to release glucocorticoids (Figure 1). Glucocorticoids act on various target tissues and negatively feedback on ACTH secretion, thereby limiting their own effects. The SNS is stimulated by projections from the locus ceruleus and the paraventricular nucleus, causing the release of norepinephrine (NE) from the SNS and NE and epinephrine (EPI) from the adrenal medulla (Figure 1). The HPA axis and the SNS also feedback on each other.

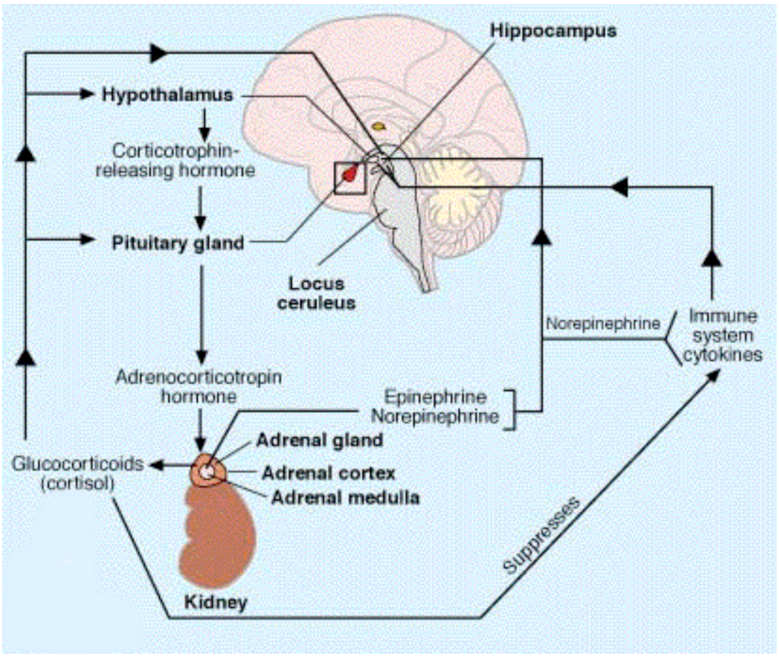

Figure 1. Stress activates the HPA axis and the SNS. Figure adapted from Ember, 1998.

Signals from these pathways dictate the body's response to stress and are generally considered adaptive to survive threat. On the contrary, extended exposure to glucocorticoids and catecholamines can negatively affect the body. McEwen termed the negative impact of stress the "allostatic load" and defined it as "the cost of chronic exposure to fluctuating or heightened neural or neuroendocrine response resulting from repeated or chronic environmental challenge that an individual reacts to as being particularly stressful" (McEwen & Stellar, 1993). Allostatic load can be a result of frequent stress, inability to adapt to repeated, similar stressors, inability to stop physiologic responses once the stressor is removed, or overcompensation by one system when another does not respond adequately (McEwen, 1998).

Stress has been associated with increased incidence of many diseases such as coronary artery disease (Ramachandruni *et al.*, 2004), asthma (Vig *et al.*, 2006) and chronic widespread pain (McBeth *et al.*, 2007). Psychosocial distress and life event stress are linked to increases in functional dyspepsia and irritable bowel syndrome (Locke *et al.*, 2004; Mizuta *et al.*, 2006). Depression and social isolation predict mortality in heart failure patients (Friedmann *et al.*, 2006). Social stress reactivates latent herpes simplex virus in infected mice (Padgett *et al.*, 1998), and examination stress reactivates Epstein-Barr virus in students (Glaser *et al.*, 1999).

Stress and the immune system

Through the activation of the HPA axis and SNS, stress affects the immune system. The resulting outcome, however, is highly complex. The age and sex of the subject experiencing stress as well as the intensity, duration and perception of stress all

influence changes in immune parameters. In earlier work, acute stress was shown to increase corticosterone and depress lymphocyte stimulation and cytotoxicity in rats, while long-term stress enhances lymphocyte measures and corticosterone levels return to baseline (Monjan & Collector, 1977). Increased circulating corticosterone can induce lymphocytopenia, thymus involution and loss of tissue mass from the spleen and peripheral lymph nodes (Keller *et al.*, 1981; Riley, 1981). It was later demonstrated that stress-induced lymphocytopenia is adrenal-dependent but suppression of lymphocyte stimulation is independent of corticosterone (Keller *et al.*, 1983). Acute stress has been shown to both suppress and enhance immune parameters. An acute, variable tailshock paradigm suppresses T-cell proliferation in both splenic and peripheral blood populations, while after multiple sessions, splenic T-cell responses return to baseline and peripheral blood responses remain suppressed (Lysle *et al.*, 1987). Another study using acute, intermittent footshock established that stress-induced suppression of lymphocyte proliferation and NK activity is modulated by β-endorphin but not corticosterone (Panerai *et al.*, 1997). Using a model of delayed-type hypersensitivity (DTH), acute stress enhanced the antigen-specific DTH response measured by ear swelling, and increased plasma corticosterone and leukocyte redistribution from the peripheral blood to the skin, while chronic stress suppressed these measures (Dhabhar & McEwen, 1996, 1997). Interestingly, stress did not affect the nonspecific immune response as measured by irritant contact sensitivity, indicating that behavioral stress affects cell-mediated immunity (Dhabhar & McEwen, 1996). Adrenalectomy abolishes the acute stress-induced leukocyte redistribution from peripheral blood and spleen to bone marrow, and

exogenous corticosterone administration mimics the effects of stress (Dhabhar *et al.*, 1996).

Social confrontation studies using rats have demonstrated an activation of both the HPA axis and the SNS. Subdominant males have decreased body mass, increased plasma levels of EPI and NE and while corticosterone levels are not significantly elevated, corticosteroid-binding capacity is decreased, thereby increasing the free, biologically active proportion of corticosterone (Stefanski, 2000). Submissive and subdominant males have a drastically reduced number of circulating CD4+ and CD8+ cells and a decreased proliferative response of T-cells in blood and mesenteric lymph nodes (Stefanski, 1998; Stefanski & Engler, 1999). Submissive males show an increase in B cells while subdominant males show either no change or a decrease. Additionally, NK activity is reduced in subdominant males (Stefanski & Engler, 1999).

Different stress exposure results in different changes in immune parameters. Restraint stress for 24 h increases serum corticosterone and decreases NK cell activity in mice and these levels recover within 48 h following release (Iwakabe *et al.*, 1998). This restraint stress also decreases interferon (IFN)-γ, involved in cell-mediated immunity, but did not affect levels of interleukin (IL)-4, involved in humoral immunity. This suggests a shift away from a T helper (Th)-1 response to a Th-2 immune response. Mice restrained for three hours per day for eleven days show no change in NK activity but lymphocytes are significantly decreased and neutrophils and large granular lymphocytes are increased (Steplewski & Vogel, 1986). Following twelve days of recovery from stress, NK activity is enhanced, T-cells and large granular lymphocytes are increased but the percentage of neutrophils is below baseline.

Chronic stress in humans also affects immune parameters. In a striking prospective study, subjects completed a questionnaire to assess psychological stress, were given nose drops with either one of five respiratory viruses or saline and monitored for symptoms (Cohen *et al.*, 1991). Viral infection was confirmed by viral isolation or an increase in virus-specific antibodies. Infection rates were positively correlated with the degree of psychological stress for all five viruses. Later work demonstrated that people with higher perceived stress are at increased risk for upper respiratory infections and levels of cortisol, NK cell cytotoxicity and CD8+ cell numbers correlated with perceived stress and risk of infection while SNS markers including blood pressure, heart rate and catecholamine levels did not (Cohen *et al.*, 2002). A study of people who care for a spouse with progressive dementia found that this chronic stress decreases the antibody response and induction of IL-2 and IL-1β following influenza virus vaccination (KiecoltGlaser *et al.*, 1996). This may lead to increased vulnerability to infection. A later study examining caregiver stress found both stress and age to be correlated with enhanced IL-10 production in CD4+ and CD8+ cells while neither affected IFN-γ or IL-2 production in CD4+ and CD8+ cells (Glaser *et al.*, 2001). This suggests a shift toward Th-2 mediated immunity.

The stress-induced shift from Th-1 to Th-2 mediated immunity, seen both in animal and human studies, plays an important role in disease resistance and progression. Both glucocorticoids and catecholamines promote this Th-2 shift (Elenkov, 2004). By limiting the number and activity of T and NK cells, stress decreases the host's resistance to many diseases.

Animal studies of stress and cancer

There have been a number of investigations on the effects of stress on cancer progression in animal models. In a mouse model of mastocytoma, inescapable shock was found to reduce the time to tumor appearance, enhance tumor size and decrease survival although these effects diminished with increased stress duration (Sklar & Anisman, 1979). On the contrary, escapable shock did not affect tumor growth or mortality. A few years later, Riley published striking results on a mouse model of stress-induced cancer progression (Riley, 1981). C3H/He mice were injected subcutaneously with murine 6C3HED lymphosarcoma cells on day zero. Mice were then subjected to rotational stress of forty-five revolutions per minute for ten minutes out of every hour while in their home cage on days four, five and six. Control animals, not subjected to the rotational stress, exhibited stabilized tumor growth after approximately twelve days but tumors in stressed mice continued to enlarge over thirty days. Unfortunately, there were no error bars or statistical tests of the data, so while the results appear quite dramatic, it is difficult to assess the significance of the effects.

Corticosterone plays a key role in regulating tumor growth. Aged rats that display a delay in extinguishing their stress response hypersecrete corticosterone and have increased vulnerability to tumor growth compared to non-stressed age-matched controls, stressed young mice and nonstressed young mice (Sapolsky & Donnelly, 1985). In this study, mice were subjected to variable stressors for one week and tumor growth was assessed at multiple time points. Young, stressed mice also had increased tumor growth compared to age-matched controls. A separate group of young stressed mice who were administered corticosterone following each stressor displayed increased tumor growth

over the group receiving stress alone. Corticosterone, however, does not account for all stress-induced increases in tumor growth. One interesting study tested the effects of three different stressors, inescapable footshock, escapable footshock and a psychological stressor during which mice received no footshock but heard the response of mice receiving inescapable footshock (Palermo-Neto *et al.*, 2003). Mice that experienced inescapable footshock or psychological stress had increased Ehrlich tumor growth as well as decreased macrophage spreading and phagocytosis following injection of Mycobacterium bovis, while mice that experienced escapable footshock were comparable to controls in these measures. Interestingly, all stressed mice had elevated serum corticosterone indicating that other factors besides HPA axis activation are involved.

To assess the effect of social confrontation stress on tumor metastasis and NK activity, MADB 106 tumor cells were injected intravenously into rats two days after the start of confrontation (Stefanski & Ben-Eliyahu, 1996). It was previously demonstrated that MADB106 cells selectively metastasize to the lung, and the number of metastases is inversely proportional to the activity of NK cells (Ben-Eliyahu *et al.*, 1991; Ben-Eliyahu & Page, 1992). Subdominant males displayed a dramatic increase in tumor burden in the lungs, indicating reduced NK activity. While termed metastasis, this model only measures the neocolonization component of metastasis and does not address metastasis originating from an *in situ* tumor. Another study found that the SNS mediates NK cell activity, thereby influencing tumor metastasis in the MADB 106 model (Shakhar & Ben-Eliyahu, 1998). In an experiment to mimic the effects of stress, the β-adrenergic agonist, metaproterenol, suppressed NK activity in a concentration-dependent manner. This resulted in a 10-fold increase in the number of MADB 106 cells in the lungs after 1 day,

and the enhanced colonization continued for three weeks. Nadolol, a β-adrenergic antagonist, blocked both metapreterenol-induced NK cell activity and increased tumor burden (Shakhar & Ben-Eliyahu, 1998). Additional investigation supported these findings and further implicated the SNS in stress-induced tumor growth. Swim stress enhanced lung colonization by MADB 106 cells and this effect was blocked by chlorisondamine (a ganglionic blocker), adrenal demedullation and β-adrenergic antagonists (Ben-Eliyahu *et al.*, 2000). Swim stress and adrenaline administration concomitantly decreased NK activity, and adrenal demedullation prevented the stress effect. Pain from surgery as a stressor also enhances metastasis in this model and an analgesic dose of morphine blocks this effect (Page *et al.*, 1993).

Stress from crowded housing and, to a lesser extent, isolated housing enhanced early melanoma tumor growth but this effect did not last over time (Hasegawa & Saiki, 2002). Social stress increased serum corticosterone and lung metastasis in a melanoma mouse model, while decreasing splenocyte proliferation in response to Concanavalin A (Vegas *et al.*, 2006). Another experiment with a melanoma model found that in mouse pairs with a stable social hierarchy, submissives had increased corticosterone and metastasis and decreased NK activity compared to dominant mice (Sa-Rocha *et al.*, 2006). This study indicates that submissive animals experience a perpetual anxiety-like state since no additional stressor was administered. In a model of ultraviolet B radiation-induced squamous cell carcinoma, tumors appeared earlier in stressed mice, which also had lower serum IFN-γ and a lower number of infiltrating CD4+ cells (Saul *et al.*, 2005).

In addition to modulating immune parameters, stress may more directly affect tumor progression. Physiological concentrations of cortisol induced proliferation in five

out of ten primary cultures of metastatic breast cancer (Simon *et al.*, 1984). In a study of prostate cancer, cortisol and its metabolite, cortisone, increased tumor cell proliferation and prostate-specific antigen secretion by binding a mutated androgen receptor (Zhao *et al.*, 2000). Stress may also promote cancer by affecting angiogenesis and invasive potential. In ovarian cancer cell lines, NE, EPI and isoproterenol, a β-adrenergic agonist, stimulate vascular endothelial growth factor (VEGF) production, an important stimulator of angiogenesis, and this effect is blocked by propranolol, a β-adrenergic antagonist (Lutgendorf *et al.*, 2003). Cortisol had mixed effects in this study, stimulating VEGF production in one cell line at a higher pharmacologic and stimulating VEGF production in another cell line at a 100-fold lower dose, while inhibiting VEGF at the higher dose. NE also enhanced migration of breast cancer and colon cancer cells (Masur *et al.*, 2001; Drell *et al.*, 2003). NE increased *in vitro* membrane invasion of three ovarian cancer cell lines and this effect was blocked by propranolol (Sood *et al.*, 2006). In this study, NE enhanced matrix metalloproteinase (MMP)-2 and MMP-9 levels, key components in tumor invasion, and an MMP inhibitor blocked NE-enhanced tumor invasion. EPI also enhanced the *in vitro* invasion of three ovarian cancer cell lines and cortisol increased invasion of one. Interestingly, cortisol has been shown to increase the density of β-adrenergic receptors in human lung cancer cells (Nakane *et al.*, 1990). This suggests that a stressor that increases both glucocorticoids and catecholamines would have the greatest impact on cancer progression.

Recently, Thaker and colleagues demonstrated that chronic restraint stress enhanced ovarian tumor growth and angiogenesis in nude mice (Thaker *et al.*, 2006). Restraint stress increased HeyA8 tumor weight and the number of tumor nodules *in vivo*

and this effect was mimicked with isoproterenol, a nonspecific β-adrenergic agonist, and terbutaline, a β_2-adrenergic agonist, but not xamoterol, a β_1-adrenergic agonist. Propranolol blocked the effects of stress and isoproterenol on tumor growth. Mice injected with β-adenoreceptor-null ovarian cancer lines did not show increased tumor growth in response to stress. Moreover, mice injected with HeyA8 cells in which β_2-adenoreceptor expression had been decreased with small interfering RNA also did not demonstrate enhanced tumor growth following stress. In addition to growth, stress also induced angiogenesis and VEGF mRNA and protein levels. Again, stress effects were mimicked by isoproterenol and terbutaline but not xamoterol, and were blocked by propranolol. VEGF mRNA was also dramatically increased by NE administration. Stress also increased levels of MMP-2 and MMP-9, thereby increasing invasion potential. VEGF inhibitors blocked the stress-induced tumor growth indicating that VEGF and the neovasculature played a key role in tumor growth.

Stress also interacts with known causes and therapies of cancer. Stress enhanced carcinogen action, inducing higher hepatocellular carcinoma incidence than with carcinogen alone (Laconi *et al.*, 2000). Stress reduced the effectiveness of chemotherapy in a mouse model of lung cancer, decreasing the proportion of long-term survivors and overall survival (Zorzet *et al.*, 1998). Dexamethasone, a synthetic glucocorticoid, suppresses chemotherapy-induced apoptosis in a xenograft mouse model of lung cancer, and in cervical carcinoma and breast cancer cell lines (Herr *et al.*, 2003; Wu *et al.*, 2004).

Nonetheless, not all studies have demonstrated a correlation between stress and cancer. In a melanoma mouse model, restraint stress induced changes in cytokine levels, decreasing IFN-γ but not IL-4, but had no effect on tumor growth (Li *et al.*, 1997).

Another study found that restraint stress did not consistently alter any immune parameters in two different strains of mice, and melanoma cells injected after stress did not show enhanced growth or metastasis compared to controls (Posevitz *et al.*, 2003).

Animal models used to investigate the effects of behavioral stress on cancer progression are highly complex and timing of the stressor may play a critical role. Using the murine lymphosarcoma model, Riley demonstrated that injection of a synthetic glucocorticoid had similar effects on tumor growth as rotational stress but the consequence of the glucocorticoid injection was critically dependent upon its timing (Riley, 1981). Given one week prior to lymphosarcoma injection, dexamethasone suppressed tumor growth compared to controls, while given one week after lymphosarcoma implantation, dexamethasone enhanced tumor growth. Riley also injected slow-release depots of fluocinolone acetonide (FCA), another synthetic corticoid, at one, two or three weeks after lymphosarcoma implantation and found that, while tumor growth was enhanced above controls at all times, delayed administration of FCA decreased promotion of tumor growth. Increased age also positively correlated with increased tumor growth in the absence of any stressors. As in Riley's previously mentioned experiments, no error bars or statistics accompany the data. In another study of timing effects, stress was initiated before MADB 106 tumor injection and still increased tumor burden (Stefanski & Ben-Eliyahu, 1996). Another factor in stress experimentation is housing conditions. Increasing ventilation and reducing noise lowers basal corticosterone levels, and mice show an enhanced response to stress (Riley, 1981; Neigh *et al.*, 2005).

Largely, animal studies have demonstrated that stress enhances tumor growth. The HPA axis and the SNS both play a role in these stress effects. Stress promotes tumor progression through a variety of means including, altering immune parameters, direct action on tumor cell receptors, enhancing angiogenesis and increasing invasive potential.

Stress and cancer in the clinic

Proving that stress plays a role in human cancer incidence has been difficult. Studies have reported conflicting results when assessing if stressful life events increase the risk of developing cancer (Forsen, 1991; Chen *et al.*, 1995; Protheroe *et al.*, 1999; Lillberg *et al.*, 2003; Kroenke *et al.*, 2004). With the possible exception of death of a spouse, meta-analysis of the literature has not revealed a strong correlation between stressful life events and cancer onset (Duijts *et al.*, 2003). Death of a child increased the incidence risk of certain cancers, and it shortened survival if the death occurred after the cancer diagnosis in one study (Levav *et al.*, 2000), but showed no effect on incidence risk in another (Kvikstad & Vatten, 1996). Moreover, no association between stressful life events and cancer relapse has been demonstrated (Forsen, 1991; Kvikstad & Vatten, 1996; Levav *et al.*, 2000; Lillberg *et al.*, 2003)

Studies attempting to link patient outlook and disease progression and survival have yielded mixed results. An investigation of non-small cell lung carcinoma found no evidence that a high level of optimism before treatment influenced progression-free survival or overall survival (Schofield *et al.*, 2004). Another study of lung cancer found that depressive coping and emotional distress predicted shorter survival (Faller *et al.*, 1999). A study of mixed cancer types found no association between optimism and

survival but did find a positive correlation between pessimism and mortality in younger patients (Schulz *et al.*, 1996). Unfortunately, this study did not control for the extent or sites of metastases, and this may have influenced the statistical analysis. A recent study used the National Comprehensive Cancer Network (NCCN) Distress Thermometer to assess distress levels in breast cancer patients (Dabrowski *et al.*, 2007). The survey asked patients to rate their distress for the past week on a ten-point scale and then classify the source of the distress on a problem list with five major categories, including practical problems, family problems, emotional problems, spiritual or religious concerns and physical problems. Of 286 patients that completed the questionnaires, 96 subjects (34%) reported high distress classified as five or more on the ten-point scale. No correlation was found between high distress and stage of disease. An interesting study that examined psychological well-being and circulating VEGF levels found that increased social support correlated with decreased VEGF levels, and feelings of helplessness or worthlessness were correlated with increased VEGF levels (Lutgendorf *et al.*, 2002). This could impact cancer neoangiogenesis.

If stress plays a role in cancer progression, psychosocial intervention may alleviate stress and thereby reduce cancer occurrence or progression. One study of metastatic breast cancer patients found that a one-year intervention of weekly group therapy and self-hypnosis for pain positively correlated with overall survival (Spiegel *et al.*, 1989), while a later study found no significant effects (Cunningham *et al.*, 1998). A large, multicenter investigation found that while supportive group therapy improved mood and pain perception in metastatic breast cancer patients, it did not correlate with survival (Goodwin *et al.*, 2001). Studies of malignant melanoma patients also

demonstrated a positive correlation between group therapy and immune parameters as well as survival and reduced recurrence (Fawzy *et al.*, 1990; Fawzy *et al.*, 1993). A ten-year follow-up found that participation in the intervention was still predictive of survival although the benefit had weakened from the five-to-six year follow-up (Fawzy *et al.*, 2003). Presurgical psychosocial intervention showed promise in breast cancer patients (Larson *et al.*, 2000). While control patients experienced increased distress and decreased IFN-γ levels over time, patients who participated in the intervention experienced less distress and stable levels of IFN-γ. Unfortunately, no follow-up was conducted to establish if psychosocial therapy had long-lasting effects on disease progression, recurrence or survival. Another study of breast cancer patients undergoing chemotherapy found that psychological group therapy improved outlook and healthful behaviors and maintained baseline levels of T-cell proliferative responses, while these levels declined in control patients (Andersen *et al.*, 2004). Improved lymphocyte proliferation and emotional well-being was linked to cognitive-behavioral stress management intervention in post-surgical early-stage breast cancer patients at a three-month follow-up (McGregor *et al.*, 2004). One study even found that, in healthy subjects, mirthful laughter while viewing a humorous video correlated with increased NK cell activity compared to those who viewed a tourism video (Bennett *et al.*, 2003). While many studies agree that psychosocial intervention improves patient outlook and healthy behaviors, it is not as clear if these and other immune-related changes result in better long-term outcomes. More studies with extended follow-up are needed.

References

Andersen BL, Farrar WB, Golden-Kreutz DM, Glaser R, Emery CF, Crespin TR, Shapiro CL & Carson WE, 3rd. (2004). Psychological, behavioral, and immune changes after a psychological intervention: a clinical trial. *J Clin Oncol* **22,** 3570-3580.

Antoni MH, Lutgendorf SK, Cole SW, Dhabhar FS, Sephton SE, McDonald PG, Stefanek M & Sood AK. (2006). The influence of bio-behavioural factors on tumour biology: pathways and mechanisms. *Nat Rev Cancer* **6,** 240-248.

Ben-Eliyahu S & Page GG. (1992). Invivo Assessment of Natural-Killer-Cell Activity in Rats. *Progress in Neuroendocrinimmunology* **5,** 199-214.

Ben-Eliyahu S, Shakhar G, Page GG, Stefanski V & Shakhar K. (2000). Suppression of NK cell activity and of resistance to metastasis by stress: a role for adrenal catecholamines and beta-adrenoceptors. *Neuroimmunomodulation* **8,** 154-164.

Ben-Eliyahu S, Yirmiya R, Liebeskind JC, Taylor AN & Gale RP. (1991). Stress increases metastatic spread of a mammary tumor in rats: evidence for mediation by the immune system. *Brain Behav Immun* **5,** 193-205.

Bennett MP, Zeller JM, Rosenberg L & McCann J. (2003). The effect of mirthful laughter on stress and natural killer cell activity. *Altern Ther Health Med* **9,** 38-45.

Chen CC, David AS, Nunnerley H, Michell M, Dawson JL, Berry H, Dobbs J & Fahy T. (1995). Adverse life events and breast cancer: case-control study. *Bmj* **311,** 1527-1530.

Chrousos GP. (1998). Stressors, Stress, and Neuroendocrine Integration of the Adaptive Response: The 1997 Hans Selye Memorial Lecture. *Annals of the New York Academy of Sciences* **851,** 311-335.

Cohen S, Hamrick N, Rodriguez MS, Feldman PJ, Rabin BS & Manuck SB. (2002). Reactivity and vulnerability to stress-associated risk for upper respiratory illness. *Psychosom Med* **64,** 302-310.

Cohen S, Tyrrell DA & Smith AP. (1991). Psychological stress and susceptibility to the common cold. *N Engl J Med* **325,** 606-612.

Cunningham AJ, Edmonds CV, Jenkins GP, Pollack H, Lockwood GA & Warr D. (1998). A randomized controlled trial of the effects of group psychological therapy on survival in women with metastatic breast cancer. *Psychooncology* **7,** 508-517.

Dabrowski M, Boucher K, Ward JH, Lovell MM, Sandre A, Bloch J, Carlquist L, Porter M, Norman L & Buys SS. (2007). Clinical experience with the NCCN distress thermometer in breast cancer patients. *J Natl Compr Canc Netw* **5,** 104-111.

Dhabhar FS & McEwen BS. (1996). Stress-induced enhancement of antigen-specific cell-mediated immunity. *J Immunol* **156,** 2608-2615.

Dhabhar FS & McEwen BS. (1997). Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: a potential role for leukocyte trafficking. *Brain Behav Immun* **11,** 286-306.

Dhabhar FS, Miller AH, McEwen BS & Spencer RL. (1996). Stress-induced changes in blood leukocyte distribution. Role of adrenal steroid hormones. *J Immunol* **157,** 1638-1644.

Drell TLt, Joseph J, Lang K, Niggemann B, Zaenker KS & Entschladen F. (2003). Effects of neurotransmitters on the chemokinesis and chemotaxis of MDA-MB-468 human breast carcinoma cells. *Breast Cancer Res Treat* **80,** 63-70.

Duijts SF, Zeegers MP & Borne BV. (2003). The association between stressful life events and breast cancer risk: a meta-analysis. *Int J Cancer* **107,** 1023-1029.

Elenkov IJ. (2004). Glucocorticoids and the Th1/Th2 Balance. *Annals of the New York Academy of Sciences* **1024,** 138-146.

Ember LR. (1998). Surviving stress. *Chemical and engineering news* **76,** 12.

Faller H, Bulzebruck H, Drings P & Lang H. (1999). Coping, distress, and survival among patients with lung cancer. *Arch Gen Psychiatry* **56,** 756-762.

Fawzy FI, Canada AL & Fawzy NW. (2003). Malignant melanoma: effects of a brief, structured psychiatric intervention on survival and recurrence at 10-year follow-up. *Arch Gen Psychiatry* **60,** 100-103.

Fawzy FI, Fawzy NW, Hyun CS, Elashoff R, Guthrie D, Fahey JL & Morton DL. (1993). Malignant melanoma. Effects of an early structured psychiatric intervention, coping, and affective state on recurrence and survival 6 years later. *Arch Gen Psychiatry* **50,** 681-689.

Fawzy FI, Kemeny ME, Fawzy NW, Elashoff R, Morton D, Cousins N & Fahey JL. (1990). A structured psychiatric intervention for cancer patients. II. Changes over time in immunological measures. *Arch Gen Psychiatry* **47,** 729-735.

Forsen A. (1991). Psychosocial stress as a risk for breast cancer. *Psychother Psychosom* **55,** 176-185.

Friedmann E, Thomas SA, Liu F, Morton PG, Chapa D & Gottlieb SS. (2006). Relationship of depression, anxiety, and social isolation to chronic heart failure outpatient mortality. *Am Heart J* **152,** 940 e941-948.

Glaser R, Friedman SB, Smyth J, Ader R, Bijur P, Brunell P, Cohen N, Krilov LR, Lifrak ST, Stone A & Toffler P. (1999). The differential impact of training stress and final examination stress on herpesvirus latency at the United States Military Academy at West Point. *Brain Behav Immun* **13,** 240-251.

Glaser R, MacCallum RC, Laskowski BF, Malarkey WB, Sheridan JF & Kiecolt-Glaser JK. (2001). Evidence for a shift in the Th-1 to Th-2 cytokine response associated with chronic stress and aging. *J Gerontol A Biol Sci Med Sci* **56,** M477-482.

Goodwin PJ, Leszcz M, Ennis M, Koopmans J, Vincent L, Guther H, Drysdale E, Hundleby M, Chochinov HM, Navarro M, Speca M & Hunter J. (2001). The effect of group psychosocial support on survival in metastatic breast cancer. *N Engl J Med* **345,** 1719-1726.

Hasegawa H & Saiki I. (2002). Psychosocial stress augments tumor development through beta-adrenergic activation in mice. *Jpn J Cancer Res* **93,** 729-735.

Herr I, Ucur E, Herzer K, Okouoyo S, Ridder R, Krammer PH, von Knebel Doeberitz M & Debatin KM. (2003). Glucocorticoid cotreatment induces apoptosis resistance toward cancer therapy in carcinomas. *Cancer Res* **63,** 3112-3120.

Iwakabe K, Shimada M, Ohta A, Yahata T, Ohmi Y, Habu S & Nishimura T. (1998). The restraint stress drives a shift in Th1/Th2 balance toward Th2-dominant immunity in mice. *Immunol Lett* **62,** 39-43.

Keller SE, Weiss JM, Schleifer SJ, Miller NE & Stein M. (1981). Suppression of immunity by stress: effect of a graded series of stressors on lymphocyte stimulation in the rat. *Science* **213,** 1397-1400.

Keller SE, Weiss JM, Schleifer SJ, Miller NE & Stein M. (1983). Stress-induced suppression of immunity in adrenalectomized rats. *Science* **221,** 1301-1304.

KiecoltGlaser JK, Glaser R, Gravenstein S, Malarkey WB & Sheridan J. (1996). Chronic stress alters the immune response to influenza virus vaccine in older adults. *Proceedings of the National Academy of Sciences of the United States of America* **93,** 3043-3047.

Kroenke CH, Hankinson SE, Schernhammer ES, Colditz GA, Kawachi I & Holmes MD. (2004). Caregiving stress, endogenous sex steroid hormone levels, and breast cancer incidence. *Am J Epidemiol* **159,** 1019-1027.

Kvikstad A & Vatten LJ. (1996). Risk and prognosis of cancer in middle-aged women who have experienced the death of a child. *Int J Cancer* **67,** 165-169.

Laconi E, Tomasi C, Curreli F, Diana S, Laconi S, Serra G, Collu M & Pani P. (2000). Early exposure to restraint stress enhances chemical carcinogenesis in rat liver. *Cancer Lett* **161,** 215-220.

Larson MR, Duberstein PR, Talbot NL, Caldwell C & Moynihan JA. (2000). A presurgical psychosocial intervention for breast cancer patients. psychological distress and the immune response. *J Psychosom Res* **48,** 187-194.

Levav I, Kohn R, Iscovich J, Abramson JH, Tsai WY & Vigdorovich D. (2000). Cancer incidence and survival following bereavement. *Am J Public Health* **90,** 1601-1607.

Li T, Harada M, Tamada K, Abe K & Nomoto K. (1997). Repeated restraint stress impairs the antitumor T cell response through its suppressive effect on Th1-type CD4+ T cells. *Anticancer Res* **17,** 4259-4268.

Lillberg K, Verkasalo PK, Kaprio J, Teppo L, Helenius H & Koskenvuo M. (2003). Stressful life events and risk of breast cancer in 10,808 women: a cohort study. *Am J Epidemiol* **157,** 415-423.

Locke GR, 3rd, Weaver AL, Melton LJ, 3rd & Talley NJ. (2004). Psychosocial factors are linked to functional gastrointestinal disorders: a population based nested case-control study. *Am J Gastroenterol* **99,** 350-357.

Lutgendorf SK, Cole S, Costanzo E, Bradley S, Coffin J, Jabbari S, Rainwater K, Ritchie JM, Yang M & Sood AK. (2003). Stress-related mediators stimulate vascular endothelial growth factor secretion by two ovarian cancer cell lines. *Clin Cancer Res* **9,** 4514-4521.

Lutgendorf SK, Johnsen EL, Cooper B, Anderson B, Sorosky JI, Buller RE & Sood AK. (2002). Vascular endothelial growth factor and social support in patients with ovarian carcinoma. *Cancer* **95,** 808-815.

Lysle DT, Lyte M, Fowler H & Rabin BS. (1987). Shock-induced modulation of lymphocyte reactivity: suppression, habituation, and recovery. *Life Sci* **41,** 1805-1814.

Masur K, Niggemann B, Zanker KS & Entschladen F. (2001). Norepinephrine-induced migration of SW 480 colon carcinoma cells is inhibited by beta-blockers. *Cancer Res* **61,** 2866-2869.

McBeth J, Silman AJ, Gupta A, Chiu YH, Ray D, Morriss R, Dickens C, King Y & Macfarlane GJ. (2007). Moderation of psychosocial risk factors through dysfunction of the hypothalamic-pituitary-adrenal stress axis in the onset of chronic widespread musculoskeletal pain: findings of a population-based prospective cohort study. *Arthritis Rheum* **56,** 360-371.

McEwen BS. (1998). Protective and damaging effects of stress mediators. *N Engl J Med* **338,** 171-179.

McEwen BS & Stellar E. (1993). Stress and the individual. Mechanisms leading to disease. *Arch Intern Med* **153,** 2093-2101.

McGregor BA, Antoni MH, Boyers A, Alferi SM, Blomberg BB & Carver CS. (2004). Cognitive-behavioral stress management increases benefit finding and immune function among women with early-stage breast cancer. *J Psychosom Res* **56,** 1-8.

Mizuta Y, Shikuwa S, Isomoto H, Mishima R, Akazawa Y, Masuda J, Omagari K, Takeshima F & Kohno S. (2006). Recent insights into digestive motility in functional dyspepsia. *J Gastroenterol* **41,** 1025-1040.

Monjan AA & Collector MI. (1977). Stress-induced modulation of the immune response. *Science* **196,** 307-308.

Moynihan JA. (2003). Mechanisms of stress-induced modulation of immunity. *Brain, Behavior, and Immunity* **17,** 11-16.

Nakane T, Szentendrei T, Stern L, Virmani M, Seely J & Kunos G. (1990). Effects of IL-1 and cortisol on beta-adrenergic receptors, cell proliferation, and differentiation in cultured human A549 lung tumor cells. *J Immunol* **145,** 260-266.

Neigh GN, Bowers SL, Korman B & Nelson RJ. (2005). Housing environment alters delayed-type hypersensitivity and corticosterone concentrations of individually housed male C57BL/6 mice. *Animal Welfare* **14,** 249-257.

Padgett DA, Sheridan JF, Dorne J, Berntson GG, Candelora J & Glaser R. (1998). Social stress and the reactivation of latent herpes simplex virus type 1. *Proceedings of the National Academy of Sciences of the United States of America* **95,** 7231-7235.

Page GG, Ben-Eliyahu S, Yirmiya R & Liebeskind JC. (1993). Morphine attenuates surgery-induced enhancement of metastatic colonization in rats. *Pain* **54,** 21-28.

Palermo-Neto J, de Oliveira Massoco C & Robespierre de Souza W. (2003). Effects of physical and psychological stressors on behavior, macrophage activity, and Ehrlich tumor growth. *Brain Behav Immun* **17,** 43-54.

Panerai AE, Sacerdote P, Bianchi M & Manfredi B. (1997). Intermittent but not continuous inescapable footshock stress and intracerebroventricular interleukin-1 similarly affect immune responses and immunocyte beta-endorphin concentrations in the rat. *Int J Clin Pharmacol Res* **17,** 115-116.

Posevitz V, Vizler C, Benyhe S, Duda E & Borsodi A. (2003). Restraint stress and anti-tumor immune response in mice. *Acta Biol Hung* **54,** 167-176.

Protheroe D, Turvey K, Horgan K, Benson E, Bowers D & House A. (1999). Stressful life events and difficulties and onset of breast cancer: case-control study. *Bmj* **319,** 1027-1030.

Ramachandruni S, Handberg E & Sheps DS. (2004). Acute and chronic psychological stress in coronary disease. *Curr Opin Cardiol* **19,** 494-499.

Riley V. (1981). Psychoneuroendocrine influences on immunocompetence and neoplasia. *Science* **212,** 1100-1109.

Sa-Rocha VM, Sa-Rocha LC & Palermo-Neto J. (2006). Variations in behavior, innate immunity and host resistance to B16F10 melanoma growth in mice that present social stable hierarchical ranks. *Physiol Behav* **88,** 108-115.

Sapolsky RM & Donnelly TM. (1985). Vulnerability to stress-induced tumor growth increases with age in rats: role of glucocorticoids. *Endocrinology* **117,** 662-666.

Saul AN, Oberyszyn TM, Daugherty C, Kusewitt D, Jones S, Jewell S, Malarkey WB, Lehman A, Lemeshow S & Dhabhar FS. (2005). Chronic stress and susceptibility to skin cancer. *J Natl Cancer Inst* **97,** 1760-1767.

Schofield P, Ball D, Smith JG, Borland R, O'Brien P, Davis S, Olver I, Ryan G & Joseph D. (2004). Optimism and survival in lung carcinoma patients. *Cancer* **100,** 1276-1282.

Schulz R, Bookwala J, Knapp JE, Scheier M & Williamson GM. (1996). Pessimism, age, and cancer mortality. *Psychol Aging* **11,** 304-309.

Shakhar G & Ben-Eliyahu S. (1998). In vivo beta-adrenergic stimulation suppresses natural killer activity and compromises resistance to tumor metastasis in rats. *Journal of Immunology* **160,** 3251-3258.

Simon WE, Albrecht M, Trams G, Dietel M & Holzel F. (1984). In vitro growth promotion of human mammary carcinoma cells by steroid hormones, tamoxifen, and prolactin. *J Natl Cancer Inst* **73,** 313-321.

Sklar LS & Anisman H. (1979). Stress and Coping Factors Influence Tumor-Growth. *Science* **205,** 513-515.

Sood AK, Bhatty R, Kamat AA, Landen CN, Han L, Thaker PH, Li Y, Gershenson DM, Lutgendorf S & Cole SW. (2006). Stress hormone-mediated invasion of ovarian cancer cells. *Clin Cancer Res* **12,** 369-375.

Spiegel D, Bloom JR, Kraemer HC & Gottheil E. (1989). Effect of psychosocial treatment on survival of patients with metastatic breast cancer. *Lancet* **2,** 888-891.

Stefanski V. (1998). Social stress in loser rats: opposite immunological effects in submissive and subdominant males. *Physiol Behav* **63,** 605-613.

Stefanski V. (2000). Social stress in laboratory rats: hormonal responses and immune cell distribution. *Psychoneuroendocrinology* **25,** 389-406.

Stefanski V & Ben-Eliyahu S. (1996). Social confrontation and tumor metastasis in rats: defeat and beta-adrenergic mechanisms. *Physiol Behav* **60,** 277-282.

Stefanski V & Engler H. (1999). Social stress, dominance and blood cellular immunity. *J Neuroimmunol* **94,** 144-152.

Steplewski Z & Vogel WH. (1986). Total leukocytes, T cell subpopulation and natural killer (NK) cell activity in rats exposed to restraint stress. *Life Sci* **38,** 2419-2427.

Thaker PH, Han LY, Kamat AA, Arevalo JM, Takahashi R, Lu C, Jennings NB, Armaiz-Pena G, Bankson JA, Ravoori M, Merritt WM, Lin YG, Mangala LS, Kim TJ, Coleman RL, Landen CN, Li Y, Felix E, Sanguino AM, Newman RA, Lloyd M, Gershenson DM, Kundra V, Lopez-Berestein G, Lutgendorf SK, Cole SW & Sood AK. (2006). Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma. *Nat Med* **12,** 939-944.

Vegas O, Fano E, Brain PF, Alonso A & Azpiroz A. (2006). Social stress, coping strategies and tumor development in male mice: behavioral, neuroendocrine and immunological implications. *Psychoneuroendocrinology* **31,** 69-79.

Vig RS, Forsythe P & Vliagoftis H. (2006). The role of stress in asthma: insight from studies on the effect of acute and chronic stressors in models of airway inflammation. *Ann N Y Acad Sci* **1088,** 65-77.

Wu W, Chaudhuri S, Brickley DR, Pang D, Karrison T & Conzen SD. (2004). Microarray analysis reveals glucocorticoid-regulated survival genes that are associated with inhibition of apoptosis in breast epithelial cells. *Cancer Res* **64,** 1757-1764.

Zhao XY, Malloy PJ, Krishnan AV, Swami S, Navone NM, Peehl DM & Feldman D. (2000). Glucocorticoids can promote androgen-independent growth of prostate cancer cells through a mutated androgen receptor. *Nat Med* **6,** 703-706.

Zorzet S, Perissin L, Rapozzi V & Giraldi T. (1998). Restraint stress reduces the antitumor efficacy of cyclophosphamide in tumor-bearing mice. *Brain Behav Immun* **12,** 23-33.

Chapter II

Behavioral Stress and Tumor Progression

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Behavioral Stress and Tumor Progression

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Abstract. *Background: A number of laboratories have reported a possible link between behavioral stress and cancer progression. Previously published findings demonstrated a stress-induced increase in tumor growth of implanted lymphosarcoma in C3H mice. Here, two mouse models were utilized to investigate whether stress alters the growth of solid tumors. Materials and Methods: We developed a stress paradigm that involves alternating established stressors for 12 days. FVB mice implanted with melanoma were subjected to this stress protocol. We also attempted to duplicate Riley's finding. Results: Our stress paradigm markedly increased serum corticosterone levels and thymus involution. No alteration in the growth of the melanoma tumors was observed. There was also no significant effect on lymphosarcoma progression using either our own or Riley's stress protocol. Conclusion: Under the conditions used in this study, strong behavioral stress did not influence tumor progression.*

Psychosocial stress is increasingly recognized as an important public health issue. It is implicated in diseases such as irritable bowel syndrome (1, 2), coronary artery disease (3) and cancer (4). In humans, linking stress with cancer progression and outcome has proven to be difficult, with many contradictory reports in the literature. For instance, studies investigating the effects of group therapy on metastasis and survival in breast cancer patients reported conflicting results, with some studies demonstrating a protective effect and others seeing no effect (5, 6). An investigation of patient optimism prior to treatment and survival in non-small cell lung carcinoma found no correlation between the two (7). In addition, another study found no correlation between the stress of caregiving and breast cancer incidence (8). While the connection between stress and cancer has also been investigated in animals, a causal relationship has not been proven. Riley (9) reported that the increase in glucocorticoids caused by stress in mice enhanced tumor growth for tumors that are thought to be at least partially controlled by the immune response. Additional studies have indicated that behavioral stress increases tumor progression in rodents (10-14) and can also decrease the efficacy of a cytotoxic antitumor drug (15). Other studies, however, showed variable results, indicating a complex interaction between stress and cancer, including differences resulting from differential housing (16, 17).

Possible mechanisms linking stress and tumor growth have also been investigated. Behavioral stress increases corticosteroid levels, leading to lymphocytopenia, thymus involution and a decrease in spleen and peripheral lymph node mass (9). These effects may be mediated through the autonomic innervation of primary and secondary lymphoid organs, as well as through modulation of adrenergic and glucocorticoid receptors on immune cells. Nonetheless, studies on the effect of stress on the immune system have led to contradictory results. While low NK activity has been linked to decreased disease resistance, stress can decrease NK cell cytotoxicity (12, 13, 18-22) or have no effect (23-25). Another study in humans found that mirthful laughter appeared to reduce stress and increase natural killer (NK) cell activity (26).

Using two separate stress-inducing paradigms, we set out to investigate the mechanisms whereby stress regulates tumor growth, but were unable to demonstrate any effect on tumor growth or replicate an important prior finding of such regulation.

Materials and Methods

Animals. Six-week-old male FVB mice were obtained from Taconic (Germantown, NY, USA) and used in the melanoma experiments. Six-week-old female C3H/He mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and used in the lymphosarcoma experiments. The mice were housed individually to prevent differential stress levels associated with social hierarchy. All the mice were allowed to recover from transport and adjust to the Caltech animal facility for 2 weeks prior to use. Mice were stressed using a combination of 3 established stressors: rotation at 45 rpm for 45 minutes, forced cold-water swimming at 10°C for 3 minutes, and restraint for 1 hour. Rotation occurred in a

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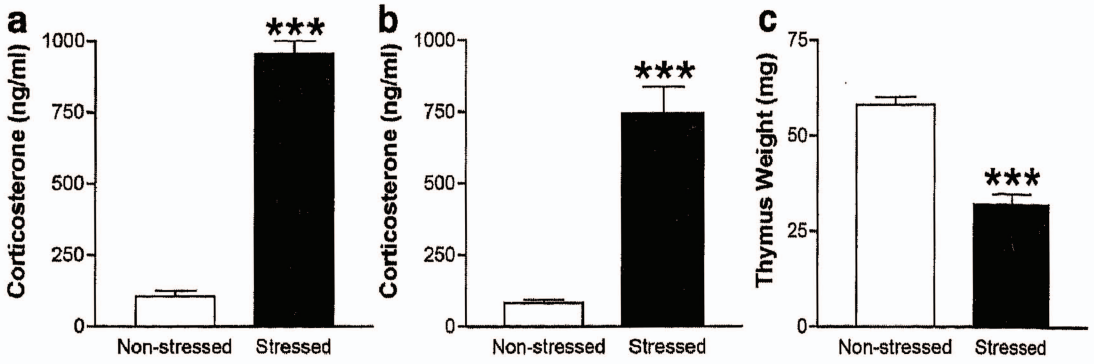

Figure 1. *Stress increases serum corticosterone and decreases thymus weight. a. FVB mice, without tumors, were subjected to the variable stress protocol for 12 days and blood was taken immediately following the last stressor. Mean corticosterone levels were 106.6±19.6 ng/ml (n=25) and 953.7±46.3 ng/ml (n=10) for non-stressed and stressed mice, respectively (p<0.0001). b. FVB mice were injected with B16F10 melanoma cells and subjected to the variable stress protocol for 12 days and blood was taken immediately following the last stressor. Mean corticosterone levels were 82.6±11.8 ng/ml (n=10) and 744.6±90.8 (n=10) for non-stressed and stressed mice, respectively (p<0.0001). c. FVB mice were injected with B16F10 melanoma cells and subjected to the variable stress protocol for 12 days and the thymus weight was measured following the last stressor. The mean thymus weights were 58±2 mg (n=9) and 32±3 mg (n=9) for non-stressed and stressed mice, respectively (p<0.0001).*

translucent plastic box, 3 3/8"x 4 1/4"x 2 1/2" with a ventilated lid, that was secured on a level GlasCol rotator (Fisher Scientific, Pittsburgh, PA, USA). Restraint occurred in a ventilated 50-ml conical centrifuge tube. The animals experienced each stressor once per day for a total of 3 stresses a day, and rested for 3-4 hours between each stress. The order of stressors was rotated each day to minimize habituation. In the melanoma experiments, mice were stressed for 12 days following tumor cell implantation, and sacrificed after the first stress on the 12th day. In the lymphosarcoma experiments, mice were stressed from days 4-6 after tumor implantation and sacrificed on the 23rd day. Tumor size was measured at the end of the experiment using calipers in 3-dimensions to calculate volume.

Cell culture. Mouse melanoma B16F10 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in RPMI 1640 (ATCC) supplemented with 10% fetal bovine serum (Gemini Biological Products, Calabasas, CA, USA) in a humidified incubator with 5% CO_2 at 37°C. Mouse lymphosarcoma cells, 6C3HED, were obtained from the NCI-Frederick Cancer DCT Tumor Repository, USA, and cultured as above. In each case, $5x10^5$ cells were injected subcutaneously into the right flank.

Corticosterone assay. Blood was collected immediately following the last stress by cardiac puncture in a serum separation tube (Becton, Dickson & Company, Franklin Lakes, NJ, USA), allowed to clot at room temperature and centrifuged at 1200 xg for 10 minutes. Serum samples were stored at -80°C until analysis. The corticosterone concentration (ng/ml) was determined using a radioimmunoassay kit following the manufacturer's instructions (MP Biomedicals, Costa Mesa, CA, USA).

Statistics. Data is represented in all cases as mean ± standard error (SEM). Statistical significance was determined in all cases by an unpaired *t*-test.

Results

Behavioral stress increases serum corticosterone and causes thymus involution. To maximize the stress response and limit habituation, a rotating schedule of 3 modes of behavioral stress to activate the hypothalamic-pituitary-adrenal axis were used. These stressors included rotation, forced cold-water swimming and restraint. The efficacy of this paradigm was illustrated by the dramatic increase in serum corticosterone levels assessed immediately following the last stressor (Figure 1a). In fact, many of the stressed mice displayed levels that surpassed the maximal sensitivity of the assay (1000 ng/ml). When this stress paradigm was applied to mice injected subcutaneously with melanoma cells, a similar corticosterone response was seen (Figure 1b). The stress response was also illustrated by a striking decrease in thymus weight (Figure 1c).

Behavioral stress does not alter melanoma progression in vivo. The mice were injected with melanoma cells on day 0, stressed on days 1-12 and sacrificed immediately following the first stress on day 12. Despite a few animals with increased tumor size, the mean tumor volume of the stressed group did not significantly differ from the non-stressed group (Figure 2). There were also no visible metastases in any of the mice.

Behavioral stress does not alter lymphosarcoma progression in vivo. We also attempted to duplicate the reported stress-induced increase in 6C3HED lymphosarcoma tumor growth in C3H mice cells (9). As previously published, tumor cells

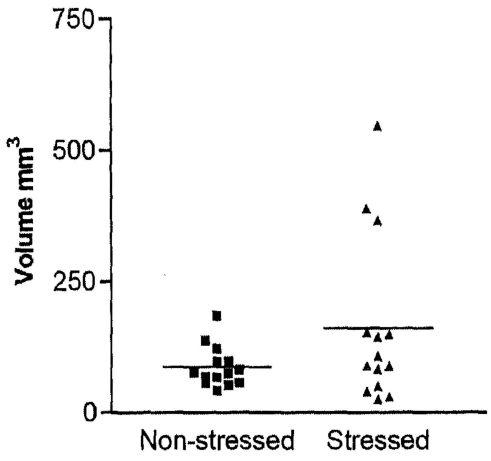

Figure 2. *Stress did not alter melanoma tumor growth in vivo. FVB mice were injected with B16F10 melanoma cells and subjected to the variable stress protocol for 12 days. Tumor volume was determined following the last stressor. Mean tumor volumes were 86.5±10.4 mm3 (n=14) and 160.7±42.2 (n=14) for non-stressed and stressed mice, respectively (p=0.1).*

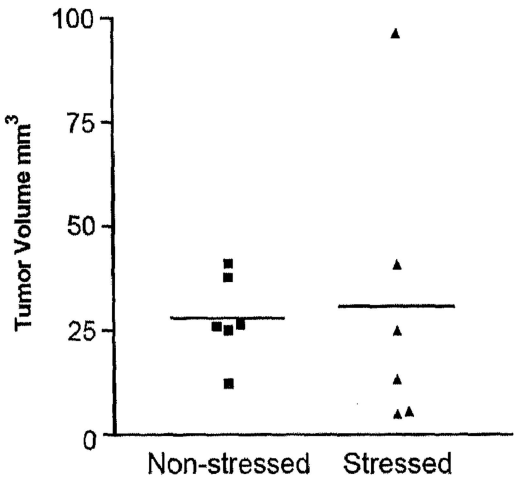

Figure 3. *Stress did not alter lymphosarcoma progression in vivo. C3H mice were injected with 6C3HED lymphosarcoma cells and subjected to rotational stress for 10 minutes of every hour on days 4-6. Tumor volume was determined on day 23. Mean tumor volumes were 28.1±4.2 (n=6) and 30.9±14.2 (n=6) for non-stressed mice and stressed mice, respectively (p=0.85).*

were injected subcutaneously on day 0 and the mice stressed on days 4-6. In this case, the stress was solely rotational. The tumors were then allowed to continue growing until day 23, when the animals were sacrificed. Riley's prior work (9) showed similar tumor growth curves for stressed and non-stressed mice until day 11 or 12, at which time the tumors in the non-stressed mice plateaued in size, while tumors in the stressed mice continued to increase. Thus, by day 23, there was a significant difference in the tumor size between the groups. Our replication of this work did not duplicate this effect of stress (Figure 3). We also repeated this experiment using our stronger, alternating stress paradigm during days 4-6. This approach also did not lead to increased tumor growth in the stressed mice (data not shown). In both cases, the serum corticosterone had returned to control levels by day 23. Riley did not report, nor did we see, any evidence of metastasis.

Discussion

Our experiments did not demonstrate any effect of behavioral stress on tumor growth. The stress paradigm that we developed, consisting of alternating rotation, restraint and cold-water swimming, caused striking increases in serum corticosterone levels and thymus involution, indicating very high levels of behavioral stress. Applied to two models of solid tumors, melanoma and lymphosarcoma, this method did not significantly alter on tumor progression. While three mice in the melanoma experiment displayed increased tumor size, there was no significant difference between the stressed and control groups. Moreover, the mice with the largest tumors did not display higher levels of corticosterone compared to the mice with smaller tumors (data not shown). In addition, we failed to duplicate the differential tumor growth findings of Riley (9) using his stress paradigm of rotation alone.

Our conditions differed from those of Riley's in one respect. While we housed our animals in a conventional animal facility, Riley employed a low-stress environment involving enclosed, individually ventilated shelves that reduced sound and odor transfer between the cages. This low-stress housing reduced the baseline serum corticosterone to 0-35 ng/ml (9). Our control mice had a higher basal level of stress that could have masked any changes similar to those seen by Riley. If this were the case, it would suggest that stress only affects tumor progression when the baseline stress approaches zero. Such a baseline stress level is unrealistic when translating Riley's results to humans. In support of our results, another investigation into the effect of stress on primary tumor growth and metastasis also concluded that mice in conventional animal housing did not display a stress-induced immunomodulation of tumor progression (27).

That we were not able to replicate Riley's finding could alternatively be explained by a possible genetic drift in the mouse strain or an alteration in the tumor cell line. On the other hand, we also found no effect of stress on melanoma growth.

In summary, no association was found between behavioral stress and tumor progression using two mouse models. While our stress paradigm significantly increased serum corticosterone levels and caused thymus involution, it did not influence the growth of either melanoma or lymphosarcoma tumors. Additionally, we could not replicate a previously published finding (9) that demonstrated increased tumor growth following behavioral stress.

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References

1 Locke GR III, Weaver AL, Melton LJ III and Talley NJ: Psychosocial factors are linked to functional gastrointestinal disorders: a population based nested case-control study. Am J Gastroenterol *99*: 350-357, 2004.

2 Murray CD, Flynn J, Ratcliffe L, Jacyna MR, Kamm MA and Emmanuel AV: Effect of acute physical and psychological stress on gut autonomic innervation in irritable bowel syndrome. Gastroenterology *127*: 1695-1703, 2004.

3 Ramachandruni S, Handberg E and Sheps DS: Acute and chronic psychological stress in coronary disease. Curr Opin Cardiol *19*: 494-499, 2004.

4 Jacobs JR and Bovasso GB: Early and chronic stress and their relation to breast cancer. Psychol Med *30*: 669-678, 2000.

5 Spiegel D, Sephton SE, Terr AI and Stites DP: Effects of psychosocial treatment in prolonging cancer survival may be mediated by neuroimmune pathways. Ann NY Acad Sci *840*: 674-683, 1998.

6 Goodwin PJ, Leszcz M, Ennis M, Koopmans J, Vincent L, Guther H, Drysdale E, Hundleby M, Chochinov HM, Navarro M, Speca M and Hunter J: The effect of group psychosocial support on survival in metastatic breast cancer. N Engl J Med *345*: 1719-1726, 2001.

7 Schofield P, Ball D, Smith JG, Borland R, O'Brien P, Davis S, Olver I, Ryan G and Joseph D: Optimism and survival in lung carcinoma patients. Cancer *100*: 1276-1282, 2004.

8 Kroenke CH, Hankinson SE, Schernhammer ES, Colditz GA, Kawachi I and Holmes MD: Caregiving stress, endogenous sex steroid hormone levels, and breast cancer incidence. Am J Epidemiol *159*: 1019-1027, 2004.

9 Riley V: Psychoneuroendocrine influences on immunocompetence and neoplasia. Science *212*: 1100-1109, 1981.

10 Kanno J, Wakikawa A, Utsuyama M and Hirokawa K: Effect of restraint stress on immune system and experimental B16 melanoma metastasis in aged mice. Mech Ageing Dev *93*: 107-117, 1997.

11 Sapolsky RM and Donnelly TM: Vulnerability to stress-induced tumor growth increases with age in rats: role of glucocorticoids. Endocrinology *117*: 662-666, 1985.

12 Stefanski V: Social stress in laboratory rats: behavior, immune function, and tumor metastasis. Physiol Behav *73*: 385-391, 2001.

13 Ben-Eliyahu S, Yirmiya R, Liebeskind JC, Taylor AN and Gale RP: Stress increases metastatic spread of a mammary tumor in rats: evidence for mediation by the immune system. Brain Behav Immun *5*: 193-205, 1991.

14 Hasegawa H and Saiki I: Psychosocial stress augments tumor development through beta-adrenergic activation in mice. Jpn J Cancer Res *93*: 729-735, 2002.

15 Zorzet S, Perissin L, Rapozzi V and Giraldi T: Restraint stress reduces the antitumor efficacy of cyclophosphamide in tumor-bearing mice. Brain Behav Immun *12*: 23-33, 1998.

16 Neigh G, Bowers SL, Korman B and Nelson RJ: Housing environment alters delayed-type hypersensitivity and corticosterone concentrations of individually housed male C57BL/6 mice. Animal Welfare *14*: 249-257, 2005.

17 Strange KS, Kerr LR, Andrews HN, Emerman JT and Weinberg J: Psychosocial stressors and mammary tumor growth: an animal model. Neurotoxicol Teratol *22*: 89-102, 2000.

18 Kurihara H, Koda H, Asami S, Kiso Y and Tanaka T: Contribution of the antioxidative property of astaxanthin to its protective effect on the promotion of cancer metastasis in mice treated with restraint stress. Life Sci *70*: 2509-2520, 2002.

19 Iwakabe K, Shimada M, Ohta A, Yahata T, Ohmi Y, Habu S and Nishimura T: The restraint stress drives a shift in Th1/Th2 balance toward Th2-dominant immunity in mice. Immunol Lett *62*: 39-43, 1998.

20 Manfredi B, Sacerdote P, Gaspani L, Poli V and Panerai AE: IL-6 knock-out mice show modified basal immune functions, but normal immune responses to stress. Brain Behav Immun *12*: 201-211, 1998.

21 Li JM, Kong LD, Wang YM, Cheng CH, Zhang WY and Tan WZ: Behavioral and biochemical studies on chronic mild stress models in rats treated with a Chinese traditional prescription Banxia-houpu decoction. Life Sci *74*: 55-73, 2003.

22 Ben-Eliyahu S, Shakhar G, Page GG, Stefanski V and Shakhar K: Suppression of NK cell activity and of resistance to metastasis by stress: a role for adrenal catecholamines and beta-adrenoceptors. Neuroimmunomodulation *8*: 154-164, 2000.

23 Li T, Harada M, Tamada K, Abe K and Nomoto K: Repeated restraint stress impairs the antitumor T cell response through its suppressive effect on Th1-type CD4+ T cells. Anticancer Res *17*: 4259-4268, 1997.

24 Steplewski Z and Vogel WH: Total leukocytes, T cell subpopulation and natural killer (NK) cell activity in rats exposed to restraint stress. Life Sci *38*: 2419-2427, 1986.

25 Velazquez-Moctezuma J, Dominguez-Salazar E, Cortes-Barberena E., Najera-Medina O, Retana-Marquez S, Rodriguez-Aguilera E, Jimenez-Anguiano A, Cortes-Martinez L and Ortiz-Muniz R: Differential effects of rapid eye movement sleep deprivation and immobilization stress on blood lymphocyte subsets in rats. Neuroimmunomodulation *11*: 261-267, 2004.

26 Bennett MP, Zeller JM, Rosenberg L and McCann J: The effect of mirthful laughter on stress and natural killer cell activity. Altern Ther Health Med *9*: 38-45, 2003.

27 Posevitz V, Vizler C, Benyhe S, Duda E and Borsodi A: Restraint stress and anti-tumor immune response in mice. Acta Biol Hung *54*: 167-176, 2003.

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Chapter III

Concluding Remarks

Modeling the effects of stress on tumor progression is complex and it is not clear what elicits positive results. The work by Thaker and colleagues, previously mentioned in the introduction and published after the work conducted here, raises an interesting question regarding the role of β-adrenergic receptors in stress-induced tumor growth (Thaker *et al.*, 2006). Are β-adrenergic receptors necessary to elicit enhanced tumor growth following stress? In their work, the answer is yes. The same stress paradigm that increases tumor weight and nodules in cell lines that have β-adrenergic receptors has no effect on cell lines that are β-adrenergic receptor-null and stress effects were mimicked by nonspecific β-adrenergic agonist, isoproterenol, and β2-specific agonist, terbutaline, and blocked by nonspecific β-adrenergic antagonist propranolol. This implicates the stress-induced activation of the SNS as the key component regulating stress-related tumor growth. Other work has implicated the activation of the HPA by demonstrating that exogenous corticosterone or synthetic glucocorticoid, dexamethasone, induces tumor growth (Riley, 1981; Sapolsky & Donnelly, 1985). These studies did not, however, investigate the role of the SNS in their models and it is possible that the glucocorticoids were influencing β-adrenergic receptors given the evidence that cortisol increases the density of β-adrenergic receptors in lung cancer (Nakane *et al.*, 1990). Future work should investigate the necessity of HPA versus SNS activation in stress-induced tumor growth. This could include investigating the presence and activation of β-adrenergic receptors in Riley's and Sapolsky's exogenous glucocorticoid models as well as my melanoma model. If β-adrenergic receptors are integral to stress-induced tumor growth then antagonists such as propranolol, already FDA approved and in common use for various conditions, may have therapeutic benefits. Pending a positive effect of

propranolol, a non-selective β-adrenergic receptor antagonist, the scope of the experiments could be focused further by administering the specific β_1-adrenergic antagonist atenolol and the specific β_2-adrenergic antagonist butoxamine to determine through which receptor the effect was mediated (Brennan *et al.*, 1996; Emilien & Maloteaux, 1998).

Another therapy currently being investigated to combat stress-induced tumor growth is group therapy (Goodwin, 2005). As discussed in the introduction, this has met with mixed results. According to the literature, an animal model of stress reduction effects on tumor growth has never been developed. It would be interesting to investigate if environmental enrichment could counteract the effects of stress on tumor growth. Environment enrichment is associated with decreased anxiety behavior and serum corticosterone levels (Fox *et al.*, 2006). If an animal model proved successful this would lend substantial credibility to human psychosocial interventions.

References

Brennan FX, Jr., Cobb CL, Silbert LH, Watkins LR & Maier SF. (1996). Peripheral beta-adrenoreceptors and stress-induced hypercholesterolemia in rats. *Physiol Behav* **60,** 1307-1310.

Emilien G & Maloteaux JM. (1998). Current therapeutic uses and potential of beta-adrenoceptor agonists and antagonists. *Eur J Clin Pharmacol* **53,** 389-404.

Goodwin PJ. (2005). Support groups in advanced breast cancer. *Cancer* **104,** 2596-2601.

Nakane T, Szentendrei T, Stern L, Virmani M, Seely J & Kunos G. (1990). Effects of IL-1 and cortisol on beta-adrenergic receptors, cell proliferation, and differentiation in cultured human A549 lung tumor cells. *J Immunol* **145,** 260-266.

Riley V. (1981). Psychoneuroendocrine influences on immunocompetence and neoplasia. *Science* **212,** 1100-1109.

Sapolsky RM & Donnelly TM. (1985). Vulnerability to stress-induced tumor growth increases with age in rats: role of glucocorticoids. *Endocrinology* **117,** 662-666.

Thaker PH, Han LY, Kamat AA, Arevalo JM, Takahashi R, Lu C, Jennings NB, Armaiz-Pena G, Bankson JA, Ravoori M, Merritt WM, Lin YG, Mangala LS, Kim TJ, Coleman RL, Landen CN, Li Y, Felix E, Sanguino AM, Newman RA, Lloyd M,

Gershenson DM, Kundra V, Lopez-Berestein G, Lutgendorf SK, Cole SW & Sood AK. (2006). Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma. *Nat Med* **12,** 939-944.

Section II

Chapter IV

Evidence for an Association Between the Endothelin Axis and Cancer

The endothelin system

Endothelin (ET), later termed endothelin-1 (ET-1), is a highly potent vasoactive compound originally isolated and characterized in 1988 (Yanagisawa *et al.*, 1988). Preproendothelin (ppET-1) is cleaved to a 39-amino acid intermediate, big-ET, that is then processed to mature ET by endothelin converting enzyme (ECE-1)(Figure 1). The initial cleavage to proendothelin (pET-1), also known as big ET, is made by a neutral endopeptidase (Laporte *et al.*, 1993) and ECE-1, a membrane-bound neutral metalloprotease, cleaves big ET-1 both intracellularly and extracellularly (Xu *et al.*, 1994). Endothelin has regional homology to α-scorpion toxins and its vasocontrictive activity depends on the presence of extracellular calcium (Ca^{2+}). It was therefore postulated that ET-1 interacts directly with membrane ion channels, possibly the dihydropyridine-sensitive Ca^{2+} channels (Yanagisawa *et al.*, 1988). Three distinct genes were soon identified and found to encode three highly similar 21-amino acid peptides, ET-1, ET-2 and ET-3 (Inoue *et al.*, 1989)(Figure 1). Others demonstrated the high homology between endothelins and sarafotoxins, a group

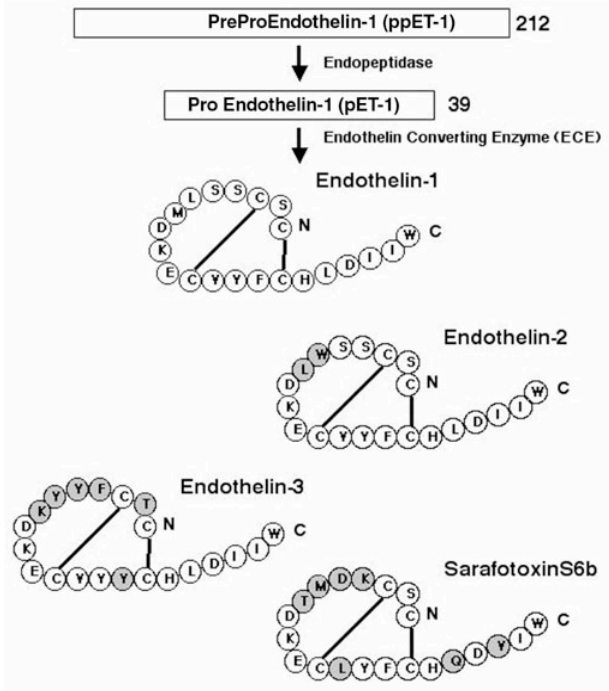

Figure 1. Biosynthesis and amino acid sequence and structure of endothelin-1, endothelin-2, and endothelin-3 and related sarafotoxins. ET-2 and ET-3 differ from ET-1 by two and five amino acids, respectively, while sarafotoxin differs by seven amino acids. Figure reproduced from Fagan *et al.*, 2001.

of vasoactive and cardiotoxic peptides derived from snake venom (Kloog & Sokolovsky, 1989)(Figure 1). The substantially different pharmacologic profiles of these three peptides suggested more than one ET receptor (ETR) subtype. Two G-protein coupled receptors were later identified, endothelin receptor type A (ETRA) that binds ET-1 and ET-2 preferentially over ET-3 (Arai *et al.*, 1990), and endothelin receptor type B (ETRB) that binds all three isopeptides with equal affinity (Sakurai *et al.*, 1990). Binding of any of the ETs results in the production of inositol phosphates and increases intracellular Ca^{2+} (Sakurai *et al.*, 1990). In addition to vasoconstriction, ET-1 and ET-3 also exhibit depressor effects, inducing the release of endothelium-derived relaxing factor/nitric oxide (EDRF/NO) and prostacyclin in an autocrine manner through ETRB (de Nucci *et al.*, 1988; Emori *et al.*, 1991a; Emori *et al.*, 1991b; Clozel *et al.*, 1992). ET-1 also acts on other tissues, inducing atrial natriuretic peptide and thromboxane A2 secretion (Fukuda *et al.*, 1988), inhibiting renin release (Rakugi *et al.*, 1988) and stimulating aldosterone biosynthesis (Morishita *et al.*, 1989). ET-1 synthesis and secretion is stimulated by a variety of other compounds and processes including itself, arginine vasopressin, angiotensin II, catecholamines, cytokines, growth factors, hypoxia, ischemia and shear stress (Emori *et al.*, 1991c; Masaki *et al.*, 1991; Levin, 1995; Love & McMurray, 1996; Jougasaki *et al.*, 2002). ET-1 production is inhibited by nitric oxide, heparin, atrial natriuretic peptide, prostaglandin E2 and prostacyclin (Boulanger & Luscher, 1990; Hu *et al.*, 1992; Yokokawa *et al.*, 1993; Prins *et al.*, 1994). ET-1 is believed to function in an autocrine or paracrine mechanism and not as a circulating hormone (Wagner *et al.*, 1992). Plasma ET-1 concentrations represent only a small percentage of the ET-1 production and clearance from circulation is very rapid (Sirvio *et al.*, 1990; Vierhapper *et al.*, 1990).

In fact, working through ETRB, the pulmonary circulation has the capacity to clear approximately 50% of circulating ET-1 in one pass (Dupuis *et al.*, 1996a; Dupuis *et al.*, 1996b). All three ETs and their receptors have been found in a variety of vascular and nonvascular tissues (Simonson & Dunn, 1990; Rubanyi & Polokoff, 1994). While expression overlaps, ET-1 is found predominantly in endothelial cells, ET-2 in the kidneys and intestines and ET-3 in the brain (Levin, 1995). Yet another role for ET-1 is that of mitogen. ET-1 induces c-fos and c-myc expression and increases proliferation in vascular smooth muscle cells (Komuro *et al.*, 1988). A similar effect is seen in fibroblasts along with the production of inositol trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) and the stimulation of phospholipase C (PLC) (Takuwa *et al.*, 1989). ET-1 has comparable effects on other cell types such as rat glomerular mesangial cells (Simonson *et al.*, 1989) and rat osteoblasts (Takuwa *et al.*, 1990).

Endothelins and development

Endothelins are not solely important for vasoconstriction; they also play a role in normal development. In a targeted mutation of the ET-1 gene, heterozygous mice appear normal, but, mice deficient in ET-1 die at birth from respiratory failure and have extensive craniofacial deformities although they exhibit no other organ abnormalities (Kurihara *et al.*, 1994). These craniofacial malformations stem from problems in the development of the pharyngeal arches. Other studies also support the notion that the ET system is involved in proper development of neural crest derivatives. A null mutation of the ETRB gene in mice results in aganglionic megacolon and coat color spotting (Hosoda *et al.*, 1994), a phenotype akin to hereditary syndromes in mammals including the

piebald-lethal mutation in mice (Lane, 1966) and Waardenburg-Shah syndrome in humans (Shah *et al.*, 1981). Hirschsprung's disease, characterized by lack of enteric ganglia and failed innervation of the gastrointestinal tract, has a missense mutation in the ETRB gene (Puffenberger *et al.*, 1994). A targeted mutation of the ET-3 gene also produces a phenotype of megacolon and coat color spotting remarkably similar to the naturally occurring lethal spotting mutation in mice (Baynash *et al.*, 1994). Using a tetracycline-inducible system, another study determined that ETRB is critical to neural crest development between embryonic days 10 and 12.5, timing important for the proper migration of both enteric neuroblasts and melanoblasts (Shin *et al.*, 1999). These data indicate that ET-3 signaling through ETRB is responsible for proper development of at least two neural crest derivatives, melanocytes and enteric ganglion neurons. It further suggests that ETs do not behave like circulating hormones, since normal levels of ET-1 and ET-2 do not compensate for the lack of ET-3.

Endothelins and cancer

The ET system has been implicated in the growth and progression of several cancers; its role in melanoma and glioma will be discussed in particular detail later in this chapter. Many cell lines derived from cancerous tissue express and respond to components of the ET system. A renal adenocarcinoma cell line was found to secrete immunoreactive ET-2 along with two forms of its precursor, big ET-2 (Ohkubo *et al.*, 1990; Onda *et al.*, 1991; Yorimitsu *et al.*, 1992). In a study of five endometrial adenocarcinoma lines, two secreted ET-1, two had ET-1 mRNA but no detectable ET-1 in the cultured conditioned medium and one had no evidence at all of ET-1 expression

(Pekonen *et al.*, 1992). ET-1 production and secretion has also been documented in mammary, pancreatic and colon carcinoma cell lines (Kusuhara *et al.*, 1990). Another group found several human carcinoma cell lines secreted ET-1 and responded to exogenous ET-1 with increased intracellular Ca^{2+} suggesting an autocrine/paracrine function of ET-1 in cancer (Shichiri *et al.*, 1991a; Shichiri *et al.*, 1991b). Moreover, further investigation of two of the carcinoma lines found that ET-1 induced proliferation at a concentration comparable to that of circulating ET-1 in humans (Shichiri *et al.*, 1991b).

Production and secretion of ET-1 and big ET-1 has also been noted in primary tumors. Not surprisingly, two cases of malignant hemangionendothelioma, a tumor involving endothelial cells, were found to secrete ET-1 and had a concurrent rise in blood pressure (Yokokawa *et al.*, 1991a, b). In a study of hepatocellular carcinoma the arteriovenous difference of ET-1 and big ET-1 was measured across the tumor bed and increased concentrations of both were found in the venous circulation (Ishibashi *et al.*, 1993). Additional histochemical analysis revealed ET-1 and big ET-1 immunoreactivity in tumor tissue with no positive signal in adjacent, noncancerous tissue. In ovarian cancer cell lines and primary and metastatic tumors, ET-1 expression and secretion is increased and acts in an autocrine manner through the ETRA receptor, inducing calcium signaling and proliferation (Bagnato *et al.*, 1995; Bagnato *et al.*, 1999). Plasma ET-1 is elevated in metastatic prostate cancer and every prostate cancer cell line tested produces and secretes ET-1 (Nelson *et al.*, 1995). Moreover, ET-1 increases prostate cancer proliferation and alkaline phosphatase activity, indicating that it may be involved in bone metastasis. Additionally, it has been demonstrated that ET-1 enhances the mitogenic effects of other

known growth factors in prostate cancer and while ETRB is present in benign prostatic tissue, it is down-regulated in malignant tissue and mitogenic effects of ET-1 are mediated through ETRA (Nelson *et al.*, 1996). In colorectal cancer patients with and without metastases, plasma ET-1 is increased compared to controls, ET-1 is up-regulated, has a different distribution in the colon and is located in liver metastasis tumor cells but not normal liver cells (Shankar *et al.*, 1998; Asham *et al.*, 2001). ET-1 and ETRA has been localized to human meningiomas and ET-1 increases proliferation through ETRA in tumor cells (Kitagawa *et al.*, 1994; Pagotto *et al.*, 1995a; Pagotto *et al.*, 1995b). Similar findings have been noted in human papillomavirus-positive cervical cancer cells (Venuti *et al.*, 2000). Increased ET-1 production has also been found in an adrenocorticotropic hormone-secreting bronchial carcinoid (Murakami *et al.*, 1993), some cases of pheochromocytomas (Sone *et al.*, 1991), mammary phyllodes tumors (Yamashita *et al.*, 1992) and other breast cancers (Yamashita *et al.*, 1991). ET-1 promotes survival and proliferation through the activation and enhancement of various signaling molecules including protein kinase C (PKC), epidermal growth factor (EGF), EGF receptor, insulin-like growth factor-1 and mitogen-activated protein kinase (MAPK) (Battistini *et al.*, 1993; Bagnato, 1998; Pirtskhalaishvili & Nelson, 2000; Vacca *et al.*, 2000).

ET-1 also suppresses apoptosis, further perpetuating cancer growth. In fibroblasts, apoptosis induced by serum starvation was attenuated by ET-1 at concentrations lower than those needed for mitogeneis (Shichiri *et al.*, 1998). A similar effect, also mediated by ETRA through MAPK, was seen in vascular smooth muscle cells (Shichiri *et al.*, 2000). Bosentan, a mixed ETRA/ETRB antagonist, enhances Fas-mediated apoptosis in colon cancer cell lines expressing Fas and Fas ligand that are

generally resistant to Fas-mediated apoptosis and low doses of ET-1 blocked this effect (Eberl *et al.*, 2000b). Furthermore, BQ123, an ETRA antagonist, and bisindolylmaleimide IX, a PKC inhibitor, both induced apoptosis in colon carcinoma cells (Eberl *et al.*, 2000a). ET-1 also decreases apoptotic rates in melanocytes and melanoma cell lines that express ETRB and in a line with forced expression of ETRB (Eberle *et al.*, 2002). Additionally, BQ788, an ETRB antagonist, enhances apoptosis in melanoma (Lahav *et al.*, 1999).

Angiogenesis is another aspect of cancer progression influenced by ETs. ET-1 promotes proliferation, migration and invasion of human umbilical vein endothelial cells (HUVEC) in a dose-dependent manner and BQ788 blocked this effect (Salani *et al.*, 2000b). Moreover, ET-1 enhanced vascular-like cord formation on Matrigel and augmented vascular endothelial growth factor (VEGF)-induced angiogenesis *in vivo*. In rat corneas, ET-1 and ET-3 induce angiogenesis through ETRA activation in a higher percentage of animals than VEGF (Bek & McMillen, 2000). In ovarian tumors, ET-1 expression has been correlated with VEGF expression and neovascularization (Salani *et al.*, 2000a). Additionally, ET-1 increased VEGF production through ETRA activation in an ovarian cancer cell line. A separate study of ovarian cancer cell lines also found that ET-1 increased VEGF mRNA and protein levels, enhanced hypoxia inducible factor (HIF)-1α stabilization and activated the HIF-1 transcription complex in an ETRA-mediated manner, all factors that promote angiogenesis (Spinella *et al.*, 2002).

In addition to effects on growth and angiogenesis, ETs may also promote tumor invasion. In ovarian cancer cell lines, ET-1 induces the secretion and activation of several tumor proteinases through binding ETRA, thereby promoting their migration and

invasive capabilities measured by matrix metalloproteinase (MMP)-dependent invasion through Matrigel (Rosano *et al.*, 2001; Rosano *et al.*, 2002).

Endothelin receptor antagonists

Due to the role of the ETs in several disease processes, a wide range of ETR antagonists have been developed including peptides and now more recently bioavailable small molecules. The first ETR antagonist was identified a mere two years after ETs were first characterized although this compound, [D-Arg1,D-Phe5,D-Trp7,9,Leu11] substance P, is actually a nonspecific neuropeptide antagonist that also blocks the effects of bombesin, vasopressin and bradykinin (Fabregat & Rozengurt, 1990). Because of the ETRB's role in clearance of ET-1 from circulation, administration of ETRB antagonists and ETRA/ETRB nonselective antagonists increase plasma ET-1 (Donckier *et al.*, 1995; Dupuis *et al.*, 1996a; Hemsen *et al.*, 1996; Reinhart *et al.*, 2002). Interestingly, while ETRA is not known to be involved in ET-1 clearance from circulation and ETRA antagonists generally do not affect plasma ET-1 levels there has been one case where circulating ET-1 has decreased after administration of sitaxsentan, a specific ETRA antagonist (Givertz *et al.*, 2000). While the mechanism for this ETRA antagonist-induced decrease in plasma ET-1 is not known, it was postulated that ET-1 production may have decreased or the antagonist may have displaced ET-1 from ETRA to ETRB for clearance. Another study demonstrated an increase in plasma ET-1 following administration of Atrasentan, an ETRA specific antagonist, from an unknown mechanism (Verhaar *et al.*, 2000). ETR antagonists have been most thoroughly studied in treatment of cardiovascular diseases. In fact, several drugs are in clinical trials and Bosentan (trade

name, Tracleer®), a dual ETRA/ETRB antagonist, was approved by the U.S. Food and Drug Administration (FDA) for treatment of pulmonary arterial hypertension in 2001 (Table 1). While ETR antagonists are generally well tolerated, side effects do occur. Most side effects reported appear to be related to nonspecific vasodilating effects and include headache, dizziness, chest pain, fatigue, nasal congestion, flushing and peripheral edema (Barst *et al.*, 2002; Luscher *et al.*, 2002; Rubin *et al.*, 2002; Anand *et al.*, 2004; Dingemanse & van Giersbergen, 2004). These effects were generally not significantly different from placebo-treated control groups. One side effect that did differ between treatment groups was concentration-dependent liver toxicity, which was generally reversible upon drug discontinuation (Channick *et al.*, 2001; Rubin *et al.*, 2002; Dingemanse & van Giersbergen, 2004). This toxicity appears to be a result of impaired bile salt transport leading to bile salt accumulation and increases in liver enzyme levels (Fattinger *et al.*, 2001; Fouassier *et al.*, 2002). ETR antagonists are also being studied as possible therapeutics for cancer. Effects of ETR antagonists include blocking ET-1-induced mitogenesis, inhibiting tumor growth *in vitro* and *in vivo*, promoting apoptosis, decreasing tumor proteinases to inhibit invasion, decreasing angiogenesis and enhancing other chemotherapeutic agents (Table 2). The role of ETR antagonists in melanoma and glioma is discussed in further detail in their respective sections.

Endothelins and melanoma

In accord with the previously mentioned role ETs play in neural crest development, ET-1 and ET-3 induce proliferation of neural crest cells and promote their differentiation into mature, pigmented melanocytes (Lahav *et al.*, 1996; Reid *et al.*,

1996). Links between ETs and developing melanocytes naturally led to the investigation of the ET system in melanoma since malignant cells resemble progenitors in their high proliferation rate, high motility, low degree of differentiation or lack of contact inhibition. In melanoma cell lines, ET-1, ET-3 and ETRB are all functionally expressed and binding of ET-1 or ET-3 to ETRB increases proliferation (Yohn *et al.*, 1994; Kikuchi *et al.*, 1996). ET-1 also reduces basal apoptotic levels in melanoma cell lines expressing ETRB (Eberle *et al.*, 2002). On the contrary, one study found that ET-1 inhibited growth and induced apoptosis in synchronized A375 human melanoma cells (Okazawa *et al.*, 1998).

ET-1 is also produced by human keratinocytes, cells that surround melanocytes *in situ*, and its secretion is enhanced in a concentration-dependent manner following ultraviolet B (UVB) irradiation (Imokawa *et al.*, 1992). In human skin, UVB appears to stimulate ET-1 and ETRB expression via stem cell factor (SCF). UVB irradiation enhanced SCF expression after 3 days and ET-1 and ETRB expression after 5-10 days, and exogenous SCF application to cultured human melanocytes increased ETRB expression and ET-1 binding (Hachiya *et al.*, 2004). Moreover, melanocytes exposed to ET-1 following UVB were able to overcome the UVB-induced G1 growth arrest (Tada *et al.*, 1998). Through paracrine signaling, ET-1 could promote melanoma development following sunburn. ETs role in transforming melanocytes to melanoma was further supported using a skin graft model (Berking *et al.*, 2004). Using newborn human skin grafts on severe combined immunodeficient mice, combination treatment of SCF, basic fibroblast growth factor, and ET-3 caused clustering of melanocytes and migration in the upper layers of the epidermis and the epidermo-dermal junction. Individually, the growth

factors had no effect. Addition of UVB irradiation to the combined growth factor treatment resulted in pigmented lesions, *in situ* and invasive melanomas capable of forming colonies in soft agar. Using adult human skin grafts, *in situ* melanomas formed with the treatment of combined growth factor independent of UVB irradiation although the melanomas were less severe and noninvasive. All lesions regressed upon removal of growth factors and colonies on soft agar also required growth factor supplementation and displayed a finite life span. It was postulated that additional factors or time of stimulation might be required to induce stable alterations allowing for autonomous growth.

ETRA expression is inversely correlated with degree of malignancy but ETRB expression patterns are not as clear. In one study, cell lines derived from primary melanoma had a more robust growth response to ET-1 and higher ETRB mRNA expression than those cell lines derived from metastatic melanoma (Kikuchi *et al.*, 1996). Correlating evidence demonstrated that ETRB was down-regulated in melanoma cell lines compared to normal melanocytes and pigment genes for tyrosinase correlated positively with ETRB and negatively with ETRA (Eberle *et al.*, 1999). Tyrosinase genes, TRP-1 and TRP-2, were previously demonstrated to be down-regulated in melanoma cell lines compared to melanocytes (Eberle *et al.*, 1995). On the contrary, a study examining nevi and malignant melanoma lesions found increased ETRB expression in malignant and metastatic melanomas over common and dysplastic nevi and ETRB expression correlated positively with the level of invasion in malignant lesions (Demunter *et al.*, 2001). Interestingly, forced differentiation of melanoma cell line A375 resulted in induction of ETRA while, untreated, this cell line expresses ETRB nearly exclusively (Ohtani *et al.*, 1997).

Our laboratory previously demonstrated that the ETRB-specific antagonist, BQ788, inhibits melanoma cell growth and induces apoptosis both *in vitro* and in a xenograft mouse model (Lahav *et al.*, 1999). *In vitro*, BQ788 reduced the number of viable cells in several human melanoma cell lines in a dose-dependent manner but had no effect on a human kidney cell line. BQ123, an ETRA-specific antagonist, had no effect on cell number despite ETRA expression. BQ788 also induced phenotypic changes in some melanoma cell lines, enhancing pigmentation and dendritic outgrowth, and resembling differentiated melanocytes. BQ788 also induced apoptosis in some melanoma cell lines measured by terminal deoxynucleotidyltransferase-mediated UTP nick-end labeling (TUNEL). Additionally, sarafotoxin 6c, an ETRB-specific agonist, blocked the effects of BQ788 on cell number. Both intratumor and systemic injection of BQ788 inhibited the growth of A375 cells in nude mice. Moreover, BQ788 administration resulted in tumor regression in half of the mice treated systemically. Increased TUNEL staining in the excised tumors treated with BQ788 indicated induction of apoptosis.

Application of bosentan to five primary cultures of melanoma cells decreased viable cell number by inhibiting DNA synthesis and inducing apoptosis (Berger *et al.*, 2006). While all cultures displayed decreased DNA synthesis, one derived from a primary melanoma did not show a significant reduction in cell number. Two cultures, one from a primary melanoma and another from a lymph node metastasis, did not display increased apoptosis although two different cultures from primary melanoma and lymph node metastasis did, indicating that this response was not a function of the degree of malignancy. Binding studies using BQ123 and BQ788 determined that ET-1 binding is

mediated through ETRB although exogenous ET-1 did not induce proliferation in this case. Bosentan application also displayed additive effects in reducing cell number when combined with dacarbazine, an alkylating agent, in a line derived from a cutaneous metastasis.

Another study using primary cultures of melanoma derived from various stages of disease found that BQ788 was most effective at reducing cell number in a lymph node metastasis compared to a cutaneous metastasis or a primary lesion (Lahav *et al.*, 2004). BQ788 reduced expression of anti-apoptotic factor, BCL-2A1, DNA repair enzyme, poly(ADP-ribose) polymerase 3 (PARP-3), and ETRB in metastatic melanomas after two days of treatment and increased apoptosis after five days of treatment. The primary melanoma culture did not display these responses to BQ788. Thus, reduction of BCL-2A1 and PARP-3 expression paralleled reduction in cell numbers. A pan-caspase inhibitor and a caspase-6 inhibitor blocked the apoptotic effects of BQ788, supporting BQ788 induction of caspase-mediated apoptosis. Reduction of ETRB by small interfering RNA (siRNA) reduced cell viability in metastatic melanoma. This effect was positively correlated with degree of malignancy.

An additional function of ET-1 and ET-3 is to down-regulate mRNA and protein levels of E-cadherin as well as α- and β-catenins (Jamal & Schneider, 2002; Rosano *et al.*, 2004b). Furthermore, ET-1 and ET-3 lead to a gain of N-cadherin and induce integrin expression and activation of MMP-2, MMP-9, membrane type-1-MMP, and tissue inhibitor MMP-2 leading to increased cell motility and invasion (Bagnato *et al.*, 2004). It had been previously shown that there is a switch from E-cadherin to N-cadherin expression during melanoma development and forced E-cadherin expression by

melanoma cells leads to cell adhesion with keratinocytes, restoring the normal regulatory dominance of keratinocytes and inhibiting invasion of melanoma cells (Hsu *et al.*, 2000a; Hsu *et al.*, 2000b). Further work found that ET-1 and ET-3, functioning through ETRB, also signal HIF-1α and cyclooxygenase (COX) to promote melanoma invasiveness (Spinella *et al.*, 2007). Under normoxic conditions, ET-1 and ET-3 stimulate VEGF, HIF-1α, prostaglandin E_2 (PGE_2), COX-1 and -2 expression, and COX-2 promoter activity. Hypoxic conditions amplified this effect. COX-1/-2 inhibitors blocked ET-1 induction of VEGF and PGE_2 secretion as well as MMP activation and cell invasion capabilities. Reducing ETRB expression with siRNA and inhibiting ETRB activation with BQ788 individually blocked ET-induced effects. Reduction of HIF-1α by siRNA also attenuated ET-3 induction of VEGF, PGE_2, COX activity and MMP activation. This indicates that ETs play a role in the survival and growth of melanoma as well as the interactions involved in disease progression.

Mutations in ETRB may also play a role in melanoma development. Nonsynonymous variants of ETRB have been linked to an increased risk for malignant melanoma (Soufir *et al.*, 2005). Nonetheless, a separate investigation that focused on only one of the more common ETRB variants, but which screened a larger patient pool, failed to find a correlation to melanoma risk (Thirumaran *et al.*, 2006).

Endothelins and glioma

Shortly after ETs had been identified, it was established that while ET-1 does not cross the blood-brain barrier (Koseki *et al.*, 1989), it is located in the brain (Lee *et al.*, 1990), spinal cord neurons and dorsal root ganglia (Giaid *et al.*, 1989) and nonvascular

binding sites were identified (Jones *et al.*, 1989; Koseki *et al.*, 1989; Kurihara *et al.*, 1990; Kohzuki *et al.*, 1991). ET-3 was also localized to the brain (Shinmi *et al.*, 1989). ET-1 and ET-3 binding sites have similar distribution throughout the brain (Nambi *et al.*, 1990). It was subsequently suggested that in addition to its other roles, ET-1 might act as a neurotransmitter or neuromodulator (Giaid *et al.*, 1989; Jones *et al.*, 1989; Koseki *et al.*, 1989; Yanagisawa & Masaki, 1989; Lee *et al.*, 1990; Kohzuki *et al.*, 1991). ETRA and ETRB are differentially expressed throughout the body and ETRB is the predominant receptor type in nonvascular neural tissue (Williams *et al.*, 1991; Hori *et al.*, 1992; Fernandez-Durango *et al.*, 1994).

Considerably less has been studied about ET's role in glioma progression compared to other cancers. It is known that human glioblastoma and neuroblastoma cell lines express ETRA and ETRB (Takahashi *et al.*, 2002) and produce and secrete ET-1 (Hamroun *et al.*, 2000; Sone *et al.*, 2000; Takahashi *et al.*, 2002). Glioblastoma cell lines also express ppET-1, ppET-3 and ECE-1, in addition to ETRA and ETRB (Egidy *et al.*, 2000). Another investigation examined one low grade human astrocytoma cell line and eight human glioblastoma cell lines (Paolillo *et al.*, 2006). This work detected ppET-1 and ETRB mRNA in all nine lines but ppET-1 and ETRA were only found in the astrocytoma cells and four of the glioblastoma lines. In four of the five cell lines that coexpressed ETRA and ETRB, ETRB mRNA levels were approximately 3.5 times higher than ETRA. The ppET-1 mRNA levels correlated extremely well with the levels of secreted ET-1 and exogenous ET-1 induced ERK phosphorylation (Paolillo *et al.*, 2006). In a neuroblastoma cell line, ET-1, binding ETRA, induces an increase in intracellular

$Ca2^{+}$ and inositol phosphate and inhibits cyclic adenosine monophosphate (cAMP) formation (Heinroth-Hoffmann *et al.*, 1998).

Several human glioma surgical specimens have also demonstrated ET-1 immunoreactivity with a positive correlation to degree of malignancy (Stiles *et al.*, 1997). Another study found glioblastoma multiforme tumors express ETRA on the neovasculature and ETRB on nonvascular, tumor cells (Harland *et al.*, 1998). ET-1, ECE-1, ETRA and ETRB have also all been detected in several human astrocytoma specimens (Naidoo *et al.*, 2005). Surgical samples of oligodendrogliomas, oligoastrocytomas, and glioblastomas also displayed ETRB immunoreactivity (Anguelova *et al.*, 2005). Interestingly, the oligodendroglioma and oligoastrocytoma samples were varied in the location of ETRB, some had nuclear staining, some had cytoplasmic and others had both, although the astrocytic component of the oligoastrocytomas only stained cytoplasmically. In the glioblastoma specimens, ETRB was present in the cytoplasm in limited cells. Neovasculature was postitive for ETRB in oligodendrogliomas and oligoastrocytomas but negative for ETRB in glioblastomas. ETRA immunoreactivity was only found in some glioblastoma cells and control neurons. In primary cultures of oligodendrogliomas, ET-1 induced the transient phosphorylation of ERK and focal adhesion kinase indicating that ETRB was functionally coupled to mitogenic pathways. BQ788 administration induced cytotoxicity and severe morphological changes associated with cytoplasmic vacuoles while BQ123 had no effect on the cells. ETRB-positive reactive astrocytes were also found in surrounding tissue. This is not surprising given previous evidence that reactive gliosis occurs around brain metastases and involves an up-regulation of ET immunoreactivity and secretion of ET-1

(Zhang & Olsson, 1995, 1997). One study took another approach to disrupting ET signaling by targeting ECE-1 (Berger *et al.*, 2005). Using small molecule inhibitors of ECE-1, they were able to inhibit DNA synthesis in human glioblastoma cells. Application of exogenous big ET-1 and, interestingly, ET-1 could not overcome the growth inhibition. These ECE-1 inhibitors, therefore, block proliferation through a mechanism independent of extracellular ET-1 production.

Bosentan administration induced apoptosis in four glioblastoma cell lines in a dose-dependent manner but ET-1 did not stimulate proliferation despite transiently phosphorylating p44/42 MAPK/extracellular signal-regulated kinase (ERK), a known step in proliferation induction (Egidy *et al.*, 2000). Recently, it has been demonstrated that ET-1 induced proliferation in astrocytoma cells involves the MAPK-, PKC- and phosphoinositide 3-kinase (PI3K)-dependent pathways (He *et al.*, 2007). Interestingly, these pathways did not demonstrate any "cross talk" as a both a PKC inhibitor and a PI3K inhibitor independently attenuated proliferation but had no effect on ERK phosphorylation. BQ788, however, did block ERK phosphorylation while BQ610, an ETRA-specific antagonist, had no effect. Another molecule receiving attention in glioblastoma is HIF-1. In hypoxic tumor areas, HIF-1 is stabilized and activated in glioblastoma leading to neoangiogenesis (Kaur *et al.*, 2005). As previously mentioned, ET-1 stabilizes HIF-1α and activates the HIF-1 transcriptional complex in ovarian cancer (Spinella *et al.*, 2002). It is also known that, conversely, HIF-1 can induce ET-1 (Hu *et al.*, 1998). These molecules can thereby function in a positive feedback loop promoting the growth of glioblastoma. Together, these data demonstrate a potential role of ETs in

regulating glioma progression and highlight the need for further investigation of the pathways by which the act in this type of cancer.

Table 1. Endothelin receptor antagonists in clinical trials

Name	Target	Disease	Comments
Bosentan (Tracleer®) (RO 47-0203) (Clozel *et al.*, 1994)	ETRA/ ETRB	Pulmonary arterial hypertension	FDA approved (Motte *et al.*, 2006)
		Systemic sclerosis • Digital ulcers • Raynaud's phenomenon • Myocardial perfusion and function	Clinical improvement noted (Korn *et al.*, 2004; Allanore *et al.*, 2006; Moore *et al.*, 2007)
		Metastatic melanoma	Disease stabilization in some patients (Kefford *et al.*, 2006)
Enrasentan (SB-217242) (Ohlstein *et al.*, 1996)	ETRA/ ETRB	Congestive heart failure	No clinical benefit (Louis *et al.*, 2001)
Tezosentan (Veletri™) (RO 61-0612) (Clozel *et al.*, 1999)	ETRA/ ETRB	Acute heart failure	Mixed results (Cotter *et al.*, 2004)
		Hepatorenal syndrome	Clinical study ongoing (Motte *et al.*, 2006)
Ambrisentan (LU-209075) (Billman, 2002)	ETRA	Pulmonary arterial hypertension	Clinical improvement noted (Galie *et al.*, 2005)
Atrasentan (Xinlay™) (ABT-627) (Opgenorth *et al.*, 1996; Winn *et al.*, 1996)	ETRA	Prostate cancer	Delayed progression and improved quality of life (Fisher, 2002; Carducci *et al.*, 2003; Michaelson *et al.*, 2006)
Clazosentan (RO 61-7790) (Roux *et al.*, 1997)	ETRA	Subarachnoid hemorrhage	Phase IIa looked promising (Vajkoczy *et al.*, 2005), in phase IIb (Motte *et al.*, 2006)

Name	Target	Disease	Comments
Darusentan (LU-135252) (Raschack *et al.*, 1995)	ETRA	Systemic hypertension	Promising outcomes (Nakov *et al.*, 2002)
		Chronic heart failure	Mixed outcomes (Anand *et al.*, 2004; Bergler-Klein *et al.*, 2004)
Edonentan (BMS-207940) (Hulpke-Wette & Buchhorn, 2002)	ETRA	Congestive heart failure	Entered phase I trials (Hulpke-Wette & Buchhorn, 2002)
Sitaxsentan (Thelin™) (TBC-11251) (Wu *et al.*, 1997)	ETRA	Pulmonary arterial hypertension	Clinical improvements noted (Barst *et al.*, 2004; Barst *et al.*, 2006)
TBC-3711 (Wu *et al.*, 2004)	ETRA	Pulmonary arterial hypertension & cardiovascular diseases	Entered phase I trials (Motte *et al.*, 2006)
YM598 (Yuyama *et al.*, 2003)	ETRA	Prostate cancer	Entered phase I trials (Nelson *et al.*, 2003)

Table 2. Select endothelin receptor antagonists in the treatment of cancer

Name	Target	Cancer	Effect
A-127722	ETRA	Prostate	Inhibits ET-1 stimulated growth (Nelson *et al.*, 1996)
BQ610	ETRA	Glioblastoma	Decreases cell number (Takahashi *et al.*, 2002)
Atrasentan (ABT-627)	ETRA	Cervical	Inhibits basal growth rate and ET-1 stimulated growth (Venuti *et al.*, 2002) Inhibits growth and neoangiogenesis in xenografts, additive effects with paclitaxel (Bagnato *et al.*, 2002)
		Ovarian	Inhibits ET-1 stimulated growth (Salani *et al.*, 2002) Inhibits xenograft tumor growth, enhances cytotoxic agents (Rosano *et al.*, 2004a)
		Prostate	In clinical trials (Table 1)
BQ123 (Ihara *et al.*, 1992)	ETRA	Meningioma	Inhibits ET-1 stimulated growth (Pagotto *et al.*, 1995a)
		Cervical	Inhibits growth in culture (Venuti *et al.*, 2000)
		Colorectal	Decreases liver metastasis tumor weight in rats (Asham *et al.*, 2001)
		Ovarian	Inhibits ET-1 induced increase in tumor proteinase activity, decreases migration and invasive potential (Rosano *et al.*, 2001; Rosano *et al.*, 2002)
Bosentan	ETRA /ETRB	Metastatic melanoma	In clinical trials (Table 1)
		Melanoma	Decreases cell viability and DNA synthesis, induces apoptosis, additive effects to alkylating agents (Berger *et al.*, 2006)
		Glioblastoma	Induces apoptosis (Egidy *et al.*, 2000)

Name	Target	Cancer	Effect
BQ788 (Ishikawa *et al.*, 1994)	ETRB	Melanoma	Inhibits growth in culture, inhibits xenograft tumor growth, induces apoptosis (Lahav *et al.*, 1999) Blocks activation of MMPs (Bagnato *et al.*, 2004)
		Astrocytoma	Inhibits ERK phosphorylation (He *et al.*, 2007)
		Oligodendro-glioma	Induces cytotoxicity and morphological changes (Anguelova *et al.*, 2005)
A-192621 (von Geldern *et al.*, 1999)	ETRB	Melanoma	Inhibits xenograft tumor growth (Bagnato *et al.*, 2004; Rosano *et al.*, 2004b)

References

Allanore Y, Meune C, Vignaux O, Weber S, Legmann P & Kahan A. (2006). Bosentan increases myocardial perfusion and function in systemic sclerosis: a magnetic resonance imaging and Tissue-Doppler echography study. *J Rheumatol* **33,** 2464-2469.

Anand I, McMurray J, Cohn JN, Konstam MA, Notter T, Quitzau K, Ruschitzka F & Luscher TF. (2004). Long-term effects of darusentan on left-ventricular remodelling and clinical outcomes in the EndothelinA Receptor Antagonist Trial in Heart Failure (EARTH): randomised, double-blind, placebo-controlled trial. *Lancet* **364,** 347-354.

Anguelova E, Beuvon F, Leonard N, Chaverot N, Varlet P, Couraud PO, Daumas-Duport C & Cazaubon S. (2005). Functional endothelin ET B receptors are selectively expressed in human oligodendrogliomas. *Brain Res Mol Brain Res* **137,** 77-88.

Arai H, Hori S, Aramori I, Ohkubo H & Nakanishi S. (1990). Cloning and expression of a cDNA encoding an endothelin receptor. *Nature* **348,** 730-732.

Asham E, Shankar A, Loizidou M, Fredericks S, Miller K, Boulos PB, Burnstock G & Taylor I. (2001). Increased endothelin-1 in colorectal cancer and reduction of tumour growth by ET(A) receptor antagonism. *Br J Cancer* **85,** 1759-1763.

Bagnato A. (1998). Endothelins as autocrine regulators of tumor cell growth. *Trends in endocrinology and metabolism* **9,** 378-383.

Bagnato A, Cirilli A, Salani D, Simeone P, Muller A, Nicotra MR, Natali PG & Venuti A. (2002). Growth inhibition of cervix carcinoma cells in vivo by endothelin A receptor blockade. *Cancer Res* **62,** 6381-6384.

Bagnato A, Rosano L, Spinella F, Di Castro V, Tecce R & Natali PG. (2004). Endothelin B receptor blockade inhibits dynamics of cell interactions and communications in melanoma cell progression. *Cancer Res* **64,** 1436-1443.

Bagnato A, Salani D, Di Castro V, Wu-Wong JR, Tecce R, Nicotra MR, Venuti A & Natali PG. (1999). Expression of endothelin 1 and endothelin A receptor in ovarian carcinoma: evidence for an autocrine role in tumor growth. *Cancer Res* **59,** 720-727.

Bagnato A, Tecce R, Moretti C, Di Castro V, Spergel D & Catt KJ. (1995). Autocrine actions of endothelin-1 as a growth factor in human ovarian carcinoma cells. *Clin Cancer Res* **1,** 1059-1066.

Barst RJ, Langleben D, Badesch D, Frost A, Lawrence EC, Shapiro S, Naeije R & Galie N. (2006). Treatment of pulmonary arterial hypertension with the selective endothelin-A receptor antagonist sitaxsentan. *J Am Coll Cardiol* **47,** 2049-2056.

Barst RJ, Langleben D, Frost A, Horn EM, Oudiz R, Shapiro S, McLaughlin V, Hill N, Tapson VF, Robbins IM, Zwicke D, Duncan B, Dixon RA & Frumkin LR. (2004). Sitaxsentan therapy for pulmonary arterial hypertension. *Am J Respir Crit Care Med* **169,** 441-447.

Barst RJ, Rich S, Widlitz A, Horn EM, McLaughlin V & McFarlin J. (2002). Clinical efficacy of sitaxsentan, an endothelin-A receptor antagonist, in patients with pulmonary arterial hypertension: open-label pilot study. *Chest* **121,** 1860-1868.

Battistini B, Chailler P, D'Orleans-Juste P, Briere N & Sirois P. (1993). Growth regulatory properties of endothelins. *Peptides* **14,** 385-399.

Baynash AG, Hosoda K, Giaid A, Richardson JA, Emoto N, Hammer RE & Yanagisawa M. (1994). Interaction of endothelin-3 with endothelin-B receptor is essential for development of epidermal melanocytes and enteric neurons. *Cell* **79,** 1277-1285.

Bek EL & McMillen MA. (2000). Endothelins are angiogenic. *J Cardiovasc Pharmacol* **36,** S135-139.

Berger Y, Bernasconi CC & Juillerat-Jeanneret L. (2006). Targeting the endothelin axis in human melanoma: combination of endothelin receptor antagonism and alkylating agents. *Exp Biol Med (Maywood)* **231,** 1111-1119.

Berger Y, Dehmlow H, Blum-Kaelin D, Kitas EA, Loffler BM, Aebi JD & Juillerat-Jeanneret L. (2005). Endothelin-converting enzyme-1 inhibition and growth of human glioblastoma cells. *J Med Chem* **48,** 483-498.

Bergler-Klein J, Pacher R, Berger R, Bojic A & Stanek B. (2004). Neurohumoral and hemodynamic effects of the selective endothelin antagonist darusentan in advanced chronic heart failure. *J Heart Lung Transplant* **23,** 20-27.

Berking C, Takemoto R, Satyamoorthy K, Shirakawa T, Eskandarpour M, Hansson J, VanBelle PA, Elder DE & Herlyn M. (2004). Induction of melanoma phenotypes in human skin by growth factors and ultraviolet B. *Cancer Res* **64,** 807-811.

Billman GE. (2002). Ambrisentan (Myogen). *Curr Opin Investig Drugs* **3,** 1483-1486.

Boulanger C & Luscher TF. (1990). Release of endothelin from the porcine aorta. Inhibition by endothelium-derived nitric oxide. *J Clin Invest* **85,** 587-590.

Carducci MA, Padley RJ, Breul J, Vogelzang NJ, Zonnenberg BA, Daliani DD, Schulman CC, Nabulsi AA, Humerickhouse RA, Weinberg MA, Schmitt JL & Nelson JB. (2003). Effect of endothelin-A receptor blockade with atrasentan on tumor progression in men with hormone-refractory prostate cancer: a randomized, phase II, placebo-controlled trial. *J Clin Oncol* **21,** 679-689.

Channick RN, Simonneau G, Sitbon O, Robbins IM, Frost A, Tapson VF, Badesch DB, Roux S, Rainisio M, Bodin F & Rubin LJ. (2001). Effects of the dual endothelin-receptor antagonist bosentan in patients with pulmonary hypertension: a randomised placebo-controlled study. *Lancet* **358,** 1119-1123.

Clozel M, Breu V, Gray GA, Kalina B, Loffler BM, Burri K, Cassal JM, Hirth G, Muller M, Neidhart W & et al. (1994). Pharmacological characterization of bosentan, a new potent orally active nonpeptide endothelin receptor antagonist. *J Pharmacol Exp Ther* **270,** 228-235.

Clozel M, Gray GA, Breu V, Loffler BM & Osterwalder R. (1992). The endothelin ETB receptor mediates both vasodilation and vasoconstriction in vivo. *Biochem Biophys Res Commun* **186,** 867-873.

Clozel M, Ramuz H, Clozel JP, Breu V, Hess P, Loffler BM, Coassolo P & Roux S. (1999). Pharmacology of tezosentan, new endothelin receptor antagonist designed for parenteral use. *J Pharmacol Exp Ther* **290,** 840-846.

Cotter G, Kaluski E, Stangl K, Pacher R, Richter C, Milo-Cotter O, Perchenet L, Kobrin I, Kaplan S, Rainisio M, Frey A, Neuhart E, Vered Z, Dingemanse J & Torre-Amione G. (2004). The hemodynamic and neurohormonal effects of low doses of

tezosentan (an endothelin A/B receptor antagonist) in patients with acute heart failure. *Eur J Heart Fail* **6,** 601-609.

de Nucci G, Thomas R, D'Orleans-Juste P, Antunes E, Walder C, Warner TD & Vane JR. (1988). Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. *Proc Natl Acad Sci U S A* **85,** 9797-9800.

Demunter A, De Wolf-Peeters C, Degreef H, Stas M & van den Oord JJ. (2001). Expression of the endothelin-B receptor in pigment cell lesions of the skin. Evidence for its role as tumor progression marker in malignant melanoma. *Virchows Arch* **438,** 485-491.

Dingemanse J & van Giersbergen PL. (2004). Clinical pharmacology of bosentan, a dual endothelin receptor antagonist. *Clin Pharmacokinet* **43,** 1089-1115.

Donckier J, Stoleru L, Hayashida W, Van Mechelen H, Selvais P, Galanti L, Clozel JP, Ketelslegers JM & Pouleur H. (1995). Role of endogenous endothelin-1 in experimental renal hypertension in dogs. *Circulation* **92,** 106-113.

Dupuis J, Goresky CA & Fournier A. (1996a). Pulmonary clearance of circulating endothelin-1 in dogs in vivo: exclusive role of ETB receptors. *J Appl Physiol* **81,** 1510-1515.

Dupuis J, Stewart DJ, Cernacek P & Gosselin G. (1996b). Human pulmonary circulation is an important site for both clearance and production of endothelin-1. *Circulation* **94,** 1578-1584.

Eberl LP, Egidy G, Pinet F & Juillerat-Jeanneret L. (2000a). Endothelin receptor blockade potentiates FasL-induced apoptosis in colon carcinoma cells via the protein kinase C-pathway. *J Cardiovasc Pharmacol* **36,** S354-356.

Eberl LP, Valdenaire O, Saintgiorgio V, Jeannin JF & Juillerat-Jeanneret L. (2000b). Endothelin receptor blockade potentiates FasL-induced apoptosis in rat colon carcinoma cells. *Int J Cancer* **86,** 182-187.

Eberle J, Fecker LF, Orfanos CE & Geilen CC. (2002). Endothelin-1 decreases basic apoptotic rates in human melanoma cell lines. *J Invest Dermatol* **119,** 549-555.

Eberle J, Garbe C, Wang N & Orfanos CE. (1995). Incomplete expression of the tyrosinase gene family (tyrosinase, TRP-1, and TRP-2) in human malignant melanoma cells in vitro. *Pigment Cell Res* **8,** 307-313.

Eberle J, Weitmann S, Thieck O, Pech H, Paul M & Orfanos CE. (1999). Downregulation of endothelin B receptor in human melanoma cell lines parallel to differentiation genes. *J Invest Dermatol* **112,** 925-932.

Egidy G, Eberl LP, Valdenaire O, Irmler M, Majdi R, Diserens AC, Fontana A, Janzer RC, Pinet F & Juillerat-Jeanneret L. (2000). The endothelin system in human glioblastoma. *Lab Invest* **80,** 1681-1689.

Emori T, Hirata Y, Kanno K, Ohta K, Eguchi S, Imai T, Shichiri M & Marumo F. (1991a). Endothelin-3 stimulates production of endothelium-derived nitric oxide via phosphoinositide breakdown. *Biochem Biophys Res Commun* **174,** 228-235.

Emori T, Hirata Y & Marumo F. (1991b). Endothelin-3 stimulates prostacyclin production in cultured bovine endothelial cells. *J Cardiovasc Pharmacol* **17 Suppl 7,** S140-142.

Emori T, Hirata Y, Ohta K, Kanno K, Eguchi S, Imai T, Shichiri M & Marumo F. (1991c). Cellular mechanism of endothelin-1 release by angiotensin and vasopressin. *Hypertension* **18,** 165-170.

Fabregat I & Rozengurt E. (1990). [D-Arg1,D-Phe5,D-Trp7,9,Leu11]substance P, a neuropeptide antagonist, blocks binding, Ca2(+)-mobilizing, and mitogenic effects of endothelin and vasoactive intestinal contractor in mouse 3T3 cells. *J Cell Physiol* **145,** 88-94.

Fagan KA, McMurtry IF & Rodman DM. (2001). Role of endothelin-1 in lung disease. *Respir Res* **2,** 90-101.

Fattinger K, Funk C, Pantze M, Weber C, Reichen J, Stieger B & Meier PJ. (2001). The endothelin antagonist bosentan inhibits the canalicular bile salt export pump: a potential mechanism for hepatic adverse reactions. *Clin Pharmacol Ther* **69,** 223-231.

Fernandez-Durango R, de Juan JA, Zimman H, Moya FJ, Garcia de la Coba M & Fernandez-Cruz A. (1994). Identification of endothelin receptor subtype (ETB) in human cerebral cortex using subtype-selective ligands. *J Neurochem* **62,** 1482-1488.

Fisher M. (2002). The endothelin-1 antagonist, atrasentan, improves time to progression and quality of life in hormone-refractory prostate cancer. *Clin Prostate Cancer* **1,** 79-80.

Fouassier L, Kinnman N, Lefevre G, Lasnier E, Rey C, Poupon R, Elferink RP & Housset C. (2002). Contribution of mrp2 in alterations of canalicular bile formation by the endothelin antagonist bosentan. *J Hepatol* **37,** 184-191.

Fukuda Y, Hirata Y, Yoshimi H, Kojima T, Kobayashi Y, Yanagisawa M & Masaki T. (1988). Endothelin is a potent secretagogue for atrial natriuretic peptide in cultured rat atrial myocytes. *Biochem Biophys Res Commun* **155,** 167-172.

Galie N, Badesch D, Oudiz R, Simonneau G, McGoon MD, Keogh AM, Frost AE, Zwicke D, Naeije R, Shapiro S, Olschewski H & Rubin LJ. (2005). Ambrisentan therapy for pulmonary arterial hypertension. *J Am Coll Cardiol* **46,** 529-535.

Giaid A, Gibson SJ, Ibrahim BN, Legon S, Bloom SR, Yanagisawa M, Masaki T, Varndell IM & Polak JM. (1989). Endothelin 1, an endothelium-derived peptide, is expressed in neurons of the human spinal cord and dorsal root ganglia. *Proc Natl Acad Sci U S A* **86,** 7634-7638.

Givertz MM, Colucci WS, LeJemtel TH, Gottlieb SS, Hare JM, Slawsky MT, Leier CV, Loh E, Nicklas JM & Lewis BE. (2000). Acute endothelin A receptor blockade causes selective pulmonary vasodilation in patients with chronic heart failure. *Circulation* **101,** 2922-2927.

Hachiya A, Kobayashi A, Yoshida Y, Kitahara T, Takema Y & Imokawa G. (2004). Biphasic expression of two paracrine melanogenic cytokines, stem cell factor and endothelin-1, in ultraviolet B-induced human melanogenesis. *Am J Pathol* **165,** 2099-2109.

Hamroun D, Mathieu MN & Chevillard C. (2000). The human neuroblastoma SK-SY5Y cell line bears functional endothelin-A-receptors and endothelin. *J Cardiovasc Pharmacol* **36,** S66-68.

Harland SP, Kuc RE, Pickard JD & Davenport AP. (1998). Expression of endothelin(A) receptors in human gliomas and meningiomas, with high affinity for the selective antagonist PD156707. *Neurosurgery* **43,** 890-898; discussion 898-899.

He S, Dibas A, Yorio T & Prasanna G. (2007). Parallel signaling pathways in endothelin-1-induced proliferation of U373MG astrocytoma cells. *Exp Biol Med (Maywood)* **232,** 370-384.

Heinroth-Hoffmann I, Vogelsang M, Schiewe P, Morawietz H, Holtz J, Ponicke K & Brodde OE. (1998). Mechanism of ET(A)-receptor stimulation-induced increases in intracellular Ca2+ in SK-N-MC cells. *Br J Pharmacol* **125,** 1202-1211.

Hemsen A, Modin A, Wanecek M, Malmstrom RE & Weitzberg E. (1996). Effects of Ro 47-0203 and PD155080 on the plasma kinetics, receptor binding and vascular effects of endothelin in the pig. *Eur J Pharmacol* **318,** 369-376.

Hori S, Komatsu Y, Shigemoto R, Mizuno N & Nakanishi S. (1992). Distinct tissue distribution and cellular localization of two messenger ribonucleic acids encoding different subtypes of rat endothelin receptors. *Endocrinology* **130,** 1885-1895.

Hosoda K, Hammer RE, Richardson JA, Baynash AG, Cheung JC, Giaid A & Yanagisawa M. (1994). Targeted and natural (piebald-lethal) mutations of endothelin-B receptor gene produce megacolon associated with spotted coat color in mice. *Cell* **79,** 1267-1276.

Hsu M, Andl T, Li G, Meinkoth JL & Herlyn M. (2000a). Cadherin repertoire determines partner-specific gap junctional communication during melanoma progression. *J Cell Sci* **113 (Pt 9),** 1535-1542.

Hsu MY, Meier FE, Nesbit M, Hsu JY, Van Belle P, Elder DE & Herlyn M. (2000b). E-cadherin expression in melanoma cells restores keratinocyte-mediated growth control and down-regulates expression of invasion-related adhesion receptors. *Am J Pathol* **156,** 1515-1525.

Hu J, Discher DJ, Bishopric NH & Webster KA. (1998). Hypoxia regulates expression of the endothelin-1 gene through a proximal hypoxia-inducible factor-1 binding site on the antisense strand. *Biochem Biophys Res Commun* **245,** 894-899.

Hu RM, Levin ER, Pedram A & Frank HJ. (1992). Atrial natriuretic peptide inhibits the production and secretion of endothelin from cultured endothelial cells. Mediation through the C receptor. *J Biol Chem* **267,** 17384-17389.

Hulpke-Wette M & Buchhorn R. (2002). BMS-193884 and BMS-207940 Bristol-Myers Squibb. *Curr Opin Investig Drugs* **3,** 1057-1061.

Ihara M, Ishikawa K, Fukuroda T, Saeki T, Funabashi K, Fukami T, Suda H & Yano M. (1992). In vitro biological profile of a highly potent novel endothelin (ET) antagonist BQ-123 selective for the ETA receptor. *J Cardiovasc Pharmacol* **20 Suppl 12,** S11-14.

Imokawa G, Yada Y & Miyagishi M. (1992). Endothelins secreted from human keratinocytes are intrinsic mitogens for human melanocytes. *J Biol Chem* **267,** 24675-24680.

Inoue A, Yanagisawa M, Kimura S, Kasuya Y, Miyauchi T, Goto K & Masaki T. (1989). The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc Natl Acad Sci U S A* **86,** 2863-2867.

Ishibashi M, Fujita M, Nagai K, Kako M, Furue H, Haku E, Osamura Y & Yamaji T. (1993). Production and secretion of endothelin by hepatocellular carcinoma. *J Clin Endocrinol Metab* **76,** 378-383.

Ishikawa K, Ihara M, Noguchi K, Mase T, Mino N, Saeki T, Fukuroda T, Fukami T, Ozaki S, Nagase T & et al. (1994). Biochemical and pharmacological profile of a

potent and selective endothelin B-receptor antagonist, BQ-788. *Proc Natl Acad Sci U S A* **91,** 4892-4896.

Jamal S & Schneider RJ. (2002). UV-induction of keratinocyte endothelin-1 downregulates E-cadherin in melanocytes and melanoma cells. *J Clin Invest* **110,** 443-452.

Jones CR, Hiley CR, Pelton JT & Mohr M. (1989). Autoradiographic visualization of the binding sites for [125I]endothelin in rat and human brain. *Neurosci Lett* **97,** 276-279.

Jougasaki M, Larsen AM, Cataliotti A, Christiansen DC & Burnett JC, Jr. (2002). Cardiotrophin-1 stimulates endothelin-1 via gp130 in vascular endothelial cells. *Peptides* **23,** 1441-1447.

Kaur B, Khwaja FW, Severson EA, Matheny SL, Brat DJ & Van Meir EG. (2005). Hypoxia and the hypoxia-inducible-factor pathway in glioma growth and angiogenesis. *Neuro-oncol* **7,** 134-153.

Kefford R, Beith JM, Van Hazel GA, Millward M, Trotter JM, Wyld DK, Kusic R, Shreeniwas R, Morganti A, Ballmer A, Segal E, Nayler O & Clozel M. (2006). A Phase II study of bosentan, a dual endothelin receptor antagonist, as monotherapy in patients with stage IV metastatic melanoma. *Invest New Drugs*.

Kikuchi K, Nakagawa H, Kadono T, Etoh T, Byers HR, Mihm MC & Tamaki K. (1996). Decreased ET(B) receptor expression in human metastatic melanoma cells. *Biochem Biophys Res Commun* **219,** 734-739.

Kitagawa N, Tsutsumi K, Niwa M, Himeno A, Yamashita K, Shibata S, Taniyama K, Kurihara M, Kawano T, Yasunaga A & et al. (1994). Expression of a functional endothelin (ETA) receptor in human meningiomas. *J Neurosurg* **80,** 723-731.

Kloog Y & Sokolovsky M. (1989). Similarities in mode and sites of action of sarafotoxins and endothelins. *Trends Pharmacol Sci* **10,** 212-214.

Kohzuki M, Chai SY, Paxinos G, Karavas A, Casley DJ, Johnston CI & Mendelsohn FA. (1991). Localization and characterization of endothelin receptor binding sites in the rat brain visualized by in vitro autoradiography. *Neuroscience* **42,** 245-260.

Komuro I, Kurihara H, Sugiyama T, Yoshizumi M, Takaku F & Yazaki Y. (1988). Endothelin stimulates c-fos and c-myc expression and proliferation of vascular smooth muscle cells. *FEBS Lett* **238,** 249-252.

Korn JH, Mayes M, Matucci Cerinic M, Rainisio M, Pope J, Hachulla E, Rich E, Carpentier P, Molitor J, Seibold JR, Hsu V, Guillevin L, Chatterjee S, Peter HH, Coppock J, Herrick A, Merkel PA, Simms R, Denton CP, Furst D, Nguyen N,

Gaitonde M & Black C. (2004). Digital ulcers in systemic sclerosis: prevention by treatment with bosentan, an oral endothelin receptor antagonist. *Arthritis Rheum* **50,** 3985-3993.

Koseki C, Imai M, Hirata Y, Yanagisawa M & Masaki T. (1989). Autoradiographic distribution in rat tissues of binding sites for endothelin: a neuropeptide? *Am J Physiol* **256,** R858-866.

Kurihara M, Ochi A, Kawaguchi T, Niwa M, Kataoka Y & Mori K. (1990). Localization and characterization of endothelin receptors in human gliomas: a growth factor? *Neurosurgery* **27,** 275-281.

Kurihara Y, Kurihara H, Suzuki H, Kodama T, Maemura K, Nagai R, Oda H, Kuwaki T, Cao WH, Kamada N & et al. (1994). Elevated blood pressure and craniofacial abnormalities in mice deficient in endothelin-1. *Nature* **368,** 703-710.

Kusuhara M, Yamaguchi K, Nagasaki K, Hayashi C, Suzaki A, Hori S, Handa S, Nakamura Y & Abe K. (1990). Production of endothelin in human cancer cell lines. *Cancer Res* **50,** 3257-3261.

Lahav R, Heffner G & Patterson PH. (1999). An endothelin receptor B antagonist inhibits growth and induces cell death in human melanoma cells in vitro and in vivo. *Proc Natl Acad Sci U S A* **96,** 11496-11500.

Lahav R, Suva ML, Rimoldi D, Patterson PH & Stamenkovic I. (2004). Endothelin receptor B inhibition triggers apoptosis and enhances angiogenesis in melanomas. *Cancer Res* **64,** 8945-8953.

Lahav R, Ziller C, Dupin E & Le Douarin NM. (1996). Endothelin 3 promotes neural crest cell proliferation and mediates a vast increase in melanocyte number in culture. *Proc Natl Acad Sci U S A* **93,** 3892-3897.

Lane PW. (1966). Association of megacolon with two recessive spotting genes in the mouse. *J Hered* **57,** 29-31.

Laporte S, Denault JB, D'Orleans-Juste P & Leduc R. (1993). Presence of furin mRNA in cultured bovine endothelial cells and possible involvement of furin in the processing of the endothelin precursor. *J Cardiovasc Pharmacol* **22 Suppl 8,** S7-10.

Lee ME, de la Monte SM, Ng SC, Bloch KD & Quertermous T. (1990). Expression of the potent vasoconstrictor endothelin in the human central nervous system. *J Clin Invest* **86,** 141-147.

Levin ER. (1995). Endothelins. *N Engl J Med* **333,** 356-363.

Louis A, Cleland JG, Crabbe S, Ford S, Thackray S, Houghton T & Clark A. (2001). Clinical Trials Update: CAPRICORN, COPERNICUS, MIRACLE, STAF, RITZ-2, RECOVER and RENAISSANCE and cachexia and cholesterol in heart failure. Highlights of the Scientific Sessions of the American College of Cardiology, 2001. *Eur J Heart Fail* **3,** 381-387.

Love MP & McMurray JJ. (1996). Endothelin in chronic heart failure: current position and future prospects. *Cardiovasc Res* **31,** 665-674.

Luscher TF, Enseleit F, Pacher R, Mitrovic V, Schulze MR, Willenbrock R, Dietz R, Rousson V, Hurlimann D, Philipp S, Notter T, Noll G & Ruschitzka F. (2002). Hemodynamic and neurohumoral effects of selective endothelin A (ET(A)) receptor blockade in chronic heart failure: the Heart Failure ET(A) Receptor Blockade Trial (HEAT). *Circulation* **106,** 2666-2672.

Masaki T, Kimura S, Yanagisawa M & Goto K. (1991). Molecular and cellular mechanism of endothelin regulation. Implications for vascular function. *Circulation* **84,** 1457-1468.

Michaelson MD, Kaufman DS, Kantoff P, Oh WK & Smith MR. (2006). Randomized phase II study of atrasentan alone or in combination with zoledronic acid in men with metastatic prostate cancer. *Cancer* **107,** 530-535.

Moore TL, Vail A & Herrick AL. (2007). Assessment of digital vascular structure and function in response to bosentan in patients with systemic sclerosis-related Raynaud's phenomenon. *Rheumatology (Oxford)* **46,** 363-364.

Morishita R, Higaki J & Ogihara T. (1989). Endothelin stimulates aldosterone biosynthesis by dispersed rabbit adreno-capsular cells. *Biochem Biophys Res Commun* **160,** 628-632.

Motte S, McEntee K & Naeije R. (2006). Endothelin receptor antagonists. *Pharmacol Ther* **110,** 386-414.

Murakami O, Takahashi K, Sone M, Totsune K, Ohneda M, Itoi K, Yoshinaga K & Mouri T. (1993). An ACTH-secreting bronchial carcinoid: presence of corticotropin-releasing hormone, neuropeptide Y and endothelin-1 in the tumor tissue. *Acta Endocrinol (Copenh)* **128,** 192-196.

Naidoo V, Naidoo S, Mahabeer R & Raidoo DM. (2005). Localization of the endothelin system in human diffuse astrocytomas. *Cancer* **104,** 1049-1057.

Nakov R, Pfarr E & Eberle S. (2002). Darusentan: an effective endothelinA receptor antagonist for treatment of hypertension. *Am J Hypertens* **15,** 583-589.

Nambi P, Pullen M & Feuerstein G. (1990). Identification of endothelin receptors in various regions of rat brain. *Neuropeptides* **16,** 195-199.

Nelson J, Bagnato A, Battistini B & Nisen P. (2003). The endothelin axis: emerging role in cancer. *Nat Rev Cancer* **3,** 110-116.

Nelson JB, Chan-Tack K, Hedican SP, Magnuson SR, Opgenorth TJ, Bova GS & Simons JW. (1996). Endothelin-1 production and decreased endothelin B receptor expression in advanced prostate cancer. *Cancer Res* **56,** 663-668.

Nelson JB, Hedican SP, George DJ, Reddi AH, Piantadosi S, Eisenberger MA & Simons JW. (1995). Identification of endothelin-1 in the pathophysiology of metastatic adenocarcinoma of the prostate. *Nat Med* **1,** 944-949.

Ohkubo S, Ogi K, Hosoya M, Matsumoto H, Suzuki N, Kimura C, Ondo H & Fujino M. (1990). Specific expression of human endothelin-2 (ET-2) gene in a renal adenocarcinoma cell line. Molecular cloning of cDNA encoding the precursor of ET-2 and its characterization. *FEBS Lett* **274,** 136-140.

Ohlstein EH, Nambi P, Lago A, Hay DW, Beck G, Fong KL, Eddy EP, Smith P, Ellens H & Elliott JD. (1996). Nonpeptide endothelin receptor antagonists. VI: Pharmacological characterization of SB 217242, a potent and highly bioavailable endothelin receptor antagonist. *J Pharmacol Exp Ther* **276,** 609-615.

Ohtani T, Ninomiya H, Okazawa M, Imamura S & Masaki T. (1997). Bromodeoxyuridine-induced expression of endothelin A in A375 human melanoma cells. *Biochem Biophys Res Commun* **234,** 526-530.

Okazawa M, Shiraki T, Ninomiya H, Kobayashi S & Masaki T. (1998). Endothelin-induced apoptosis of A375 human melanoma cells. *J Biol Chem* **273,** 12584-12592.

Onda H, Ohkubo S, Kosaka T, Yasuhara T, Ogi K, Hosoya M, Matsumoto H, Suzuki N, Kitada C, Ishibashi Y & et al. (1991). Expression of endothelin-2 (ET-2) gene in a human renal adenocarcinoma cell line: purification and cDNA cloning of ET-2. *J Cardiovasc Pharmacol* **17 Suppl 7,** S39-43.

Opgenorth TJ, Adler AL, Calzadilla SV, Chiou WJ, Dayton BD, Dixon DB, Gehrke LJ, Hernandez L, Magnuson SR, Marsh KC, Novosad EI, Von Geldern TW, Wessale JL, Winn M & Wu-Wong JR. (1996). Pharmacological characterization of A-127722: an orally active and highly potent ETA-selective receptor antagonist. *J Pharmacol Exp Ther* **276,** 473-481.

Pagotto U, Arzberger T, Hopfner U, Sauer J, Renner U, Newton CJ, Lange M, Uhl E, Weindl A & Stalla GK. (1995a). Expression and localization of endothelin-1 and

endothelin receptors in human meningiomas. Evidence for a role in tumoral growth. *J Clin Invest* **96,** 2017-2025.

Pagotto U, Arzberger T, Hopfner U, Weindl A & Stalla GK. (1995b). Cellular localization of endothelin receptor mRNAs (ETA and ETB) in brain tumors and normal human brain. *J Cardiovasc Pharmacol* **26 Suppl 3,** S104-106.

Paolillo M, Barbieri A, Zanassi P & Schinelli S. (2006). Expression of endothelins and their receptors in glioblastoma cell lines. *J Neurooncol* **79,** 1-7.

Pekonen F, Saijonmaa O, Nyman T & Fyhrquist F. (1992). Human endometrial adenocarcinoma cells express endothelin-1. *Mol Cell Endocrinol* **84,** 203-207.

Pirtskhalaishvili G & Nelson JB. (2000). Endothelium-derived factors as paracrine mediators of prostate cancer progression. *Prostate* **44,** 77-87.

Prins BA, Hu RM, Nazario B, Pedram A, Frank HJ, Weber MA & Levin ER. (1994). Prostaglandin E2 and prostacyclin inhibit the production and secretion of endothelin from cultured endothelial cells. *J Biol Chem* **269,** 11938-11944.

Puffenberger EG, Hosoda K, Washington SS, Nakao K, deWit D, Yanagisawa M & Chakravart A. (1994). A missense mutation of the endothelin-B receptor gene in multigenic Hirschsprung's disease. *Cell* **79,** 1257-1266.

Rakugi H, Nakamaru M, Saito H, Higaki J & Ogihara T. (1988). Endothelin inhibits renin release from isolated rat glomeruli. *Biochem Biophys Res Commun* **155,** 1244-1247.

Raschack M, Unger L, Riechers H & Klinge D. (1995). Receptor selectivity of endothelin antagonists and prevention of vasoconstriction and endothelin-induced sudden death. *J Cardiovasc Pharmacol* **26 Suppl 3,** S397-399.

Reid K, Turnley AM, Maxwell GD, Kurihara Y, Kurihara H, Bartlett PF & Murphy M. (1996). Multiple roles for endothelin in melanocyte development: regulation of progenitor number and stimulation of differentiation. *Development* **122,** 3911-3919.

Reinhart GA, Preusser LC, Burke SE, Wessale JL, Wegner CD, Opgenorth TJ & Cox BF. (2002). Hypertension induced by blockade of ET(B) receptors in conscious nonhuman primates: role of ET(A) receptors. *Am J Physiol Heart Circ Physiol* **283,** H1555-1561.

Rosano L, Salani D, Di Castro V, Spinella F, Natali PG & Bagnato A. (2002). Endothelin-1 promotes proteolytic activity of ovarian carcinoma. *Clin Sci (Lond)* **103 Suppl 48,** 306S-309S.

Rosano L, Spinella F, Di Castro V, Natali PG & Bagnato A. (2004a). Therapeutic Targeting of the Endothelin-A Receptor in Human Ovarian Carcinoma: Efficacy of Cytotoxic Agents is Markedly Enhanced by Co-administration with Atrasentan. *J Cardiovasc Pharmacol* **44,** S132-S135.

Rosano L, Spinella F, Genovesi G, Di Castro V, Natali PG & Bagnato A. (2004b). Endothelin-B Receptor Blockade Inhibits Molecular Effectors of Melanoma Cell Progression. *J Cardiovasc Pharmacol* **44,** S136-S139.

Rosano L, Varmi M, Salani D, Di Castro V, Spinella F, Natali PG & Bagnato A. (2001). Endothelin-1 induces tumor proteinase activation and invasiveness of ovarian carcinoma cells. *Cancer Res* **61,** 8340-8346.

Roux S, Breu V, Giller T, Neidhart W, Ramuz H, Coassolo P, Clozel JP & Clozel M. (1997). Ro 61-1790, a new hydrosoluble endothelin antagonist: general pharmacology and effects on experimental cerebral vasospasm. *J Pharmacol Exp Ther* **283,** 1110-1118.

Rubanyi GM & Polokoff MA. (1994). Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. *Pharmacol Rev* **46,** 325-415.

Rubin LJ, Badesch DB, Barst RJ, Galie N, Black CM, Keogh A, Pulido T, Frost A, Roux S, Leconte I, Landzberg M & Simonneau G. (2002). Bosentan therapy for pulmonary arterial hypertension. *N Engl J Med* **346,** 896-903.

Sakurai T, Yanagisawa M, Takuwa Y, Miyazaki H, Kimura S, Goto K & Masaki T. (1990). Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature* **348,** 732-735.

Salani D, Di Castro V, Nicotra MR, Rosano L, Tecce R, Venuti A, Natali PG & Bagnato A. (2000a). Role of endothelin-1 in neovascularization of ovarian carcinoma. *Am J Pathol* **157,** 1537-1547.

Salani D, Rosano L, Di Castro V, Spinella F, Venuti A, Padley RJ, Nicotra MR, Natali PG & Bagnato A. (2002). ABT-627, a potent endothelin receptor A antagonist, inhibits ovarian carcinoma growth in vitro. *Clin Sci (Lond)* **103 Suppl 48,** 318S-321S.

Salani D, Taraboletti G, Rosano L, Di Castro V, Borsotti P, Giavazzi R & Bagnato A. (2000b). Endothelin-1 induces an angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo. *Am J Pathol* **157,** 1703-1711.

Shah KN, Dalal SJ, Desai MP, Sheth PN, Joshi NC & Ambani LM. (1981). White forelock, pigmentary disorder of irides, and long segment Hirschsprung disease: possible variant of Waardenburg syndrome. *J Pediatr* **99,** 432-435.

Shankar A, Loizidou M, Aliev G, Fredericks S, Holt D, Boulos PB, Burnstock G & Taylor I. (1998). Raised endothelin 1 levels in patients with colorectal liver metastases. *Br J Surg* **85,** 502-506.

Shichiri M, Hirata Y & Marumo F. (1991a). Endothelin-1 as an autocrine/paracrine factor for human tumor cell lines. *J Cardiovasc Pharmacol* **17 Suppl 7,** S76-78.

Shichiri M, Hirata Y, Nakajima T, Ando K, Imai T, Yanagisawa M, Masaki T & Marumo F. (1991b). Endothelin-1 is an autocrine/paracrine growth factor for human cancer cell lines. *J Clin Invest* **87,** 1867-1871.

Shichiri M, Sedivy JM, Marumo F & Hirata Y. (1998). Endothelin-1 is a potent survival factor for c-Myc-dependent apoptosis. *Mol Endocrinol* **12,** 172-180.

Shichiri M, Yokokura M, Marumo F & Hirata Y. (2000). Endothelin-1 inhibits apoptosis of vascular smooth muscle cells induced by nitric oxide and serum deprivation via MAP kinase pathway. *Arterioscler Thromb Vasc Biol* **20,** 989-997.

Shin MK, Levorse JM, Ingram RS & Tilghman SM. (1999). The temporal requirement for endothelin receptor-B signalling during neural crest development. *Nature* **402,** 496-501.

Shinmi O, Kimura S, Sawamura T, Sugita Y, Yoshizawa T, Uchiyama Y, Yanagisawa M, Goto K, Masaki T & Kanazawa I. (1989). Endothelin-3 is a novel neuropeptide: isolation and sequence determination of endothelin-1 and endothelin-3 in porcine brain. *Biochem Biophys Res Commun* **164,** 587-593.

Simonson MS & Dunn MJ. (1990). Cellular signaling by peptides of the endothelin gene family. *Faseb J* **4,** 2989-3000.

Simonson MS, Wann S, Mene P, Dubyak GR, Kester M, Nakazato Y, Sedor JR & Dunn MJ. (1989). Endothelin stimulates phospholipase C, Na+/H+ exchange, c-fos expression, and mitogenesis in rat mesangial cells. *J Clin Invest* **83,** 708-712.

Sirvio ML, Metsarinne K, Saijonmaa O & Fyhrquist F. (1990). Tissue distribution and half-life of 125I-endothelin in the rat: importance of pulmonary clearance. *Biochem Biophys Res Commun* **167,** 1191-1195.

Sone M, Takahashi K, Totsune K, Murakami O, Arihara Z, Satoh F, Mouri T & Shibahara S. (2000). Expression of endothelin-1 and endothelin receptors in cultured human glioblastoma cells. *J Cardiovasc Pharmacol* **36,** S390-392.

Sone M, Totsune K, Takahashi K, Ohneda M, Itoi K, Murakami O, Miura Y, Mouri T & Yoshinaga K. (1991). Immunoreactive endothelin in pheochromocytomas. *J Cardiovasc Pharmacol* **17 Suppl 7,** S427-429.

Soufir N, Meziani R, Lacapere JJ, Bertrand G, Fumeron F, Bourillon A, Gerard B, Descamps V, Crickx B, Ollivaud L, Archimbaud A, Lebbe C, Basset-Seguin N, Saiag P & Grandchamp B. (2005). Association between endothelin receptor B nonsynonymous variants and melanoma risk. *J Natl Cancer Inst* **97,** 1297-1301.

Spinella F, Rosano L, Di Castro V, Decandia S, Nicotra MR, Natali PG & Bagnato A. (2007). Endothelin-1 and endothelin-3 promote invasive behavior via hypoxia-inducible factor-1alpha in human melanoma cells. *Cancer Res* **67,** 1725-1734.

Spinella F, Rosano L, Di Castro V, Natali PG & Bagnato A. (2002). Endothelin-1 induces vascular endothelial growth factor by increasing hypoxia-inducible factor-1alpha in ovarian carcinoma cells. *J Biol Chem* **277,** 27850-27855.

Stiles JD, Ostrow PT, Balos LL, Greenberg SJ, Plunkett R, Grand W & Heffner RR, Jr. (1997). Correlation of endothelin-1 and transforming growth factor beta 1 with malignancy and vascularity in human gliomas. *J Neuropathol Exp Neurol* **56,** 435-439.

Tada A, Suzuki I, Im S, Davis MB, Cornelius J, Babcock G, Nordlund JJ & Abdel-Malek ZA. (1998). Endothelin-1 is a paracrine growth factor that modulates melanogenesis of human melanocytes and participates in their responses to ultraviolet radiation. *Cell Growth Differ* **9,** 575-584.

Takahashi K, Totsune K, Kitamuro T, Sone M, Murakami O & Shibahara S. (2002). Three vasoactive peptides, endothelin-1, adrenomedullin and urotensin-II, in human tumour cell lines of different origin: expression and effects on proliferation. *Clin Sci (Lond)* **103 Suppl 48,** 35S-38S.

Takuwa N, Takuwa Y, Yanagisawa M, Yamashita K & Masaki T. (1989). A novel vasoactive peptide endothelin stimulates mitogenesis through inositol lipid turnover in Swiss 3T3 fibroblasts. *J Biol Chem* **264,** 7856-7861.

Takuwa Y, Masaki T & Yamashita K. (1990). The effects of the endothelin family peptides on cultured osteoblastic cells from rat calvariae. *Biochem Biophys Res Commun* **170,** 998-1005.

Thirumaran RK, Thoelke A, Ugurel S, Hemminki K, Schadendorf D & Kumar R. (2006). Re: Association between endothelin receptor B nonsynonymous variants and melanoma risk. *J Natl Cancer Inst* **98,** 1252-1253; author reply 1253.

Vacca F, Bagnato A, Catt KJ & Tecce R. (2000). Transactivation of the epidermal growth factor receptor in endothelin-1-induced mitogenic signaling in human ovarian carcinoma cells. *Cancer Res* **60,** 5310-5317.

Vajkoczy P, Meyer B, Weidauer S, Raabe A, Thome C, Ringel F, Breu V & Schmiedek P. (2005). Clazosentan (AXV-034343), a selective endothelin A receptor antagonist, in the prevention of cerebral vasospasm following severe aneurysmal subarachnoid hemorrhage: results of a randomized, double-blind, placebo-controlled, multicenter phase IIa study. *J Neurosurg* **103,** 9-17.

Venuti A, Salani D, Cirilli A, Simeone P, Muller A, Flamini S, Padley R & Bagnato A. (2002). Endothelin receptor blockade inhibits the growth of human papillomavirus-associated cervical carcinoma. *Clin Sci (Lond)* **103 Suppl 48,** 310S-313S.

Venuti A, Salani D, Manni V, Poggiali F & Bagnato A. (2000). Expression of endothelin 1 and endothelin A receptor in HPV-associated cervical carcinoma: new potential targets for anticancer therapy. *Faseb J* **14,** 2277-2283.

Verhaar MC, Grahn AY, Van Weerdt AW, Honing ML, Morrison PJ, Yang YP, Padley RJ & Rabelink TJ. (2000). Pharmacokinetics and pharmacodynamic effects of ABT-627, an oral ETA selective endothelin antagonist, in humans. *Br J Clin Pharmacol* **49,** 562-573.

Vierhapper H, Wagner O, Nowotny P & Waldhausl W. (1990). Effect of endothelin-1 in man. *Circulation* **81,** 1415-1418.

von Geldern TW, Tasker AS, Sorensen BK, Winn M, Szczepankiewicz BG, Dixon DB, Chiou WJ, Wang L, Wessale JL, Adler A, Marsh KC, Nguyen B & Opgenorth TJ. (1999). Pyrrolidine-3-carboxylic acids as endothelin antagonists. 4. Side chain conformational restriction leads to ET(B) selectivity. *J Med Chem* **42,** 3668-3678.

Wagner OF, Christ G, Wojta J, Vierhapper H, Parzer S, Nowotny PJ, Schneider B, Waldhausl W & Binder BR. (1992). Polar secretion of endothelin-1 by cultured endothelial cells. *J Biol Chem* **267,** 16066-16068.

Williams DL, Jr., Jones KL, Colton CD & Nutt RF. (1991). Identification of high affinity endothelin-1 receptor subtypes in human tissues. *Biochem Biophys Res Commun* **180,** 475-480.

Winn M, von Geldern TW, Opgenorth TJ, Jae HS, Tasker AS, Boyd SA, Kester JA, Mantei RA, Bal R, Sorensen BK, Wu-Wong JR, Chiou WJ, Dixon DB, Novosad EI, Hernandez L & Marsh KC. (1996). 2,4-Diarylpyrrolidine-3-carboxylic acids--potent ETA selective endothelin receptor antagonists. 1. Discovery of A-127722. *J Med Chem* **39,** 1039-1048.

Wu C, Chan MF, Stavros F, Raju B, Okun I, Mong S, Keller KM, Brock T, Kogan TP & Dixon RA. (1997). Discovery of TBC11251, a potent, long acting, orally active endothelin receptor-A selective antagonist. *J Med Chem* **40,** 1690-1697.

Wu C, Decker ER, Blok N, Bui H, You TJ, Wang J, Bourgoyne AR, Knowles V, Berens KL, Holland GW, Brock TA & Dixon RA. (2004). Discovery, modeling, and human pharmacokinetics of N-(2-acetyl-4,6-dimethylphenyl)-3-(3,4-dimethylisoxazol-5-ylsulfamoyl)thio phene-2-carboxamide (TBC3711), a second generation, ETA selective, and orally bioavailable endothelin antagonist. *J Med Chem* **47,** 1969-1986.

Xu D, Emoto N, Giaid A, Slaughter C, Kaw S, deWit D & Yanagisawa M. (1994). ECE-1: a membrane-bound metalloprotease that catalyzes the proteolytic activation of big endothelin-1. *Cell* **78,** 473-485.

Yamashita J, Ogawa M, Egami H, Matsuo S, Kiyohara H, Inada K, Yamashita S & Fujita S. (1992). Abundant expression of immunoreactive endothelin 1 in mammary phyllodes tumor: possible paracrine role of endothelin 1 in the growth of stromal cells in phyllodes tumor. *Cancer Res* **52,** 4046-4049.

Yamashita J, Ogawa M, Inada K, Yamashita S, Matsuo S & Takano S. (1991). A large amount of endothelin-1 is present in human breast cancer tissues. *Res Commun Chem Pathol Pharmacol* **74,** 363-369.

Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K & Masaki T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* **332,** 411-415.

Yanagisawa M & Masaki T. (1989). Endothelin, a novel endothelium-derived peptide. Pharmacological activities, regulation and possible roles in cardiovascular control. *Biochem Pharmacol* **38,** 1877-1883.

Yohn JJ, Smith C, Stevens T, Hoffman TA, Morelli JG, Hurt DL, Yanagisawa M, Kane MA & Zamora MR. (1994). Human melanoma cells express functional endothelin-1 receptors. *Biochem Biophys Res Commun* **201,** 449-457.

Yokokawa K, Tahara H, Kohno M, Mandal AK, Yanagisawa M & Takeda T. (1993). Heparin regulates endothelin production through endothelium-derived nitric oxide in human endothelial cells. *J Clin Invest* **92,** 2080-2085.

Yokokawa K, Tahara H, Kohno M, Murakawa K, Yasunari K, Nakagawa K, Hamada T, Otani S, Yanagisawa M & Takeda T. (1991a). Endothelin-secreting tumor. *J Cardiovasc Pharmacol* **17 Suppl 7,** S398-401.

Yokokawa K, Tahara H, Kohno M, Murakawa K, Yasunari K, Nakagawa K, Hamada T, Otani S, Yanagisawa M & Takeda T. (1991b). Hypertension associated with

endothelin-secreting malignant hemangioendothelioma. *Ann Intern Med* **114,** 213-215.

Yorimitsu K, Shinmi O, Nishiyama M, Moroi K, Sugita Y, Saito T, Inagaki Y, Masaki T & Kimura S. (1992). Effect of phosphoramidon on big endothelin-2 conversion into endothelin-2 in human renal adenocarcinoma (ACHN) cells. Analysis of endothelin-2 biosynthetic pathway. *FEBS Lett* **314,** 395-398.

Yuyama H, Sanagi M, Koakutsu A, Mori M, Fujimori A, Harada H, Sudoh K & Miyata K. (2003). Pharmacological characterization of YM598, an orally active and highly potent selective endothelin ET(A) receptor antagonist. *Eur J Pharmacol* **478,** 61-71.

Zhang M & Olsson Y. (1995). Reactions of astrocytes and microglial cells around hematogenous metastases of the human brain. Expression of endothelin-like immunoreactivity in reactive astrocytes and activation of microglial cells. *J Neurol Sci* **134,** 26-32.

Zhang M & Olsson Y. (1997). Hematogenous metastases of the human brain--characteristics of peritumoral brain changes: a review. *J Neurooncol* **35,** 81-89.

Chapter V

Endothelin Receptor B Antagonists Decrease Glioma Viability Independently of Their Cognate Receptor

Abstract

Endothelin receptor antagonists inhibit the progression of many cancers, but research into their influence on glioma has been limited. We report that two endothelin receptor type B (ETRB)-specific antagonists, A-192621 and BQ788, reduce the number of viable cells in two glioma cell lines in a dose- and time-dependent manner. We describe similar results for two melanoma cell lines. The more potent of the two antagonists, A-192621, decreases the mean number of cell divisions at least in part by inducing a G2/M arrest and apoptosis. Microarray analysis of the effects of A-192621 treatment reveals up-regulation of several DNA damage-inducible genes. These results were confirmed by real-time RT-PCR. Importantly, reducing expression of ETRB with small interfering RNAs does not abrogate the effects of either A-192621 or BQ788 in glioma or melanoma cells. Furthermore, BQ123, an endothelin receptor type A-specific antagonist, has no effect on cell viability in any of these cell lines, suggesting that the ETRB-independent effects on cell viability exhibited by A-192621 and BQ788 are not a result of ETRA inhibition. Thus, while ETRB antagonists reduce the viability of glioma cells, it appears unlikely that their therapeutic effects are mediated via ETRB inhibition or cross-reaction with ETRA.

Introduction

The endothelin (ET) family includes three 21-amino acid peptides, ET-1, ET-2 and ET-3, which bind to two G-protein-coupled receptors, endothelin receptor type A (ETRA) and endothelin receptor type B (ETRB). The ETRA binds ET-1 and ET-2 with

equal preference over ET-3, while ETRB binds all three isoforms with equal affinity (1). The ET axis is believed to play a role in various malignancies including ovarian, prostate, cervical and breast carcinomas, melanoma and central nervous system tumors (2). The influence of the ET family on cancer is multifactorial: ET-1 induces proliferation (3-7), suppresses apoptosis (8), enhances angiogenesis (9, 10) and promotes invasion (11-13).

Components of the ET system have been found in many glioma tumor specimens and cell lines, and ET expression positively correlates with the degree of malignancy (14-17). Two studies demonstrated ETRA on the neovasculature of glioblastoma tumors, while ETRB was localized to the tumor cells (18, 19). Inhibitors of endothelin converting enzyme 1, which converts ET-1 into the active form, block DNA synthesis in glioblastoma cells (20). ET-1 induces proliferation in glioblastomas through various pathways including the mitogen-activated protein kinase (MAPK) pathway, and BQ788, an ETRB-specific receptor antagonist, blocks the phosphorylation of extracellular signal-related kinase, a key step in MAPK signaling (21). This led us to consider whether potential therapeutic candidates, the ETRB antagonists, negatively impact glioma growth.

Our laboratory previously showed that high doses of BQ788 inhibit melanoma proliferation both *in vitro* and *in vivo* (22). We are currently investigating the effects of ETRB antagonists on melanoma and glioma, with particular interest in two ETRB-specific antagonists, BQ788, a peptide, and A-192621, an orally bio-available small molecule. In the present work we demonstrate that both ETRB antagonists decrease the number of viable cells in melanoma and glioma cultures, while an ETRA-specific antagonist, BQ123, has no effect. In glioma cells, A-192621 induces cell cycle arrest, apoptosis and expression of DNA-damage associated genes. Importantly, the presence of

ETRB appears to not be required for the reduction in cell number by either ETRB antagonist.

Materials and Methods

Cells and cell culture conditions. The human glioma cell lines LN-229 and SW1088 and the human melanoma cell line A375 (American Type Culture Collection (ATCC), Manassas, VA, USA) were maintained in Dulbecco's Modification of Eagle's Medium (DMEM) (Mediatech, Inc., Herndon, VA, USA) and the human melanoma cell line WM35 (ATCC) was maintained in Eagle's Minimum Essential Medium (MEM) (Mediatech, Inc.). All cells were supplemented with 10% fetal bovine serum (FBS) (Gemini Biological Products, Calabasas, CA, USA), 100 units/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) and cultured in a humidified incubator with 5% CO_2 at 37 °C. For cell viability assays, 2.5×10^4 cells were plated onto 12-well tissue-culture treated plates (Fisher Scientific, Pittsburgh, PA, USA) using media supplemented with 1% FBS. A-192621 (Abbott Laboratories, Abbott Park, IL, USA), BQ788 (EMD Chemicals Inc., San Diego, CA, USA) and/or BQ123 (EMD) were added 24 h after plating and viable cell number was assessed using the Live/Dead Viability/Cytotoxicity Kit for mammalian cells (Invitrogen) according to the manufacturer's instructions. Fluorescent intensity was measured on an FLx800 multidetection microplate reader (BioTek, Winooski, VT, USA) and values represent the mean of a 25-point well scan.

Cell proliferation and cell death. LN-229 and SW1088 cells were plated at 5×10^5 cells per 100 mm dish in DMEM with 1% FBS, and A-192621 was added 24 h later.

Cell cycle analysis was performed with a BrdU/propidium iodide double stain using the Absolute-S Cell Proliferation Kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions allowing 40 minutes to pulse-label cells with BrdU. Fluorescence was measured using the BD FACSCalibur System (Becton Dickinson, Franklin Lakes, NJ, USA). The rate of cell proliferation was assessed using the CellTrace CFSE Cell Proliferation Kit (Invitrogen) according to the manufacturer's instructions. Cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) at the time of plating and fluorescence intensity was analyzed at 9, 24, 48 and 72 h after the addition of A-192621 using the BD FACSCalibur System. Cell death was quantified by staining cells with propidium iodide (Invitrogen) following treatment with A-192621. Fluorescence intensity was analyzed using the BD FACSCalibur System. All FACS data was analyzed with FlowJo (Tree Star, Inc., Ashland, OR, USA). Caspase 3/7 activity was measured using EnzChek Caspase-3 Assay Kit #2 (Invitrogen) according to the manufacturer's instructions and values adjusted for total cell number. Fluorescence intensity was measured using the FLx800 multi-detection microplate reader.

Microarray analysis. LN-229 and SW1088 cells were treated with A-192621 for 12 h and total RNA was prepared using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). The quality of the samples was checked using the RNA 6000 Nano LabChip kit (Agilent Technologies, Santa Clara, CA, USA). RNA samples were then processed according to the Affymetrix Eukaryotic Sample and Array Processing protocol. Hybridization of the in vitro amplified RNA to Affymetrix Human Genome U133Plus 2.0 chips (Affymetrix, Inc., Santa Clara, CA, USA), washing and scanning of the arrays were performed following standard Affymetrix protocols using a Hybridization Oven

640, a Fluidics Station 450, and a GeneChip® Scanner 3000 7G. The raw data (*.cel files) from the Affymetrix hybridizations were processed and analyzed using Resolver (Rosetta Biosoftware, Seattle, WA, USA).

Real-time reverse-transcription PCR. Total RNA was prepared using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). The cDNA was prepared from 1 μg total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturers instructions. Primer/probe design was accomplished using the Universal ProbeLibrary (UPL) Assay Design Center (Roche Applied Science). The primer sets (Integrated DNA Technologies, Coralville, IA, USA) were as follows: 5'-GGC AGA AGC TGA AAG GTC TC-3' and 5'-CAT CGA AGC ACT GTC TCA GAG T-3' (DR5), 5'-GGA GAG CAG AAG ACC GAA AG-3' and 5'-AGT GAT CGT GCG CTG ACT C-3' (GADD45A), 5'-GCT TCT GGC AGA CCG AAC-3' and 5'-GTA GCC TGA TGG GGT GCT T-3' (GADD34), 5 '-ACT GCG TCT TTG GCA TCA G-3' and 5'-GTA GCA GGC CAC TGT CTT GA-3' (Sestrin 2), 5'-AAG GCA CTG AGC GTA TCA TGT-3' and 5'-TGA AGA TAC ACT TCC TTC TTG AAC AC-3' (GADD153), 5'-TTC ATC CCG TTC AGA AGA CA-3' and 5'-CCA ATG GCA AGC AGA AAT AGA-3' (ETRB) and 5'-TGA CCT TGA TTT ATT TTG CAT ACC-3' and 5'-CGA GCA AGA CGT TCA GTC CT-3' (HPRT). The corresponding probes were UPL probe #63 (DR5), #65 (GADD45A), #28 (GADD34), #17 (Sestrin 2), #21 (GADD153), #83 (ETRB) and #73 (HPRT) (Roche Applied Science). PCR was performed and analyzed on a LightCycler 480 System (Roche Applied Science) using LightCycler 480 Probes Master (Roche Applied Science). The PCR was done under the following conditions: pre-incubation at 95 °C for 5 minutes, 45

cycles of amplification with melting at 95 °C for 8 seconds, annealing at 60 °C for 15 seconds and extension at 72 °C for 2 seconds, and 1 cycle of cooling at 40 °C for 10 seconds. All gene expression was quantified relative to HPRT expression.

Small interfering RNA (siRNA). Following the reverse transfection protocol, Lipofectamine RNAiMAX (Invitrogen) was diluted in Opti-MEM I Medium (Invitrogen) and ON-TARGETplus SMARTpool ETRB siRNA or ON-TARGETplus siCONTROL non-targeting siRNA (Dharmacon, Lafayette, CO, USA) was diluted in Opti-MEM I Medium without serum. These solutions were then combined and incubated together at room temperature for 10-20 minutes. The siRNA duplex–Lipofectamine RNAiMAX complexes were then plated and overlaid with 2.5×10^4 cells per ml in media with 1% FBS. Media was changed 4-6 h after plating and cells were treated as described above. ETRB gene expression was assessed by real-time PCR and was reduced by 70 - 93%.

Statistical Analysis. Values are expressed as mean ± SEM. Statistical analysis was done using one-way ANOVA with Tukey posthoc unless otherwise noted. $P < 0.05$ was considered statistically significant.

Results

Endothelin receptor B antagonist A-192621 reduces the number of viable glioma and melanoma cells in a dose- and time-dependent manner. To test the effectiveness of A-192621 in reducing viable glioma cells we employed two human glioma cell lines, LN-229 and SW1088. LN-229 was originally derived from a grade IV glioblastoma and SW1088 was derived from an anaplastic astrocytoma. A-192621 was added at concentrations of 1, 25, 50, 75 or 100 μM to nonconfluent cells 24 h after plating and

incubated for 24, 48 or 72 h. Viable cells were identified by the capacity of their intracellular esterases to convert non-fluorescent calcein AM into green-fluorescent calcein. This assay reveals a decrease in viable cell number with increasing A-192621 concentration (Fig. 1A). This decrease in viable cell number is enhanced with longer incubation times (Fig. 1A). In addition, A-192621 is effective in reducing viable cell number in the melanoma cell lines A375 and WM35 in a dose- and time-dependent manner (Fig. 1A). This finding with melanoma cells is consistent with previously published data reporting that A-192621 inhibits melanoma growth in nude mice (13). We also tested the ability of endothelin receptor B-specific antagonist BQ788 to reduce the number of viable cells in glioma cell lines LN-229 and SW1088. This agent causes a significant decrease in the number of viable LN-229 cells after 48 or 72 h of treatment but no significant change in SW1088 cell number (Fig. 1B). BQ788 also reduces cell viability in both melanoma cell lines in a dose- and time-dependent manner (Fig. 1B). This result is consistent with previous findings in our laboratory (22). To assess the involvement of ETRA in viability, cells were treated with BQ123, an endothelin receptor A-specific antagonist. BQ123 was added to cells at 1, 25 50, 75 or 100 μM and incubated for 24, 48 or 72 h. No significant changes in cell viability were observed in any of the four cell lines, at any concentration or time point (Suppl. Fig. 1). Thus, cross-reaction of A-192621 and BQ788 with ETRA does not play a role in their effects on cell viability.

A-192621 decreases glioma cell proliferation and increases cell death. To investigate how A-192621 reduces glioma cell numbers we assessed the rate of cell proliferation over time. The human glioma cell lines LN-229 and SW1088 were labeled

with carboxyfluorescein diacetate succinimidyl ester (CFSE). This non-fluorescent reagent passively diffuses into cells where the acetate groups are cleaved by intracellular esterases, producing green-fluorescent carboxyfluorescein succinimidyl ester. The succinimidyl ester groups react with intracellular amines and fluorescence intensity is exponentially diluted as cells divide. Fluorescence intensity was measured by FACS at 9, 24, 48 and 72 h. We tested two doses of A-192621, 10 nM, a concentration slightly above the IC_{50} that is calculated from radio-labeled ET-1 binding displacement studies (23), and 100 μM, a concentration that dramatically reduces the number of viable cells in both LN-229 and SW1088 (Fig. 1A). We find that 100 μM, but not 10 nM, significantly reduces CFSE fluorescence dilution in both LN-229 and SW1088 cells at 24 h, and this effect is sustained through later time points, indicating that cells treated with A-192621 are not undergoing as many cell divisions (Fig. 2A). We also examined the cell cycle status of both LN-229 and SW1088 following A-192621 treatment using a BrdU/propidium iodide (PI) double stain. Following treatment with A-192621, cultures were labeled with BrdU for 40 min to identify cells undergoing DNA synthesis and fixed immediately afterwards. Cells were analyzed by FACS and BrdU intensity was plotted against DNA content. In both LN-229 and SW1088 cells, 100 μM A-192621 significantly increases the percentage of cells in the G2/M phase compared to vehicle, or to 10 nM treatment after 24 h (data not shown). This accumulation of cells in the G2/M phase continues through 72 h and is coupled with a concomitant decrease in the G1/G0 population (Fig. 2B), indicating that A-192621 induces a G2/M phase arrest. In addition to effects on proliferation, we investigated whether A-192621 treatment also affects cell death, as measured by the percentage of total cells that stain positively with PI. A-

192621 significantly increases cell death at 48 in both LN-229 and SW1088 cells, and at 72 h in SW1088 cells (Fig. 2C). As a measure of apoptotic cell death, caspase 3/7 activity is increased at 72 and 48 h in LN-229 and SW1088 cells, respectively (Fig 2D).

Genes associated with DNA damage are up-regulated following A-192621 treatment. In order to further understand the effects of A-192621, we assessed changes in gene expression by microarray analysis following a 12 h treatment of A-192621. A striking finding is that A-192621 up-regulates several genes that are known to be induced by DNA damage (Table 1). Growth arrest- and DNA damage-inducible (GADD) 45A is induced by environmental stressors such as methylmethane sulfonate, UV or gamma irradiation (24). The subsequent expression of GADD45A, and other GADD45-like genes, activates the p38/JNK pathway and apoptosis. GADD45A has also been implicated in inducing G2/M cell cycle arrest through its interaction with Cdc2 and cyclin B1 following genotoxic stress (25-28). Two other members of the GADD family, GADD34 and GADD153, are also up-regulated by A-192621 treatment. Like other members of this family, GADD34 and GADD153 are induced by stressful growth conditions and DNA damage. Over-expression of GADD34 and GADD153, along with GADD45 and others, suppresses cell growth as measured by colony formation and induces apoptosis (29-32). Sestrin 2 is one of three closely related genes in the sestrin family (33) and is closely linked to other GADD genes since sestrin 1, also known as PA26 (34), is a member of the GADD gene family. Sestrin 2, also known as Hi95, is induced by hypoxia, oxidative stress and DNA damage (35). Over-expression of sestrin 2 leads to apoptosis approximately 24 h following induction, and the cells are hypersensitive to further insult. DR5 is one of the TRAIL receptors with a cytoplasmic

death domain that induces caspase-dependent apoptosis (36-38). DR5 is induced by DNA damaging compounds in malignant gliomas, including LN-229 (39). Taken together, this evidence suggests that A-192621 affects glioma viability by activating stress/DNA damage response pathways, which leads to cell cycle arrest and apoptosis.

A-192621 and BQ788 effects on cell viability are not mediated by endothelin receptor B. The concentrations A-192621 and BQ788 required to reduce cell viability are well above their respective IC_{50}s to displace ET-1 (23, 40). Moreover, when we examined expression levels by real-time PCR, we could not detect ETRB in the SW1088 cell line after 45 amplification cycles (Fig 3A). These data led us to question the involvement of ETRB in the reduction of viable cell numbers by ETRB antagonists. To address this question we reduced the expression level of ETRB 69-93%, using small interfering RNA (siRNA) (Fig 3B). All cell lines were transfected with siRNAs targeting ETRB or a scrambled siRNA, and then treated 24 h later with 100 μM A-192621, BQ788 or their respective vehicles. The number of viable cells was assessed at 24, 48 or 72 h. Reduced ETRB expression in vehicle-treated cells did not affect cell viability. Both A-192621 and BQ788 decreased the number of viable cells equivalently in cells transfected with ETRB-targeting siRNA and scrambled siRNA at all time points (Fig. 3C, 24 and 48 hours not shown). That ETRB knockdown does not abrogate A-192621- or BQ788-mediated reduction in cell viability indicates that antagonism of this receptor is not important for the therapeutic effects of these two drugs.

Discussion

We show here that both melanoma and glioma cell viability are sensitive to ETRB antagonists. Both BQ788 and A-192621 decrease melanoma and glioma cell number in a dose- and time-dependent manner. We find that A-192621 is more potent than BQ788, causing a greater decrease in viable cell numbers at lower concentrations and at earlier time points. In fact, within the time frame tested, only A-192621 was able to reduce the viable cell number in the astrocytoma line SW1088.

A-192621 is more attractive as a therapeutic agent since it is orally bioavailable, and considering that it is also more potent than BQ788, we investigated how it reduces glioma cell number in greater detail. CFSE labeling indicates that A-192621 inhibits mitosis. Using cell cycle analysis, treatment of LN-229 and SW1088 with A-192621 increases in the percentage of cells with G2/M DNA content over time. This is coupled with a concomitant decrease in the percentage of cells with G0/G1 DNA content, indicating an arrest in the G2/M phase of the cell cycle, which likely accounts for the reduction in cell divisions seen with CFSE labeling. We also find that A-192621 treatment induces apoptotic cell death. Reduction in viable cell number is therefore a consequence of both decreased mitogenesis and increased apoptosis. To our knowledge, this is the first evidence of ETRB antagonist-induced G2/M cell cycle arrest.

To further elucidate the actions of A-192621, we analyzed changes in gene expression using microarray technology. Notably, after 12 h of A-192621 treatment, there are significant increases in the expression of several genes known to be up-regulated following DNA damage. These genes include GADD153, GADD45A, GADD34, Sestrin 2 and DR5. Up-regulation of these genes may account for the G2/M

arrest and the apoptosis we see at later time points. This is the first evidence linking ETRB antagonist treatment to enhanced expression of DNA damage-associated genes.

We also present evidence that the reduction in both melanoma and glioma cell viability by A-192621 and BQ788 does not require the expression of ETRB. This conclusion is supported by three types of data. (1) The concentration of ETRB antagonists required to reduce cell number is approximately 20,000-fold above the reported IC_{50}s for A-192621 and BQ788 to displace ET-1. (2) At these high doses, the antagonists reduce cell viability in the glioma cell line SW1088 despite the absence of detectable ETRB expression. (3) Reduction of ETRB expression by siRNA has no effect on the ability of either antagonist to reduce cell number.

We have demonstrated that ETRB antagonists are effective agents against melanoma and glioma cell growth *in vitro*. To date, mechanisms of ETRB antagonist action in cancer treatment have focused on blocking ET-1 induced pathways. Although determining the precise mechanism by which ETRB antagonists reduce cell number in these cancers is beyond the scope of this study, the data presented here indicate that ETRB antagonists function independently from direct ETRB antagonism to mediate their effects on cancer progression.

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References

1. Levin, E. R. Endothelins. N Engl J Med, *333:* 356-363, 1995.
2. Nelson, J., Bagnato, A., Battistini, B., and Nisen, P. The endothelin axis: emerging role in cancer. Nat Rev Cancer, *3:* 110-116, 2003.
3. Bagnato, A., Salani, D., Di Castro, V., Wu-Wong, J. R., Tecce, R., Nicotra, M. R., Venuti, A., and Natali, P. G. Expression of endothelin 1 and endothelin A receptor in ovarian carcinoma: evidence for an autocrine role in tumor growth. Cancer Res, *59:* 720-727, 1999.
4. Kitagawa, N., Tsutsumi, K., Niwa, M., Himeno, A., Yamashita, K., Shibata, S., Taniyama, K., Kurihara, M., Kawano, T., Yasunaga, A., and et al. Expression of a functional endothelin (ETA) receptor in human meningiomas. J Neurosurg, *80:* 723-731, 1994.
5. Pagotto, U., Arzberger, T., Hopfner, U., Sauer, J., Renner, U., Newton, C. J., Lange, M., Uhl, E., Weindl, A., and Stalla, G. K. Expression and localization of endothelin-1 and endothelin receptors in human meningiomas. Evidence for a role in tumoral growth. J Clin Invest, *96:* 2017-2025, 1995.
6. Nelson, J. B., Chan-Tack, K., Hedican, S. P., Magnuson, S. R., Opgenorth, T. J., Bova, G. S., and Simons, J. W. Endothelin-1 production and decreased endothelin B receptor expression in advanced prostate cancer. Cancer Res, *56:* 663-668, 1996.
7. Kikuchi, K., Nakagawa, H., Kadono, T., Etoh, T., Byers, H. R., Mihm, M. C., and Tamaki, K. Decreased ET(B) receptor expression in human metastatic melanoma cells. Biochem Biophys Res Commun, *219:* 734-739, 1996.

8. Eberle, J., Fecker, L. F., Orfanos, C. E., and Geilen, C. C. Endothelin-1 decreases basic apoptotic rates in human melanoma cell lines. J Invest Dermatol, *119:* 549-555, 2002.

9. Salani, D., Di Castro, V., Nicotra, M. R., Rosano, L., Tecce, R., Venuti, A., Natali, P. G., and Bagnato, A. Role of endothelin-1 in neovascularization of ovarian carcinoma. Am J Pathol, *157:* 1537-1547, 2000.

10. Spinella, F., Rosano, L., Di Castro, V., Natali, P. G., and Bagnato, A. Endothelin-1 induces vascular endothelial growth factor by increasing hypoxia-inducible factor-1alpha in ovarian carcinoma cells. J Biol Chem, *277:* 27850-27855, 2002.

11. Rosano, L., Salani, D., Di Castro, V., Spinella, F., Natali, P. G., and Bagnato, A. Endothelin-1 promotes proteolytic activity of ovarian carcinoma. Clin Sci (Lond), *103 Suppl 48:* 306S-309S, 2002.

12. Rosano, L., Varmi, M., Salani, D., Di Castro, V., Spinella, F., Natali, P. G., and Bagnato, A. Endothelin-1 induces tumor proteinase activation and invasiveness of ovarian carcinoma cells. Cancer Res, *61:* 8340-8346, 2001.

13. Bagnato, A., Rosano, L., Spinella, F., Di Castro, V., Tecce, R., and Natali, P. G. Endothelin B receptor blockade inhibits dynamics of cell interactions and communications in melanoma cell progression. Cancer Res, *64:* 1436-1443, 2004.

14. Naidoo, V., Naidoo, S., Mahabeer, R., and Raidoo, D. M. Localization of the endothelin system in human diffuse astrocytomas. Cancer, *104:* 1049-1057, 2005.

15. Takahashi, K., Totsune, K., Kitamuro, T., Sone, M., Murakami, O., and Shibahara, S. Three vasoactive peptides, endothelin-1, adrenomedullin and

urotensin-II, in human tumour cell lines of different origin: expression and effects on proliferation. Clin Sci (Lond), *103 Suppl 48:* 35S-38S, 2002.

16. Sone, M., Takahashi, K., Totsune, K., Murakami, O., Arihara, Z., Satoh, F., Mouri, T., and Shibahara, S. Expression of endothelin-1 and endothelin receptors in cultured human glioblastoma cells. J Cardiovasc Pharmacol, *36:* S390-392, 2000.

17. Stiles, J. D., Ostrow, P. T., Balos, L. L., Greenberg, S. J., Plunkett, R., Grand, W., and Heffner, R. R., Jr. Correlation of endothelin-1 and transforming growth factor beta 1 with malignancy and vascularity in human gliomas. J Neuropathol Exp Neurol, *56:* 435-439, 1997.

18. Harland, S. P., Kuc, R. E., Pickard, J. D., and Davenport, A. P. Expression of endothelin(A) receptors in human gliomas and meningiomas, with high affinity for the selective antagonist PD156707. Neurosurgery, *43:* 890-898; discussion 898-899, 1998.

19. Egidy, G., Eberl, L. P., Valdenaire, O., Irmler, M., Majdi, R., Diserens, A. C., Fontana, A., Janzer, R. C., Pinet, F., and Juillerat-Jeanneret, L. The endothelin system in human glioblastoma. Lab Invest, *80:* 1681-1689, 2000.

20. Berger, Y., Dehmlow, H., Blum-Kaelin, D., Kitas, E. A., Loffler, B. M., Aebi, J. D., and Juillerat-Jeanneret, L. Endothelin-converting enzyme-1 inhibition and growth of human glioblastoma cells. J Med Chem, *48:* 483-498, 2005.

21. He, S., Dibas, A., Yorio, T., and Prasanna, G. Parallel signaling pathways in endothelin-1-induced proliferation of U373MG astrocytoma cells. Exp Biol Med (Maywood), *232:* 370-384, 2007.

22. Lahav, R., Heffner, G., and Patterson, P. H. An endothelin receptor B antagonist inhibits growth and induces cell death in human melanoma cells in vitro and in vivo. Proc Natl Acad Sci U S A, *96:* 11496-11500, 1999.

23. Wu-Wong, J. R., Dixon, D. B., Chiou, W. J., Sorensen, B. K., Liu, G., Jae, H. S., Tasker, A., von Geldern, T. W., Winn, M., and Opgenorth, T. J. Pharmacology of endothelin receptor antagonists ABT-627, ABT-546, A-182086 and A-192621: in vitro studies. Clin Sci (Lond), *103 Suppl 48:* 107S-111S, 2002.

24. Takekawa, M. and Saito, H. A family of stress-inducible GADD45-like proteins mediate activation of the stress-responsive MTK1/MEKK4 MAPKKK. Cell, *95:* 521-530, 1998.

25. Jin, S., Tong, T., Fan, W., Fan, F., Antinore, M. J., Zhu, X., Mazzacurati, L., Li, X., Petrik, K. L., Rajasekaran, B., Wu, M., and Zhan, Q. GADD45-induced cell cycle G2-M arrest associates with altered subcellular distribution of cyclin B1 and is independent of p38 kinase activity. Oncogene, *21:* 8696-8704, 2002.

26. Wang, X. W., Zhan, Q., Coursen, J. D., Khan, M. A., Kontny, H. U., Yu, L., Hollander, M. C., O'Connor, P. M., Fornace, A. J., Jr., and Harris, C. C. GADD45 induction of a G2/M cell cycle checkpoint. Proc Natl Acad Sci U S A, *96:* 3706-3711, 1999.

27. Zhan, Q., Antinore, M. J., Wang, X. W., Carrier, F., Smith, M. L., Harris, C. C., and Fornace, A. J., Jr. Association with Cdc2 and inhibition of Cdc2/Cyclin B1 kinase activity by the p53-regulated protein Gadd45. Oncogene, *18:* 2892-2900, 1999.

28. Jin, S., Antinore, M. J., Lung, F. D., Dong, X., Zhao, H., Fan, F., Colchagie, A. B., Blanck, P., Roller, P. P., Fornace, A. J., Jr., and Zhan, Q. The GADD45 inhibition of Cdc2 kinase correlates with GADD45-mediated growth suppression. J Biol Chem, *275:* 16602-16608, 2000.

29. Hollander, M. C., Zhan, Q., Bae, I., and Fornace, A. J., Jr. Mammalian GADD34, an apoptosis- and DNA damage-inducible gene. J Biol Chem, *272:* 13731-13737, 1997.

30. Zhan, Q., Lord, K. A., Alamo, I., Jr., Hollander, M. C., Carrier, F., Ron, D., Kohn, K. W., Hoffman, B., Liebermann, D. A., and Fornace, A. J., Jr. The gadd and MyD genes define a novel set of mammalian genes encoding acidic proteins that synergistically suppress cell growth. Mol Cell Biol, *14:* 2361-2371, 1994.

31. Adler, H. T., Chinery, R., Wu, D. Y., Kussick, S. J., Payne, J. M., Fornace, A. J., Jr., and Tkachuk, D. C. Leukemic HRX fusion proteins inhibit GADD34-induced apoptosis and associate with the GADD34 and hSNF5/INI1 proteins. Mol Cell Biol, *19:* 7050-7060, 1999.

32. Matsumoto, M., Minami, M., Takeda, K., Sakao, Y., and Akira, S. Ectopic expression of CHOP (GADD153) induces apoptosis in M1 myeloblastic leukemia cells. FEBS Lett, *395:* 143-147, 1996.

33. Peeters, H., Debeer, P., Bairoch, A., Wilquet, V., Huysmans, C., Parthoens, E., Fryns, J. P., Gewillig, M., Nakamura, Y., Niikawa, N., Van de Ven, W., and Devriendt, K. PA26 is a candidate gene for heterotaxia in humans: identification of a novel PA26-related gene family in human and mouse. Hum Genet, *112:* 573-580, 2003.

34. Velasco-Miguel, S., Buckbinder, L., Jean, P., Gelbert, L., Talbott, R., Laidlaw, J., Seizinger, B., and Kley, N. PA26, a novel target of the p53 tumor suppressor and member of the GADD family of DNA damage and growth arrest inducible genes. Oncogene, *18:* 127-137, 1999.

35. Budanov, A. V., Shoshani, T., Faerman, A., Zelin, E., Kamer, I., Kalinski, H., Gorodin, S., Fishman, A., Chajut, A., Einat, P., Skaliter, R., Gudkov, A. V., Chumakov, P. M., and Feinstein, E. Identification of a novel stress-responsive gene Hi95 involved in regulation of cell viability. Oncogene, *21:* 6017-6031, 2002.

36. Sheridan, J. P., Marsters, S. A., Pitti, R. M., Gurney, A., Skubatch, M., Baldwin, D., Ramakrishnan, L., Gray, C. L., Baker, K., Wood, W. I., Goddard, A. D., Godowski, P., and Ashkenazi, A. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science, *277:* 818-821, 1997.

37. Pan, G., Ni, J., Wei, Y. F., Yu, G., Gentz, R., and Dixit, V. M. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. Science, *277:* 815-818, 1997.

38. Walczak, H., Degli-Esposti, M. A., Johnson, R. S., Smolak, P. J., Waugh, J. Y., Boiani, N., Timour, M. S., Gerhart, M. J., Schooley, K. A., Smith, C. A., Goodwin, R. G., and Rauch, C. T. TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. Embo J, *16:* 5386-5397, 1997.

39. Nagane, M., Pan, G., Weddle, J. J., Dixit, V. M., Cavenee, W. K., and Huang, H. J. Increased death receptor 5 expression by chemotherapeutic agents in human

gliomas causes synergistic cytotoxicity with tumor necrosis factor-related apoptosis-inducing ligand in vitro and in vivo. Cancer Res, *60:* 847-853, 2000.

40. Ishikawa, K., Ihara, M., Noguchi, K., Mase, T., Mino, N., Saeki, T., Fukuroda, T., Fukami, T., Ozaki, S., Nagase, T., and et al. Biochemical and pharmacological profile of a potent and selective endothelin B-receptor antagonist, BQ-788. Proc Natl Acad Sci U S A, *91:* 4892-4896, 1994.

Tables

Table 1. Treatment of glioma and melanoma cell lines with 100 μM A-192621 up-regulates DNA damage-related genes. Genes were identified by microarray analysis and real-time RT-PCR was used to quantify the fold-increase in expression over vehicle-treated controls.

Genes	Cell Lines			
	LN-229	SW1088	A375	WM35
GADD153	28	12	10	66
GADD45A	23	5	9	8
GADD34	16	3	21	6
Sestrin 2	15	18	19	12
DR5	12	4	10	5

Figure Legends

Figure 1. Endothelin receptor B antagonists reduce the number of viable cells in a dose- and time-dependent manner. (A) A-192621 significantly reduces the number of viable cells in both glioma (LN-229 and SW1088) and melanoma (A375 and WM35) cell lines at 24, 48 and 72 h of treatment. (B) BQ788 significantly reduces the number of viable cells in melanoma at 48 and 72 h of treatment. Values are expressed as the mean of three replicates ± SEM. # $P < 0.05$, ♠ $P < 0.01$, ♣ $P < 0.001$ compared with vehicle-treated controls. Colors correspond to the cell line.

Figure 2. The endothelin B receptor antagonist A-192621 decreases glioma cell proliferation and increases cell death. (A) A-192621 suppresses cell proliferation. LN-229 and SW1088 cells were stained with CFSE and analyzed by FACS at 9, 24, 48 and 72 h after addition of A-192621. The exponential dilution of CFSE was converted to the number of cell divisions. (B) A high concentration of A-192621 (100μM) induces G2/M cell cycle arrest in both LN-229 and SW1088 cells by 72 h. Cells were pulsed-labeled with BrdU, stained with propidium iodide and analyzed by FACS. (C) A-192621 induces cell death at 48 and 72 h. LN-229 and SW1088 cells were stained with propidium iodide following treatment with A-192621 and analyzed by FACS. (D) A-192621 induces apoptosis by 72 and 48 h in LN-229 and SW1088 cell lines respectively. Caspase 3/7 activity was adjusted for total cell number. Values are expressed as means of three replicates ± SEM. # $P < 0.05$, ♠ $P < 0.01$, ♣ $P < 0.001$ compared with vehicle-treated controls.

Figure 3. Reduction of ETRB expression levels does not alter the effect of ETRB antagonists on viable cell number. (A) ETRB mRNA in untreated cell lines. ETRB mRNA was not detected in the SW1088 line. (B) Following transfection, reduction in ETRB mRNA was assessed by real-time RT-PCR. Reduction in ETRB expression is displayed as a percentage relative to ETRB mRNA in cells transfected with scrambled siRNA. (C) Cells were transfected with either scrambled siRNA or ETRB siRNA and then treated with either 100 μM A-192621 or 100 μM BQ788 for 72 h. Values are expressed as a mean of three replicates ± SEM. Significance was determined by a paired t-test # $P < 0.05$, ♠ $P < 0.01$, ♣ $P < 0.001$.

Figure 1

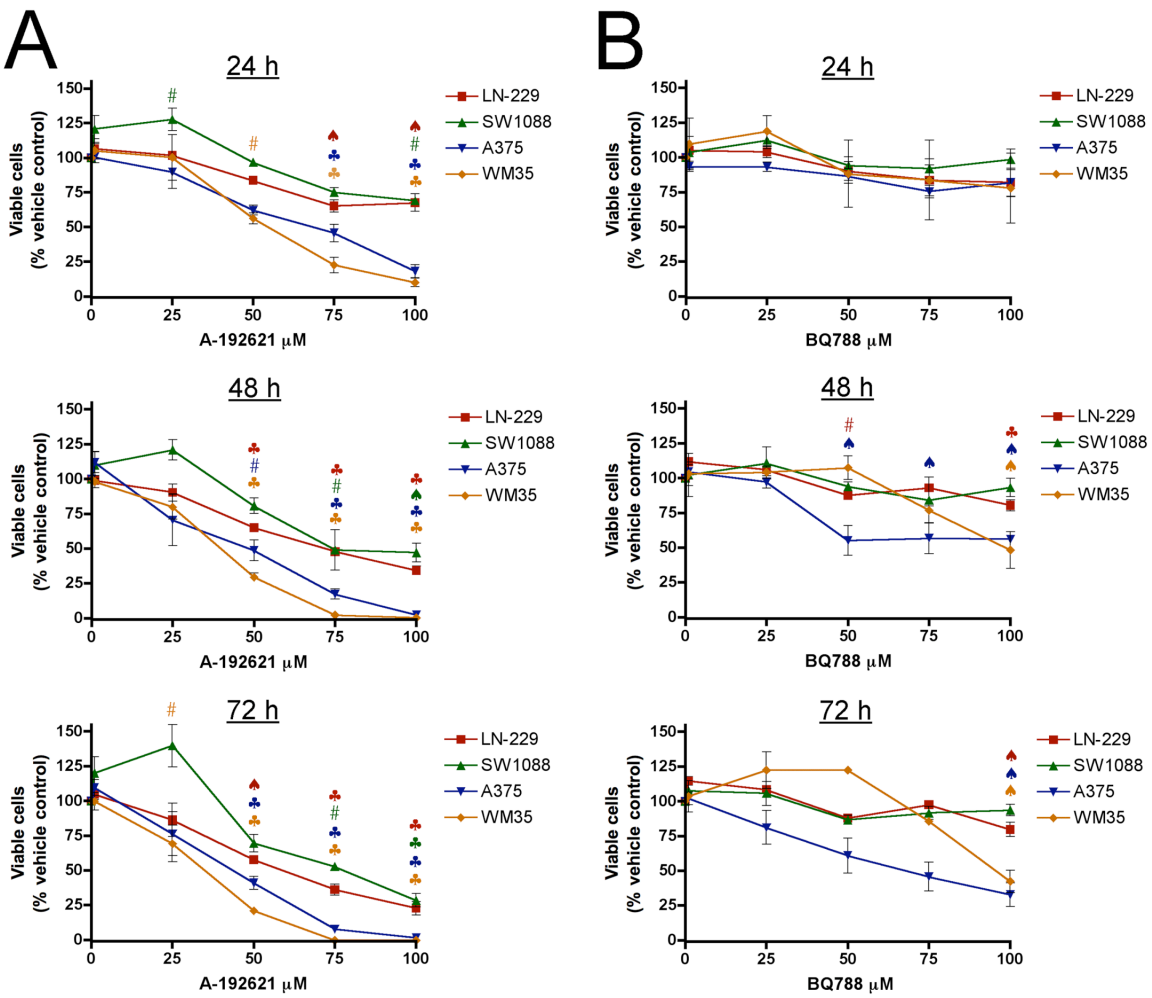

A
24 h
48 h
72 h
Viable cells (% vehicle control)
A-192621 μM
LN-229
SW1088
A375
WM35
B
24 h
48 h
72 h
Viable cells (% vehicle control)
BQ788 μM
LN-229
SW1088
A375
WM35

Figure 2

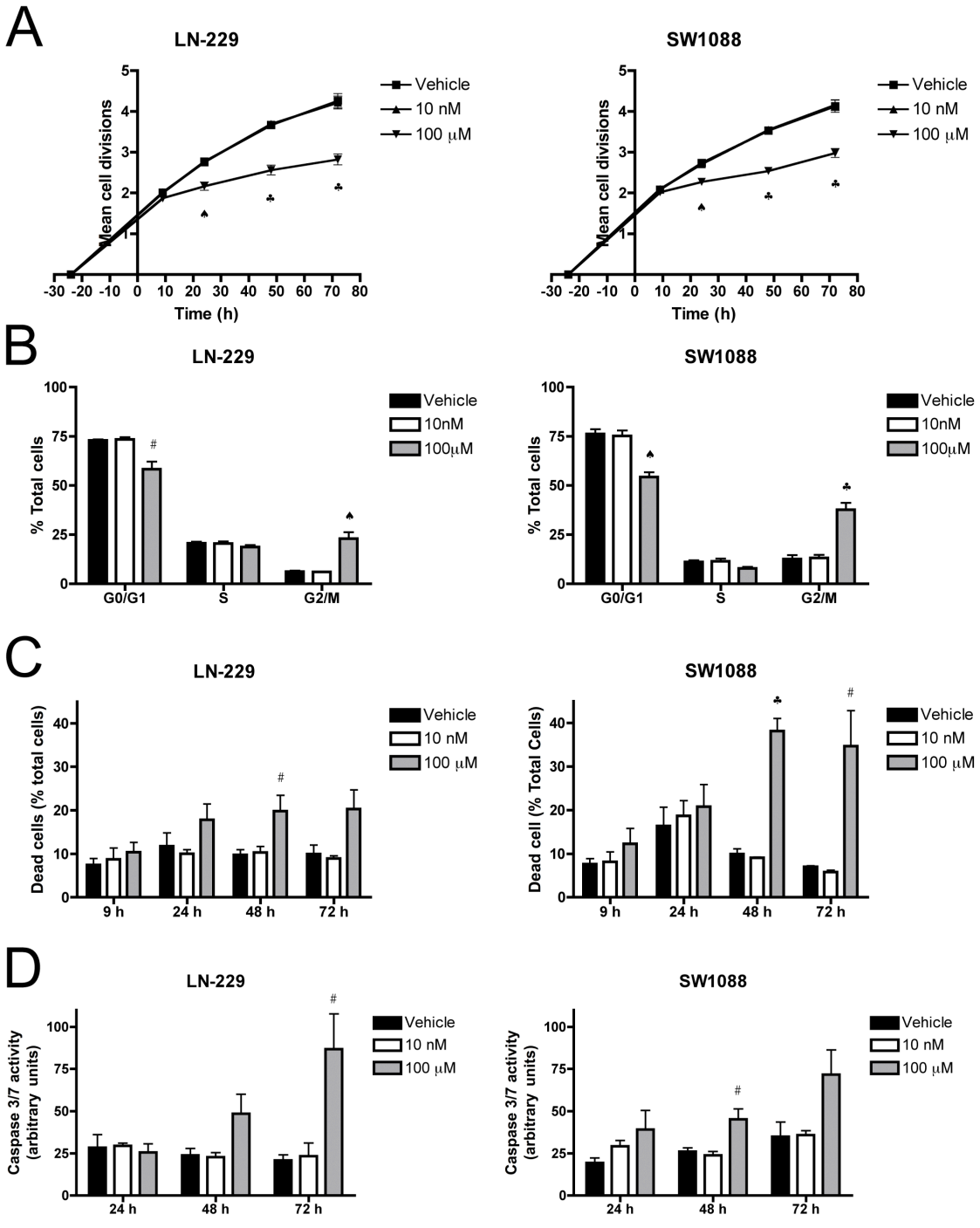
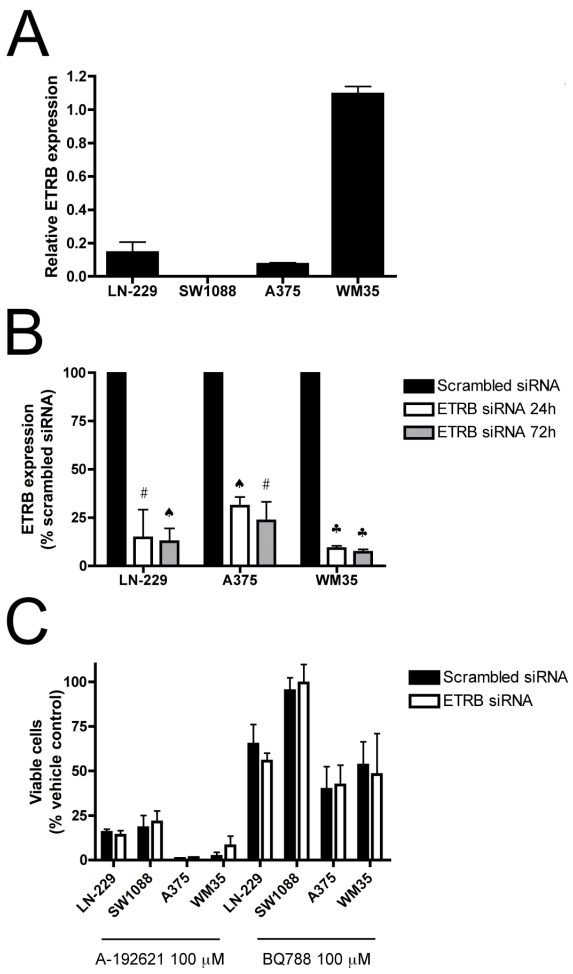

A
LN-229
SW1088
Mean cell divisions
Time (h)
Vehicle
10 nM
100 μM
B
LN-229
SW1088
% Total cells
G0/G1
S
G2/M
Vehicle
10nM
100μM
C
LN-229
SW1088
Dead cells (% total cells)
Dead cell (% Total Cells)
9 h
24 h
48 h
72 h
Vehicle
10 nM
100 μM
D
LN-229
SW1088
Caspase 3/7 activity (arbitrary units)
24 h
48 h
72 h
Vehicle
10 nM
100 μM

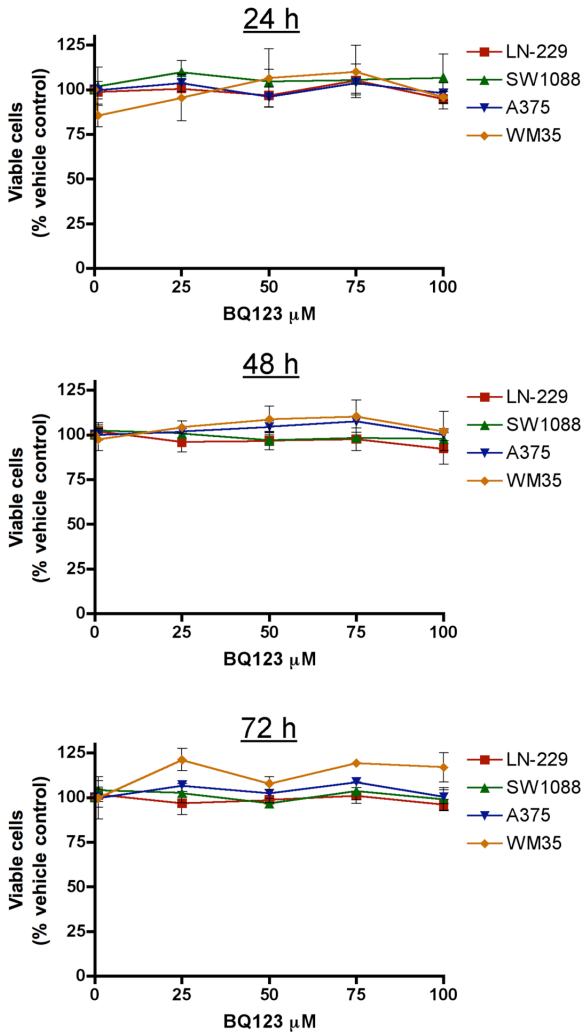
Figure 3


A
Relative ETRB expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
LN-229
SW1088
A375
WM35
B
ETRB expression
(% scrambled siRNA)
100
75
50
25
0
#
♠
#
♣
♣
LN-229
A375
WM35
Scrambled siRNA
ETRB siRNA 24h
ETRB siRNA 72h
C
Viable cells
(% vehicle control)
100
75
50
25
0
LN-229
SW1088
A375
WM35
LN-229
SW1088
A375
WM35
A-192621 100 μM
BQ788 100 μM
Scrambled siRNA
ETRB siRNA

Supplementary Data

Supplemental Figure 1. The endothelin receptor A-specific antagonist BQ123 does not affect viable cell number in glioma or melanoma cell lines. Cells were treated with BQ123 for 72 h. Values are expressed as means of three replicates ± SEM compared with vehicle-treated controls.

Supplementary Figure 1



Chapter VI

Comments and Future Considerations

Comments

When I began my investigation into the effects of endothelin receptor antagonists on cancer progression I was assessing cell viability using the TACS MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) Cell Proliferation and Viability Assay (R&D Systems, Inc., Minneapolis, MN, USA). In short, the MTT compound is added to cell culture wells and mitochondrial reductase enzymes reduce the compound to insoluble purple formazan dye crystals. Detergent is then added to solubilize the crystals and absorbance is read at 570 nm. The intensity of absorbance is proportional to the number of viable cells. This assay, and the very similar MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), is commonly used to assess cell viability, as they are rapid, relatively inexpensive and high-throughput. I found, however, that the results of the MTT assay did not correlate well with my other assays such as the CFSE labeling, cell cycle analysis and cell death quantification. This led me to reassess the viable cell numbers using the Live/Dead Viability/Cytotoxicity Kit for mammalian cells (Invitrogen, Carlsbad, CA, USA). This assay relies on intracellular esterases to cleave non-fluorescent calcein AM into green-fluorescent calcein. Fluorescent intensity is proportional to the number of viable cells. When directly compared, the two assays yield strikingly different results (Fig. 1). The MTT assay (Fig. 1A) is not as sensitive as the Live/Dead Assay (Fig. 1B).

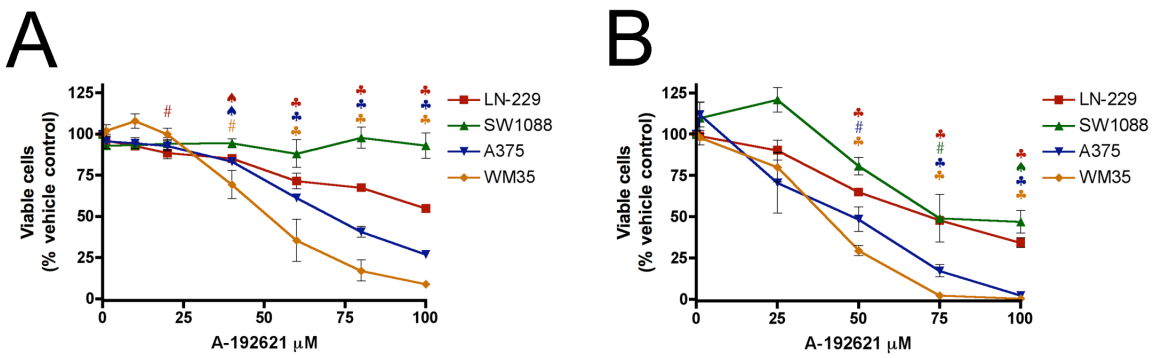


Figure 1. MTT viability assay masks effects of A-192621. (A) MTT viability assay measuring A-192621-induced reduction of cell viability at 48 hours. The lack of an observed A-192621 effect on the SW1088 line does not correlate with other assays. (B) Live/Dead viability assay measuring A-192621-induced reduction of cell viability at 48 hours. SW1088 and LN-229 cell lines have a similar response to A-192621.

The Live/Dead results indicate that LN-229 and SW1088 respond similarly to A-192621 while the MTT assay indicates that cell viability in SW1088 does is not affected by A-192621. The Live/Dead data correlate with the CFSE labeling, cell cycle analysis and cell death assays that also demonstrated a similar response in LN-229 and SW1088 cell lines following addition of A-192621. One explanation for the discrepancy could be that mitochondrial reductase activity does not accurately represent cell number. Another, more likely, explanation in this case is that cell density may have influenced the outcome of the viability assays. The MTT assays I conducted were cultured in 96-well plates as recommended by the manufacturer. Cells tend to clump in 96-well plates creating areas of higher density. In areas of higher density, growth factors secreted by cells would be in higher concentration and possibly be better able to counteract the effects of the endothelin B receptor antagonists. To avoid this occurrence, the Live/Dead experiments were conducted in 12-well plates. The smaller surface tension to surface area ratio in a 12-well plate permits an even cell distribution across the well thus eliminating any increased paracrine activity. To follow up on the effectiveness of the MTT assay it

should be scaled up to a 12-well format and compared to other assays. Currently, I recommend that investigators avoid using the MTT assay in the 96-well format and all results should be confirmed by other means. This is particularly important since in the literature claims of specificity of drug action have been made using this assay and it is clear from my work that effects may have been overlooked.

Future Considerations

For researchers with an interest in continuing the investigations described in the preceding chapter, I would recommend that the CFSE labeling, cell cycle analysis and cell death assays be repeated with the siRNA knockdown of ETRB. I hypothesize that that the results will not differ compared to a non-targeting siRNA control, confirming my findings that indicate A-192621 reduces cell numbers independently of ETRB expression. Another experiment to confirm that the cytotoxic effects of A-192621 are not mediated via ETRB would be an agonist-antagonist competition assay. Increasing concentrations of an ETRB-specific agonist, such as sarafotoxin 6c or IRL-1620, could be added to compete with the antagonist for ETRB binding sites. The effect of A-192621 on cell viability should not change in the presence of high concentrations of a competitive agonist, which will saturate the available ETRB binding sites. By their very nature ETRB agonists are mitogenic (Shichiri *et al.*, 1991); a complicating factor in these proposed experiments. Therefore, the necessary control for assessing the effects of A-192621 on cell viability of A-192621/agonist co-incubated cells will be agonist alone treated cultures. The more refined approach would be to use a neutralizing antibody to occupy the ETRB binding site but such a molecule is not available at present.

Following the confirmation that A-192621 reduces cell viability independently of ETRB, there are two important *in vivo* experiments I would recommend. 1) Assess the effect of A-192621 in a xenograft model of glioma in nude mice. A-192621 should be administered intravenously in one group of animals and orally in another to determine the most effective method of delivery. It has already been demonstrated that therapeutic doses of A-192621 are well tolerated in mice with no signs of acute or delayed toxicity (Bagnato *et al.*, 2004). 2) Confirm that A-192621 reduces tumor viability independently of ETRB *in vivo*. First, an *in vivo* tumor model that responds to A-192621 is needed. This could be the aforementioned experiment or the melanoma model described by Bagnato and colleagues (Bagnato *et al.*, 2004). Tumor cells should be stably transfected with a lentiviral shRNA vector targeting ETRB. Cells showing the greatest reduction in ETRB expression should be selected, cloned and injected into animals. Controls should include ETRB-null tumors without A-192621 treatment and animals injected with wild-type tumors with and without A-192621 treatment. A-192621 dosage should follow the same course found to be effective in wild-type tumors in proposed experiment 1. Assuming that A-192621 reduces tumor viability independently of ETRB binding one will not expect to record a difference in its effect on wild-type and ETRB-null tumor progression.

References

Bagnato A, Rosano L, Spinella F, Di Castro V, Tecce R & Natali PG. (2004). Endothelin B receptor blockade inhibits dynamics of cell interactions and communications in melanoma cell progression. *Cancer Res* **64,** 1436-1443.

Shichiri M, Hirata Y, Nakajima T, Ando K, Imai T, Yanagisawa M, Masaki T & Marumo F. (1991). Endothelin-1 is an autocrine/paracrine growth factor for human cancer cell lines. *J Clin Invest* **87,** 1867-1871.