Title

Endothelin Receptor-mediated Attenuation of Carcinoma-induced Nociception is Opioid-dependent in Mice

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Endothelin Receptor-mediated Attenuation of Carcinoma-induced Nociception is Opioid-dependent in Mice

by

Phuong Ngoc Quang

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Oral and Craniofacial Sciences

in the

DEDICATION

To my mother and my siblings, Son, Angela, Linda, Lam, Yen, Lan, and Chi

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Part of the text of this dissertation/thesis is a reprint of published materials. The co-author listed in these publications directed and supervised the research that forms the basis for the dissertation/thesis.

Chapter 3

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Chapter 4

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Abstract

Cancer pain is a frequent and disabling consequence for many patients yet the mechanism of cancer pain remains unknown largely due to its complex etiology. The objective of this thesis work was to investigate the potential of a peptide produced by carcinoma cells, endothelin, as a novel target for cancer pain management. This study provided evidence in support of **Hypothesis 1: Oral squamous carcinoma cells produce endogenous opioids in response to changes in endothelin-1 signaling.** Effects of endothelin-1 (ET-1) signaling on secretion of opioids by oral squamous cell carcinoma (SCC) were studied *in vitro*. Oral SCC cell line HSC-3 produces abundant ET-1 that can act in both an autocrine or paracrine manner. In the untreated oral SCC cells, endogenous opioids were detected in both cultured media and cell lysates. When treated with Endothelin-A receptor antagonist, secreted levels of β-endorphin and leu-enkephalin were increased; whereas treatment with Endothelin-B receptor agonist increased production of only β-endorphin. This apparent regulation of endogenous opioid levels by endothelin receptor drugs *in vitro* procured evidence suggesting that **Hypothesis 2: Endothelin-A receptor antagonist and Endothelin-B receptor agonist attenuate carcinoma-induced nociception in cancer animals through regulation of endogenous opioids.** Endothelin-A (ET-AR) antagonism has been demonstrated previously to attenuate carcinoma-mediated hyperalgesia in an orthotopic cancer pain mouse model. In this study, effects of ET-BR agonist *in vivo* were evaluated and determined to also result in significant increase in paw withdrawal thresholds, indicating attenuation of cancer-induced nociception. When peripheral μ- or δ-opioid receptor antagonists were administered following ET-AR antagonism, nociceptive attenuation was completely

reversed. Attenuation from ET-BR agonism was likewise reversed upon administration of μ-opioid receptor antagonist. Combined results demonstrate that endogenous opioids are the likely mediators responsible for attenuation of carcinoma-induced nociception with either ET-AR antagonist or ET-BR agonist.

These novel findings suggest that there exist innate modulation of pain by SCCs involving endogenous opioids that can be exploited through manipulation of endothelin activity in the cancer micro-environment. Regulation of ET-AR or ET-BR signaling may be targets for future innovations for cancer pain management.

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CHAPTER 1

INTRODUCTION

Oral cancer remains a significant national health problem affecting many patients [40,2]. In 2007 alone there were over 34,000 new oral cancer cases in the United States [37]. Cancer in the oral cavity ranks sixth worldwide amongst all other cancers [21] and oral squamous cell carcinoma (SCC) is the sixth most common malignancy among head and neck cancers [25]. Patients with oral SCC rate pain as their worst symptom and the primary determinant of a poor quality of life [19,6,16,8]. Oral cancer pain is unique for its localized, intense, and function-aggravated nature at the primary site, unlike cancer at other primary sites that are usually visceral and poorly localized [18,23,32]. Pain management can be achieved initially with opiates, but they are associated with multiple adverse side effects including tolerance [3], immunosuppression [12], nausea, vomiting, respiratory depression and even respiratory arrest. Once morphine tolerance develops, there are no other effective analgesic regimens. Significant research exists to study general pain and opiate tolerance yet little is focused specifically on pain associated with oral cancer. The mechanism of oral cancer pain remains unclear and our poor understanding of the etiology continues to hamper any improvement for patient management.

Growing evidence implicates endothelin as a mediator involved with cancer associated nociception. Endothelin is a vasoconstrictive peptide and a neuropeptide due to its function in both vascular and nervous systems. ET-1 binding to peripheral endothelin A receptors (ET-AR) induces nociception in both animals and humans [31,10]. In mice, ET-1 induced nociception is reversible with administration of ET-AR-selective antagonist drugs [10,20,36]. ET-1 binding to peripheral endothelin B receptors (ET-BR) produces equivocal results. Published data shows that ET-BR activation in rat

with trigeminal neuralgia elicits orofacial mechanical allodynia [7]. On the other hand, ET-1 binding to keratinocyte membrane-bound ET-BRs leads to an endogenous opioid-mediated analgesic response [22]. Therefore, I hypothesize that regulation of peripheral endothelin receptor activation by ET-1 modulates carcinoma-induced nociception through release of endogenous opioids in a tumor micro-environment by ultimately triggering opioid analgesia.

SIGNIFICANCE

Oral SCC continues to be a significant health problem [2,6]. Almost one-third of all oral SCC patients who undergo treatment (surgery, radiotherapy, or both), suffer the consequences of food intake restriction due to loss of tongue mobility, difficulties swallowing, and pain [38]. Opiates and non-steroidal anti-inflammatory drugs are the primary treatment regimens to manage oral SCC pain but they often render adverse complications. With about 30,000 new oral SCC cases annually, there is an obvious need for further research in carcinoma-induced pain. Growing evidence suggests a significant role for endothelin in cancer pain in both humans and animals [31,10,20,7,36]. Intradermal injection of ET-1 in humans produce a long-lasting and dose-dependent punctuate hyperalgesia [17], which further suggests the potentials for endothelin as a pain mediator in general pain, including cancer pain.

BACKGROUND

Endothelin

Endothelin is a potent vasoconstrictive peptide that was first isolated from porcine endothelial cells in 1988 [43] and initially shown to play a role in inflammatory pain [13]. It is closely related to the sarafotoxins derived from the venom of burrowing asps. There are three isoforms of endothelin peptides, ET-1, 2, and 3, of which ET-1 has been the most extensively studied. ET-1 is a 21 amino acid peptide synthesized from its precursor, pre-pro ET-1, via a proteolytic cleavage facilitated by the metalloproteinase, endothelin converting enzyme (ECE) [15]. Endothelins exert their physiological effect through binding of two receptors, endothelin A (ETAR) and B (ETBR). They are G-protein coupled transmembrane receptors found on many different types of tissue. ETAR can be found on endothelial cells, muscles, placenta, and keratinocytes [42,45]. ETBR expression is even more diverse than its A counterpart [26,27]. Remarkably, ETBRs are found on oral squamous carcinoma cells, albeit in fewer numbers than ETAR [1]. ETAR interacts preferentially with G_q and G_s while ETBR interacts preferentially with G_i and G_q [35]. ET-1 and ET-2 have greater affinity for ETAR than ET-3, whereas all three peptide isoforms have similar affinities for ETBR [33].

Endothelin in cancer pain

The peptide ET-1 has a number of different physiologic and pathologic roles, including cancer growth and pain. It is expressed by neurons of the brain [24], spinal cord, and the dorsal root ganglia [14], which implicate its role in neural transmission. In

particular, selective antagonism of endothelin A receptors has been shown to induce antinociception in various cancers, including prostate cancer [44], bone cancer [28], different types of adenocarcinomas [4], and squamous cell carcinoma [36]. Consequently, ETAR-specific antagonist under clinical trial for cancer progression results in a third of the 31 patients with tumor-related pain experiencing alleviation of symptoms [15]. Endothelin also induces primary afferent nerve ending sensitization in normal tissues. The direct subcutaneous administration of ET-1 induces nociception in noncancerous mice [31,10,11,30] and in human [17]. None of the studies, however, has shown pain modulation involving endothelin B receptors in oral squamous cell carcinoma nor elucidated the mechanistic pathway responsible for modulating endothelin-related cancer pain.

Cancer pain animal model

The cancer pain mouse model employed in this project to study carcinoma-induced nociception follows protocols previously established by studies in rats and in mice [41,34]. The application of von Frey hairs to quantitatively assess tactile allodynia is validated by Chaplan et al. in rats with surgical neuropathy [5]. In these rats, tactile allodynia can be measured reliably using von Frey hairs to stimulate the paw for up to eighty-one times within two hour. Caution is exercised when inducing mechanical allodynia by minimizing the number of stimuli and using smaller diameter hairs to prevent elevating the whole paw without stimulating a response. The same technique has

been utilized in our lab to evaluate the role of endothelin peptides in carcinoma-induced mechanical allodynia [29,36].

My proposed work will employ the cancer pain mouse model using an electronic von Frey device, an Anesthesiometer (IITC Inc., Life Science Instruments) in place of the manual hair application. The probe functions through a digital pressure-sensitive base to accurately record the maximal pressure level that evokes a paw withdrawal reflex in mice. This recording immediately gives the paw withdrawal threshold in grams of force without having to go through the complicated calculations of Dixon's up-and-down method employed by previous investigators.

To validate the use of a the electronic von Frey anesthesiometer in place of the up-down method of Dixon, effects of ET-A receptor antagonist on carcinoma-induced nociception in cancer mice were duplicated and compared between the two methods. The device consists of a rigid polypropylene pipette tip connected to a digital hand-held force-transducer for recording maximal paw pressure thresholds in grams of force. The tip is applied to the mouse midplantar paw to induce paw withdrawal reflex in a same manner as manual von Frey filaments in the up-down method. Comparing the electronic device to the classical von Frey filament mechanical test and the rat paw constant pressure test, the device is sensitive, objective, and quantitative in measuring inflammatory nociception [9,39]. To determine consistency of the digital assay in measuring carcinoma-induced nociception, paw withdrawal thresholds of oral SCC-inoculated mice using both manual von Frey filaments and electronic anesthesiometer were recorded. Nociceptive levels are graphed as percent change in paw withdrawal threshold from baseline (Fig. 1). Paired t-test reveals that measurements performed by the electronic anesthesiometer are similar to

levels obtained through the up-down method ($p > 0.05$), confirming that the electronic device can confidently replace the manual von Frey filaments.

FIGURE AND FIGURE LEGEND

Figure 1.

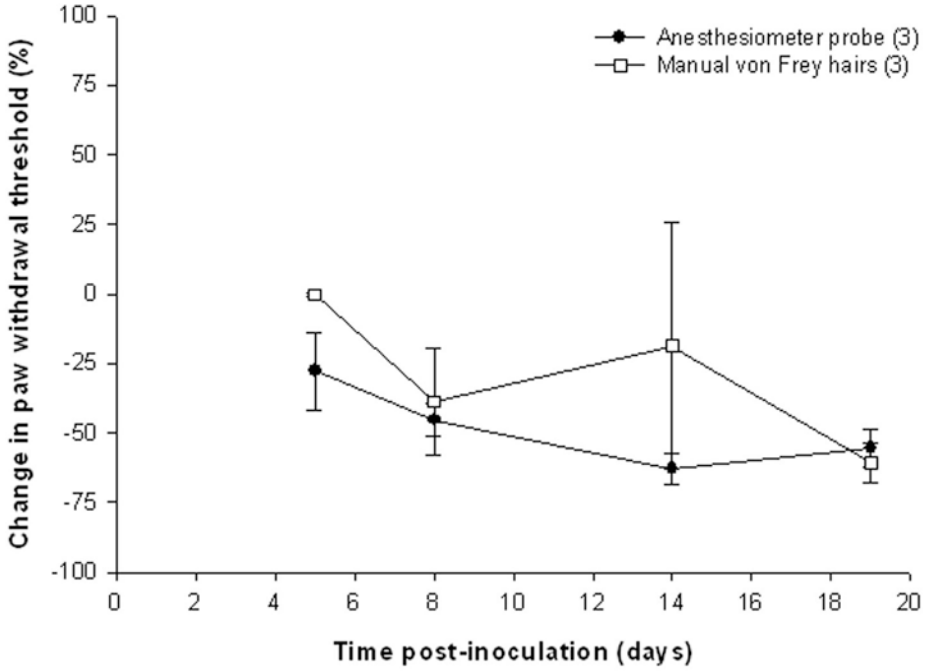

Figure 1.

Comparison of data gathered using manual von Frey hair method versus electronic Anesthesiometer probe. No significant difference detected between the two methods.

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CHAPTER 2

IN VITRO PRODUCTION OF ENDOGENOUS OPIOIDS BY ORAL SQUAMOUS CELL CARCINOMA IS REGULATED BY ENDOTHELIN

Abstract

Varying cell types can produce endogenous opioids that potentially activate innate opioid analgesic responses. Here we demonstrate that oral squamous cell carcinoma (SCC) are also capable of producing opioids, and that this production is regulated by peripheral endothelin receptor activity in an autocrine and/or paracrine manner. Oral SCC cell culture treated with endothelin-A receptor (ETAR) antagonist (BQ-123), endothelin-B receptor (ETBR) agonist (BQ-3020), or ETBR antagonist (BQ-788) were assayed for β-endorphin and leu-enkephalin peptides using an enzyme-linked immunosorbent assay (ELISA). Treatment with ETAR antagonist at 10^{-4} M and 10^{-5} M BQ-123 significantly increased β-endorphin levels while 10^{-4} M, 10^{-5} M, and 10^{-6} M BQ-123 increased leu-enkephalin levels. Oral SCC cultures treated with an ETBR agonist (10^{-4} M, 10^{-5} M, and 10^{-6} M BQ-3020) significantly increased β-endorphin production without any effects on leu-enkephalin. Specific ETBR antagonism (10^{-4} M BQ-788) reversed effects observed from activation of the receptors by significantly reducing production of β-endorphin. No change in leu-enkephalin levels were detected, which was consistent with ETBR agonist treatment. Our data demonstrate that oral SCC cells are capable of producing endogenous opioids *in vitro* in an endothelin-responsive manner.

Introduction

Endogenous opioids are known to be produced by various cell types such as leukocytes [11,4], neutrophils [6,22], erythrocytes [3], pituitary cells [8], visceral lining epithelial cells [5], skin keratinocytes [19,10] and resident cutaneous cells [21], as well as by different cancers including ovarian carcinoma [16], small cell carcinoma [12], epidermoid carcinoma [19], and malignant melanoma [14]. Immunocytes produce endogenous opioids in the blood as part of an immune response to injury, inflammation, and inflammatory pain [13]. Leukocytes, keratinocytes, and other resident cutaneous cells respond to the inflammatory signals and produce endogenous opioids that are capable of activating the innate analgesic cascade to control inflammatory pain. Production of opioids by cancer cells is not as well understood as it may involve many biological effects, including immunomodulation, pain modulation, and cancer proliferation and progression [16,12,14]. Our preliminary results demonstrating opioid production by an oral SCC cell line is the first to show opioid production by a carcinoma of oral origin. Oral squamous carcinoma cells are different from skin carcinoma in that oral cells do not have keratin, thus their cellular biology is distinct from skin cells.

Aside from having different cellular architecture, oral SCC cells produce elevated levels of endothelin-1 (ET-1) [20,17] compared to normal keratinocytes. ET-1 is one of three isoforms of the endothelin peptides comprised of a 21 amino acid vasoactive peptide synthesized from its precursor, pre-pro ET-1, through a proteolytic cleavage by endothelin converting enzyme (ECE) [7]. Two G-protein coupled receptors cloned in mammals, endothelin-A and endothelin-B receptors (ETAR and ETBR, respectively), are known to mediate the physiological actions of ET-1 [1,18]. *In vitro* immunofluorescence

has confirmed expression of both receptors on oral SCC cells, with ETAR higher than ETBR [2]. The elevated *in vitro* production of ET-1 in oral SCC cell culture can bind to ETAR and ETBR on the cell surfaces and act in an autocrine and/or paracrine manner. Since endothelin receptors are found on oral SCC and ETBR activation of normal squamous cells stimulate production of endogenous opioids [10], we hypothesize that ET-1 produced *in vitro* can bind endothelin receptors in an autocrine and/or paracrine manner to stimulate production of endogenous opioids.

Materials and Methods

Cell culture

HSC-3, an oral SCC cell line (ATCC, Manassas, VA) derived from a human tongue SCC, was cultivated in Dulbecco's Modification of Eagle's Medium (DMEM) with 4.5 g/L glucose, L-glutamine and sodium pyruvate, supplemented with 10% fetal bovine serum, 25 μg/mL fungizone, 100 μg/mL streptomycin sulfate, and 100 units/mL penicillin G.

ELISA measurement of endogenous opioids

To evaluate the effect of ETAR antagonism and ETBR agonism on opioid production and secretion in oral SCC, we used enzyme-linked immunosorbent assay (ELISA) for opioid peptide measurement. 10^5 HSC-3 cells were seeded onto 6-well tissue culture plates with 3 mL of DMEM supplemented with 10% fetal bovine serum, 25 μg/mL fungizone, 100 μg/mL streptomycin sulfate, and 100 units/mL penicillin G. HSC-3 cells were cultured for 24 h until the wells reached 70-80% confluence. Each well was washed once with PBS then incubated for 12 h in 1 mL of one of the following media: 1) DMEM alone; 2) DMEM with 10 ng/mL synthetic beta-endorphin (Sigma, St. Louis, MO) or leu-enkephalin (Phoenix Pharmaceuticals, Burlingame, CA); 3) DMEM with one of ten-fold concentrations of BQ-123 (10^{-4}M to 10^{-9}M, American Peptide Co., Sunnyvale, CA); 4) DMEM with one of ten-fold concentrations of BQ-3020 (10^{-4}M to 10^{-9}M, American Peptide Co., Sunnyvale, CA); or 5) DMEM with one of ten-fold concentrations of BQ-788 (10^{-4}M to 10^{-9}M, American Peptide Co., Sunnyvale, CA). Culture media were collected and treated with 1x HALT Protease Inhibitor Cocktail (Pierce, Rockford, IL) before performing ELISA to detect levels of β-endorphin (MD Biosciences, St. Paul,

MN) or leucine-enkephalin (leu-enk). Opioid concentrations were calculated based on a calibration curve, with 55% supernatant recovery for β-endorphin and 12% recovery for leu-enk.

Statistical Analysis

ELISA protein measurements were analyzed with one-way ANOVA with Tueky post-test. For all tests a *p* value of less than 0.05 was considered significant. Statistical analysis was performed using SigmaPlot for Windows (Version 11.0).

Results

ELISA measurement of endogenous opioids

ELISA was performed on conditioned media of SCC cells to quantify production of endogenous opioids (β-endorphin and leu-enkephalin). The concentration was calculated from a standard curve using a sigmoid logistics curve fitting program (Bio-Rad Laboratories, Inc., Hercules, CA) as appropriate for the opioid ELISA kits, followed by adjustments for percent recovery from synthetic peptide positive control treatments in culture. SCC cell culture treated with ETAR antagonists significantly increased β-endorphin and leu-enkephalin productions compared to untreated SCC cultures at 5.38 ± 0.22 ng/mL. Treatment with 10^{-4} M and 10^{-5} M BQ-123 produced 7.70 ± 0.48 and 7.60 ± 0.92 ng/mL β-endorphin, respectively ($p = 0.004$ and 0.029, respectively); 10^{-4} M, 10^{-5} M, and 10^{-6} M BQ-123 produced 3.12 ± 0.033, 2.83 ± 0.28, and 2.70 ± 0.33 ng/mL leu-enkephalin, respectively ($p = 0.006$, 0.009, and 0.024, respectively). (Fig. 1a) ETBR agonist treatment at 10^{-4} M, 10^{-5} M, and 10^{-6} M BQ-3020 produced 8.02 ± 0.45, 7.69 ± 0.53, and 6.58 ± 0.31 ng/mL β-endorphin, respectively ($p = 0.002$, 0.007, and 0.019, respectively), without any effects on leu-enkephalin. (Fig. 1b) Cell culture treatment with ETBR antagonist at 10^{-4} M BQ-788 decreased production of β-endorphin to 3.87 ± 0.74 ng/mL ($p = 0.01$), but did not affect leu-enkephalin levels. (Fig. 1c)

Discussion

In the current study, we demonstrated that oral SCC is capable of producing endogenous opioids *in vitro* and that their production is responsive to endothelin receptor agonism or antagonism. Other cell types and various cancers have been shown to produce endogenous opioids [6,8,19,14,21,9,10,22,3,11,4], but our finding is the first to show that squamous cell carcinoma from an oral origin also produces β-endorphin and leu-enkephalin. Furthermore, we demonstrated that the levels of opioid produced can be manipulated with ETAR antagonist, ETBR agonist, or ETBR antagonist treatment in cell culture.

The ability of oral SCC to produce endogenous opioids is consistent with reported data demonstrating production of proopiomelanocortin (POMC) by normal skin keratinocytes [19]. β-endorphin production in normal keratinocytes is increased upon ETBR activation [10,15]; endothelin receptors are expressed on cell surfaces of oral squamous carcinoma [2]; and oral carcinomas are a malignant form of squamous cells from which keratinocytes are derived. Since both are of the same origin, it is possible that they exhibit similar cellular functions related to endothelin.

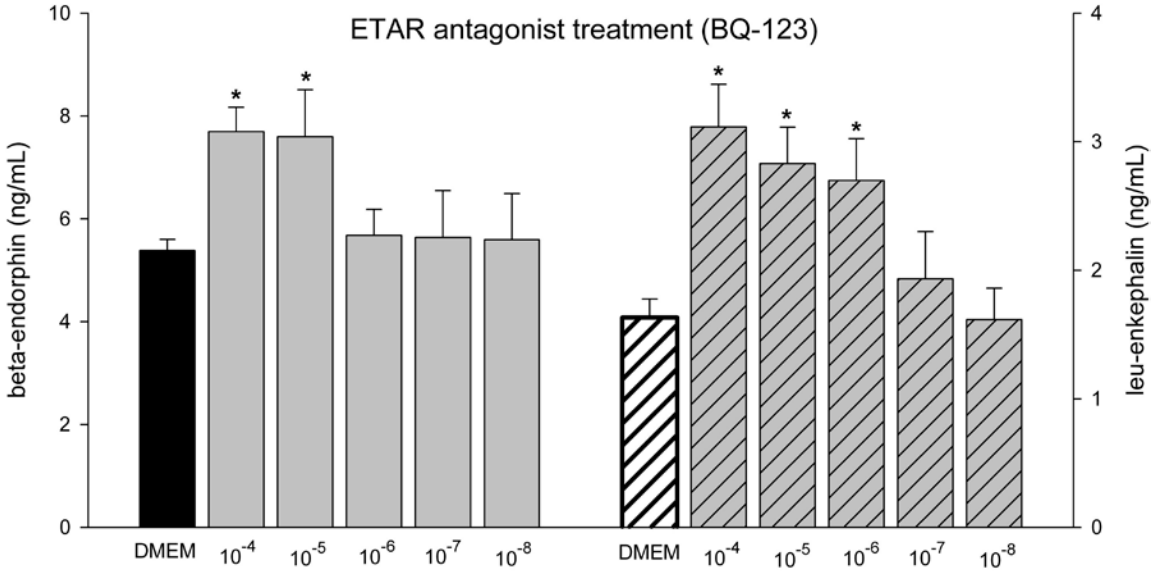
The function of opioids produced by oral SCC will require further investigation. β-endorphin peptides induced by ETBR activation in normal keratinocytes has been implicated in an opioid analgesic pathway to modulate peripheral skin injury [9,10], so we can postulate that endogenous opioids, namely β-endorphin and leu-enkephalin, produced by oral SCC cells may be responsible for modulating carcinoma-induced pain. Further studies in behavioral animal models will help to elucidate the role of opioids

produced by oral SCC upon treatment with endothelin receptor agonist or antagonist drugs.

FIGURES AND FIGURE LEGEND

Figure 1.

a



b

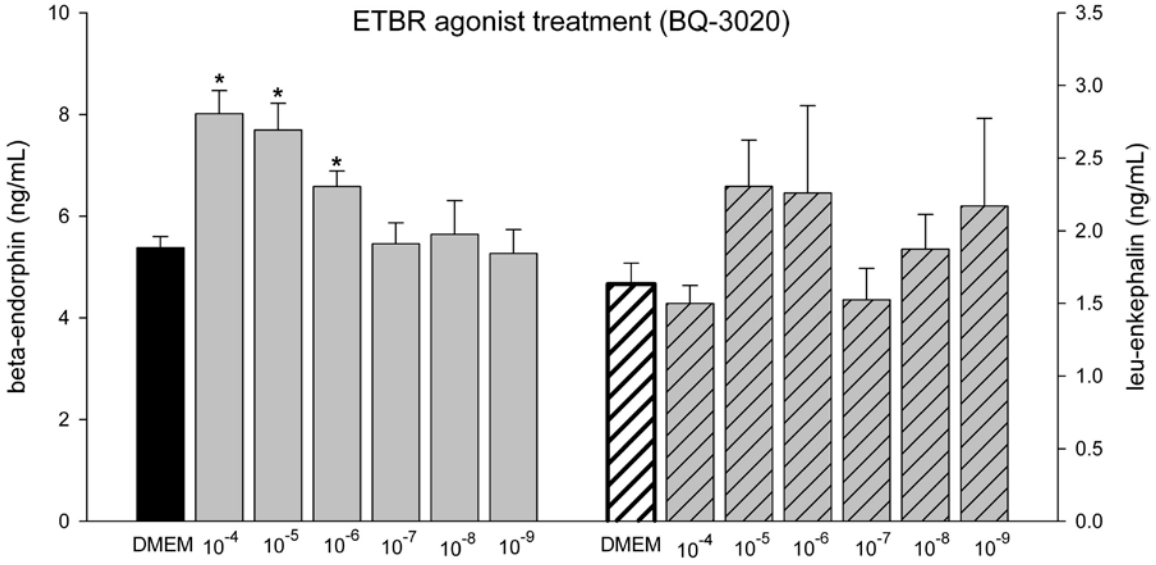


Figure 1.

c

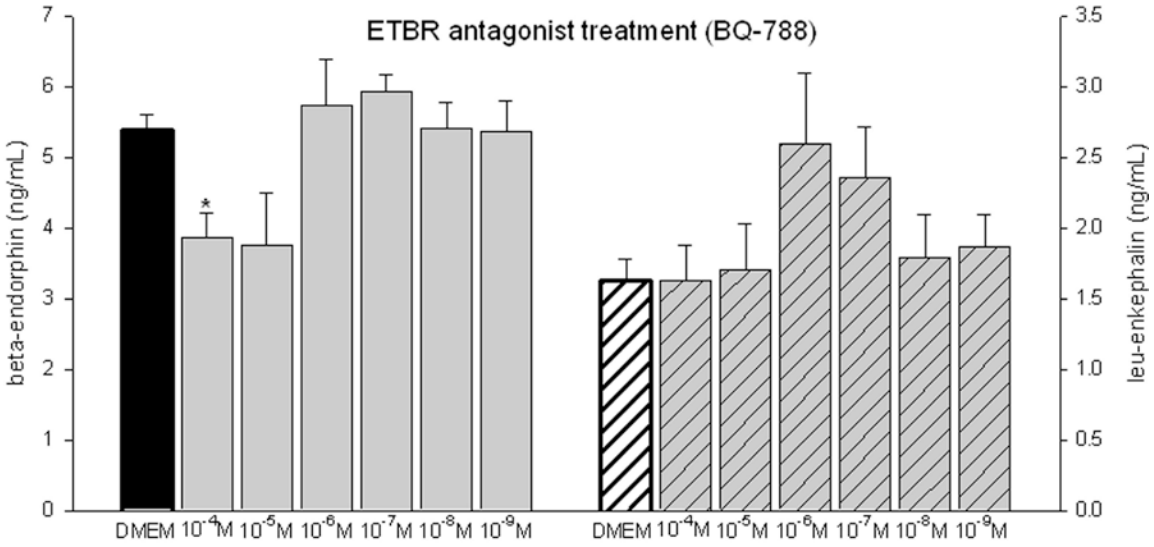


Figure 1.

ELISA quantification of endogenous opioid concentration in SCC conditioned media under different ETAR antagonist (BQ-123), ETBR agonist (BQ-3020), and ETBR antagonist (BQ-788) treatments. (**a**) β-endorphin level was significantly increased when treated with 10^{-4} M and 10^{-5} M BQ-123 compared to DMEM control (p = 0.004 and 0.029, respectively). Treatment with 10^{-4} M, 10^{-5} M, and 10^{-6} M BQ-123 also increased leu-enkephalin levels (p = 0.006, 0.009, and 0.024, respectively) (**b**) ETBR agonist treatment at 10 M, 10 M, and 10 M BQ-3020 significantly increased β-endorphin peptides (p = 0.002, 0.007, and 0.019, respectively). No changes detected in leu-enkephalin. (**c**) Cell culture treated with ETBR antagonist at 10 M BQ-788 decreased β-endorphin levels (p = 0.01) without affecting leu-enkephalin production. [* indicates significance]

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CHAPTER 3

ENDOTHELIN-A RECEPTOR ANTAGONISM ATTENUATES CARCINOMA-INDUCED PAIN THROUGH OPIOIDS IN MICE

Abstract

We previously reported that endothelin A (ET-A) receptor antagonism attenuates carcinoma-induced pain in a cancer pain mouse model. In this study, we investigated the mechanism of ET-A receptor-mediated antinociception and evaluated the role of endogenous opioid analgesia. Squamous cell carcinoma (SCC) cell culture treated with the ET-A receptor antagonist (BQ-123) at 10^{-6} M and 10^{-5} M significantly increased production and secretion of β-endorphin and leu-enkephalin, respectively. Behavioral studies were performed by inducing tumors in the hind paw of female nude mice with local injection of cells derived from a human oral SCC. Significant pain, as indicated by reduction in withdrawal thresholds in response to mechanical stimulation, began at four days after SCC inoculation and lasted to 18 days, the last day of measurement. Local administration of either naloxone methiodide (500 μg/kg), selective antagonists for μ-opioid receptor (CTOP, 500 μg/kg) or δ-opioid receptor (naltrindole, 11 mg/kg), but not κ-opioid receptor (nor-BNI, 2.5 mg/kg), significantly reversed antinociception observed from ET-A receptor antagonism (BQ-123, 92 mg/kg) in cancer animals. These results demonstrate that antagonism of peripheral endothelin-A receptor attenuates carcinoma pain by modulating release of endogenous opioids to act on opioid receptors in the cancer microenvironment.

Perspective

This article proposes a novel mechanism for endothelin-A receptor antagonist drugs in managing cancer-induced pain. An improved understanding of the role of innate opioid

analgesia in ET-AR-mediated antinociception might provide novel alternatives to morphine therapy for the treatment of cancer pain.

Introduction

Pain is a primary determinant of a poor quality of life for cancer patients, especially head and neck cancer patients.[13] Seventy-five to ninety percent of terminal cancer patients must cope with opiate-resistant pain related to cancer progression.[43,44,53,57] Eighty-five percent of cancer patients experience severe pain in their final days of life.[60] Opiates are initially effective in cancer pain management, but they are associated with a number of adverse side effects including tolerance [41,9,40,49], immunosuppression [22], nausea, vomiting, respiratory depression and even respiratory arrest. Once morphine tolerance develops, there are no effective analgesic regimens available. The etiology of cancer pain is unknown, but may involve mediator-dependent signaling by cancer cells to primary afferent sensory neurons in the cancer micro-environment.

One candidate mediator responsible for cancer pain is endothelin (ET).[51,59,52] ET is a potent vasoactive peptide first isolated from porcine aortic endothelial cells[63] and initially shown to play a role in inflammatory pain.[23] It is closely related to the sarafotoxins derived from the venom of burrowing asps.[36] Three isopeptides of endothelin, endothelin-1, 2, and 3 (ET-1, ET-2, and ET-3, respectively) have been identified.[33] ET-1 is a 21 amino acid peptide synthesized from its precursor, pre-pro ET-1, via a proteolytic cleavage facilitated by the metalloproteinase, endothelin converting enzyme (ECE).[27] ET exerts its physiological effect through binding of two G-protein coupled receptors (GPCRs), endothelin A (ET-A) and endothelin B (ET-B). ET-1 is highly expressed in different cancers [37,1,2,4] and selective antagonism of ET-A receptors has been shown to attenuate pain in prostate cancer [64], bone cancer

[18,20,51], adenomcarcinoma [12], and melanoma [25]. Moreover we have demonstrated that injection of an ET-A receptor antagonist into the site of the tumor attenuated squamous carcinoma-induced nociception in a cancer pain mouse model.[59,52] These findings indicate that ET-A receptors are implicated in cancer pain signaling and that the antinociceptive effect of ET-AR antagonists is due to antagonism of ET-A receptors in the cancer microenvironment.[19,26]

ET-A receptor activity on primary afferent neurons leads to downstream signal transduction that involve elevation in intracellular levels of calcium [56,65] and cAMP[61], activation of phospholipases (PLC-A and PLC-D) and protein kinase C (PKC), and regulation of membrane ionic channels [34], which all ultimately result in activation of peripheral nociceptors.[26] Attenuation of carcinoma pain with ET-A receptor antagonist can lead to effects on components of the signal transduction pathway. Studies in both rats and mice have demonstrated that ET-A receptor antagonist potentiates morphine analgesia and prevents development of tolerance by promoting coupling of G-proteins to opioid receptors.[8,6,28,7] Hence we hypothesize that antagonism of ET-A receptors can potentially inhibit afferent nociceptor signal transduction or potentiate innate opioid analgesic pathways to modulate cancer pain.

Opioid analgesic pathways are activated through activation of the other isotype of endothelin receptors, endothelin-B receptors (ET-BR), found on non-neuronal cells. Activation of ET-B receptors on skin keratinocytes with selective ET-B receptor agonists stimulates the release of β-endorphin, which suppresses ET-1-induced pain behavior in mice and rats.[35] Both ET-A and ET-B receptors are expressed on cell membranes of squamous cell carcinoma.[5] Since activation of ET-B receptor on squamous cells

modulates pain through endogenous opioids, we wanted to evaluate the role of opioid analgesia in ET-AR-mediated antinociceptive effects in carcinoma. In the present study, we investigated whether ET-A receptor antagonism affects endogenous opioid secretion, and whether attenuation of carcinoma pain with ET-A receptor antagonist is dependent on opioid receptors. These findings have been partly presented at an annual meeting of the American Pain Society.[54]

Materials and Methods

Cell culture

HSC-3, an oral SCC cell line (ATCC, Manassas, VA) derived from a human tongue SCC, was cultivated in Dulbecco's Modification of Eagle's Medium (DMEM) with 4.5 g/L glucose, L-glutamine and sodium pyruvate, supplemented with 10% fetal bovine serum, 25 μg/mL fungizone, 100 μg/mL streptomycin sulfate, and 100 units/mL penicillin G. Primary normal oral keratinocytes (NOK) were harvested from normal gingival tissues using a modified technique described by Hybbinette et al.[31] Collection of oral epithelium was approved by the UCSF Committee on Human Research and consent was obtained from patients. Tissues were washed in 70% sterile ethanol, cut into 5 mm square sections, and incubated in 0.25 mg/mL dispase I at 37^{o}C for approximately 3 hrs. The separated epidermis was minced and transferred to 0.25% trypsin for 5 min incubation at 37^{o}C, then stopped with 0.25-0.5 mg/mL soybean trypsin inhibitor (Invitrogen, Carlsbad, CA) and centrifuged at 1300 rpm for 8 min. The sedimented cells were resuspended in Defined Keratinocyte Serum-free media (Invitrogen, Carlsbad, CA) supplemented with 50 μg/mL gentamicin and 25 μg/mL fungizone and cultivated at 37^{o}C in 5% CO_2.

Real-time quantitative RT-PCR in cell culture

Because ET-A receptors have not been previously quantified in oral SCC we used RT-PCR to quantify and compare transcript expression levels of ET-1 and ET-A receptor

in oral SCC and normal oral keratinocytes. ET-1 and ET-A receptor mRNA expression levels were measured in the HSC-3 cell line relative to the normal oral keratinocyte control. 10^4 cells were cultivated in 300 μL of DMEM supplemented with 10% fetal bovine serum, 25 μg/mL fungizone, 100 μg/mL streptomycin sulfate, and 100 units/mL penicillin G on 96-well cell culture plates until 75%-80% confluent. Cells were then harvested and lysed for quantitative PCR analysis using the TaqMan® Gene Expression Cells-to-CT Kit (Applied Biosystems/Ambion, Austin, TX), performed at the Genome Analysis Core Facility (Helen Diller Family Comprehensive Cancer Center, UCSF). Samples were run on an ABI 7700 Prism (PE Biosystems, Foster City, CA). Relative expressions of ET-1 and ET-A receptor mRNA were calculated using the comparative Ct method as previously described.[16,59] Analysis was carried out using the software supplied with the ABI 7700 Prism. Overexpression was defined as expression >2.0 relative to the reference gene β-*N*-acetyl-glucosaminidase (β-Gus).

ELISA measurements of endogenous opioids

To evaluate the effect of ET-A receptor antagonism on opioid production and secretion in oral SCC and NOK, we used enzyme-linked immunosorbent assay (ELISA) for opioid peptide measurement. 10^5 cells of either HSC-3 or first-passaged normal keratinocytes were seeded onto 6-well tissue culture plates with 3 mL of DMEM supplemented with 10% fetal bovine serum, 25 μg/mL fungizone, 100 μg/mL streptomycin sulfate, and 100 units/mL penicillin G. HSC-3 cells were cultured for 24 hrs until the wells reached 70-80% confluency. Each well was washed once with PBS

then incubated for 12 hrs in 1 mL of one of the following media: 1) DMEM alone, 2) DMEM with 10 ng/mL synthetic beta-endorphin (Sigma, St. Louis, MO) or leu-enkephalin (Phoenix Pharmaceuticals, Burlingame, CA), or 3) DMEM with one of ten-fold concentrations of BQ-123 (10^{-5}M to 10^{-7}M, American Peptide Co., Sunnyvale, CA). Culture media were collected and treated with 1x HALT Protease Inhibitor Cocktail (Pierce, Rockford, IL) before performing ELISA to detect levels of β-endorphin (MD Biosciences, St. Paul, MN), leu-enkephalin and dynorphin (Phoenix Pharmaceuticals, Burlingame, CA). Opioid concentrations were calculated based on a calibration curve, with 55% supernatant recovery for β-endorphin and 12% recovery for leu-enkephalin. Dynorphin concentrations were calculated with a 1:1 recovery ratio since no synthetic human dynorphin was readily available for recovery test.

SCC paw model

The cancer pain mouse model was produced as previously described. [59,52] Experiments were performed on 5 weeks-old adult female *Foxn1*^{nu}, athymic, immunocompromised mice (Charles River, Wilmington, MA) weighing 16-20 g at the time of SCC inoculation. Mice were housed in a temperature-controlled room on a 12:12 h light cycle (0700–1900 h light), with *ad libitum* access to food and water; estrous cycles were not monitored. All procedures were approved by UCSF Committee on Animal Research. Researchers were trained under the Animal Welfare Assurance Program. Mice were divided into three experimental groups: those receiving an injection of squamous carcinoma cells (SCC group), those receiving an injection of normal oral

keratinocytes (negative control), and those receiving an injection of DMEM (sham operated). All injections were into the hindpaw. All groups were anesthetized by inhalation with 1-3% isofluorane. Cell injections consisted of 1.0 X 10^6 HSC-3 cells (SCC group) or NOK cells (negative control) in a vehicle consisting of 50 μL of DMEM into the plantar surface of the right hind paw. The sham-operated group received 50 μL of DMEM. Post-operative analgesia was not necessary as all injections were subcutaneous.

Behavioral testing for the SCC paw model

Behavioral testing was performed as described previously.[59] Testing was performed in the afternoons between 14:00 and 16:00 h (during the light phase). Mice were placed in a plastic cage with a wire mesh floor which allowed access to the paws. Quantitative assay guidelines were used similar to a previously described technique.[14] Fifteen minutes were allowed for acclimation prior to testing. The probe was applied to the mid-plantar right hind paw, or the tumor-front on the hind paw toward the later stages of tumor development. Paw withdrawal thresholds were determined in response to pressure from an electronic von Frey anesthesiometer (2390 series, IITC Instruments, Woodland Hills, CA). The amount of pressure (g) needed to produce a paw withdrawal response was measured three times on each paw separated by 3 minute intervals to allow resolution of previous stimuli. The results of three tests were averaged for each paw for that day. The SCC, negative control and sham-injected groups were tested under this paradigm at 4, 7, 9, 11, 14, 16, and 18 days post-inoculation of SCC, NOK or vehicle.

Drug administration and pain behavioral testing

To evaluate whether ET-A receptor antagonist-induced attenuation of carcinoma pain involves endogenous opioids, opioid receptor (OR) antagonists were administered following BQ-123 injection. Drug testing was performed on days 18-25 following inoculation of oral SCC into the hindpaw. Drugs were dissolved in a final volume of 20 μL PBS and injected subcutaneously into the mid-plantar hind paw at the site of greatest tumor development with a 30-gauge beveled needle. After a 15 min pre-injection reading, BQ-123 (92 ng/kg) was injected and paw withdrawal thresholds were recorded at post-injection times 5, 10, 15, 30, 45, 60, and 90 min. Then a second drug (PBS or OR antagonist) was injected and paw withdrawal testing was performed at 5, 10, 15, 30, 45, 60, 90, 120, 180 min, and 24 hr. Mice groups were injected with one of the following drug combinations: 1) BQ-123 (92 ng/kg) followed by PBS control, 2) BQ-123 followed by nonspecific OR antagonist (500 μg/kg naloxone methiodide, Sigma, St. Louis, MO), 3) BQ-123 followed by selective μ-OR antagonist (500 μg/kg Cys^2-Tyr^3-Orn^5-Pen^7-amide [CTOP], Sigma, St. Louis, MO), or 4) BQ-123 followed by selective δ-OR antagonist (11 mg/kg naltrindole [NTI], Sigma, St. Louis, MO). κ-OR antagonist (2.5 mg/kg nor-binaltorphimine [nor-BNI], Sigma, St. Louis, MO) was injected 12 hr prior to injection with PBS control or BQ-123. The investigators performing the injections and behavioral testing were blinded to the drugs being administered.

Statistical Analysis

A one-way Analysis of Variance (ANOVA) with a Tukey Multiple Comparisons post-test was used to compare the withdrawal threshold of the cancer inoculated mice and sham over 18 days. The same test was used to compare the percent change in withdrawal threshold of the SCC inoculated mice before and after drug or control injection. ELISA protein measurements and mRNA gene expressions were also analyzed with one-way ANOVA. When an Equal Variance Test failed, one-way ANOVA with Dunn's Method for Multiple Comparison post-test was performed. For all tests a p value of less than 0.05 was considered significant. Statistical analysis was performed using SigmaPlot for Windows (Version 11.0).

Results

ET-1 and ET-A receptor mRNA expression

ET-1 and ET-A receptor mRNA expression levels in oral SCC and NOK controls were normalized to β-Gus and compared (Fig. 1). ET-1 mRNA expression in SCC cell culture (407.69 ±68 %) was nearly doubled that in NOK controls (277.28 ±35.09%). ET-A receptor mRNA expression in SCC (2.46 ± 0.24%) was significantly less than expression in NOK controls (4.98 ± 0.67%), $p = 0.024$.

ELISA measurement of endogenous opioids in cell culture

Levels of endogenous opioids (β-endorphin, leu-enkephalin, and dynorphin) were measured in conditioned media of SCC cells by ELISA. The concentration was calculated from a standard curve using a sigmoid logistics curve fitting program (Bio-Rad Laboratories, Inc., Hercules, CA) as appropriate for the opioid ELISA kits, followed by adjustments for percent recovery from synthetic peptide positive control treatments in culture. ET-A receptor antagonist treatment induced dose-responsive production of β-endorphin in SCC cells (Fig. 2a), with 10^{-6}M BQ-123 promoting the greatest increase (7.45 ± 0.04 ng/mL, n = 3) compared to untreated control (5.83 ± 0.33 ng/mL, n = 3). Leu-enkephalin production was dose-dependent on ET-A receptor antagonist (Fig. 2b), with a significant increase at 10^{-5}M BQ-123 (4.94 ± 1.24 ng/mL, n = 3) compared to untreated control (1.84 ± 0.51 ng/mL, n = 3). ET-A receptor antagonist had no effect on dynorphin production (Fig. 2c, n = 3).

Paw withdrawal in the SCC mouse model

To determine whether SCC inoculation produced mechanical hyperalgesia in the mouse cancer model paw withdrawal thresholds for the SCC, NOK, and sham groups were compared. Mean percent change in paw withdrawal thresholds were significantly reduced in the SCC inoculated mice as compared to both NOK and sham inoculated mice on all 18 days of behavioral testing (Fig. 3). The threshold value (g) for the SCC group at baseline prior to inoculation was 4.45 ± 0.60 g and dropped at PID18 to 2.23 ± 0.97 g. Threshold values for the NOK group maintained at 5.71 ± 0.81 g on PID18 compared to its baseline at 5.46 ± 0.57 g, while the DMEM sham-injected group measured at 5.38 ± 1.08 g compared to its baseline at 4.43 ± 1.02 g.

Intra-tumor antagonist administration and nociceptive behavioral testing

To determine if endogenous opioids were involved in the attenuation of carcinoma pain with ET-A receptor antagonist we tested whether opioid receptor (OR) antagonists could reverse the antinociceptive effect. ET-A receptor antagonism with 92 ng/kg BQ-123 significantly attenuated carcinoma-induced nociception (Fig. 4). The threshold value for the PBS control group was 2.11 ± 0.43 g at baseline, 1.74 ± 0.24 g at t = 15 min, and 1.85 ± 0.15 g at t = 3 hrs post-injection. Threshold values for the BQ-123 group were 1.18 ± 0.22 g at baseline, 1.91 ± 0.22 g at t = 15 min, and 2.24 ± 0.64 g at t = 3 hrs post-injection. Since ET-A receptor antagonism attenuated carcinoma-induced nociception for up to 3 hours post-injection (Fig. 4), OR antagonists were administered at the midpoint time, immediately after the 90 min post BQ-123 recording, to evaluate the nociceptive effect of an opioid antagonist on paw withdrawal thresholds. BQ-123 (92

ng/kg) was first injected to establish the antinociceptive behavioral response to ET-A receptor antagonist (Fig. 5, up to 90 min post-injection). Administration with a non-selective opioid receptor antagonist naloxone methiodide (500 μg/kg) decreased paw withdrawal threshold (Fig. 5a) at 30 minutes after inoculation relative to drug vehicle control (PBS), lasting no more than 45 minutes. Threshold values for the PBS control group were 1.82 ± 0.37 g at baseline before BQ-123 injection, 3.47 ± 0.70 g at t = 90 min immediately prior to PBS injection, and 2.16 ± 0.34 g at t = 125 min approximately 30 min after PBS injection, compared to the naloxone methiodide-injected group which measured 1.67 ± 0.59 g, 2.40 ± 0.83 g, and 0.84 ± 0.45 g, respectively. Selective μ-opioid receptor antagonist CTOP (500 μg/kg) immediately reversed antinociception (Fig. 5b, $p < 0.001$ at t = 100 min), but rapidly dissipated within 10 min. Threshold values for the PBS control group were as detailed above, while the CTOP-injected group measured at 1.70 ± 0.35 g at baseline prior to BQ-123 injection, 3.00 ± 0.56 g at t = 90 min immediately before CTOP injection, and 0.31 ± 0.17 g at t = 100 approximately 5 minutes after CTOP administration. Specific δ-opioid receptor antagonist naltrindole (11 mg/kg) also significantly reduced paw withdrawal thresholds post-injection (Fig. 5c), lasting approximately 45 minutes. Actual threshold values for the naltrindole-injected group were 1.98 ± 0.35 g at baseline before BQ-123 injection, 2.85 ± 0.87 g at t = 90 min immediately prior to naltrindole injection, 1.36 ± 0.49 g at t = 100 min about 5 minutes after naltrindole administration, and 0.93 ± 0.31 g at t = 125 min approximately 30 minutes following naltrindole. In order to achieve selective inhibition, tumor animals were pretreated with κ-opioid receptor antagonist nor-binaltorphimine (2.5 mg/kg) 12 hours before injection with BQ-123. nor-BNI had no effect on paw withdrawal

thresholds compared to BQ-123 injection (Fig. 5d). Threshold values for the nor-BNI-treated group were 1.78 ±0.59 g at baseline before BQ-123 injection, 2.63 ± 0.22 g at t = 15 min after BQ-123 administration, and 2.18 ± 0.33 g at t = 3 hrs following BQ-123.

Discussion

In the current study we demonstrated that endogenous opioids are responsible for the attenuation of carcinoma pain with ET-A receptor antagonist BQ-123. The non-selective opioid antagonist naloxone methiodide, as well as the selective μ-opioid and δ-opioid receptor antagonists blocked antinociception induced by ET-A receptor antagonist in the mouse model of cancer pain. Our *in vitro* experiments demonstrated that secretion of β-endorphin and leu-enkephalin neuropeptides are induced upon cell culture treatment with BQ-123. These results confirm that carcinoma is capable of producing antinociception through the release of opioid peptides in the cancer microenvironment.

ET-1 is abundantly expressed by a number of carcinomas, including prostate [37], lung [48], breast [2] colorectal [4] and oral.[46] We have demonstrated increased levels of both ET-1 protein and transcript in oral SCC.[52] Endothelin-A receptor is one of two G-protein coupled receptor (GPCR) [3] isotypes that bind endothelin-1 (ET-1). ET-A receptors are localized to the oral SCC cell membrane [59]. In the current study we found that expression levels of the receptor are reduced two-fold compared to NOK. ET-AR is a G-protein coupled receptor associated with many different downstream signal transduction cascades, including protein kinase C, phosphatidylinositol-3-kinase, and mitogen-activated protein kinase.[34] Binding to ET-1 leads to ET-A receptor phosphorylation by the G-protein-coupled receptor kinase type 2 (GRK2) [24], internalization and recycling back to cell membrane surfaces.[10] Increased ET-1 may activate feedback inhibition to downregulate ET-A receptor expression similar to the negative feedback regulation of glucocorticoid receptors by glucocorticoid-induced peroxisome proliferator-activated receptor alpha (PPARα).[15]

ET-A receptor antagonist BQ-123 treatment in HSC-3 significantly increased secretion of β-endorphin and leu-enkephalin peptides. BQ-123 concentrations were selected for expected optimal effects based on its reported IC_{50} of 22 nM.[62] The higher BQ-123 concentration at 10^{-5} M had no significant effect, which may reflect a maximal effective dose for β-endorphin production. The same high concentration of BQ-123 has been shown to have no effect on maximal constrictive properties of porcine vascular smooth muscle cells.[32] BQ-123 induced leu-enkephalin production in a dose-dependent manner. While endogenous opioids are commonly produced by neuronal cells, other cell types are also capable of making opioids, such as leukocytes [11], heart and skeletal muscle cells, visceral lining epithelial cells[21], and skin keratinocytes.[35] Furthermore a number of cancers have been demonstrated to produce opioids, including malignant melanoma, benign melanocytic naevi [45], small cell lung carcinoma [42], and ovarian tumors. [48] Epidermoid carcinoma cells and human foreskin keratinocytes are shown to produce proopiomelanocortin (POMC), the precursor for melanotropic, corticotropic and opioid peptides [58], but opioid production and secretion by an oral carcinoma have not been previously demonstrated. Opioids secreted by these non-neuronal cells have potentially similar functions as peptides with neural origin. β-endorphins derived from leukocytes can enhance inhibition of inflammatory pain in both humans and animals.[39,55] Results from in vitro and our in vivo mouse model demonstrate that opioids secreted by carcinoma cells likely contribute to attenuation of cancer nociception induced by BQ-123. Intratumor injections with specific opioid receptor antagonists led to reversal of antinociception from ET-A receptor antagonist treatment. The mechanism by which ET-A receptor antagonism leads to endogenous

opioid production and secretion in carcinoma cells is unclear; however, ET-A receptors are coupled to G-proteins that affect multiple downstream signaling pathways. Moreover endothelin-A and B receptors are capable of forming homo- and hetero-dimers through linking opposing ends of the ET-1 ligand.[30] By antagonizing the ET-A receptors with BQ-123 synthetic peptides, ET-B receptors dissociate from the hetero-dimer and bind to ET-1 with a nine-fold increased affinity.[30] ET-B receptor activation in skin keratinocytes have been shown to elevate β-endorphin production.[35] Dissociated ET-B receptors in oral carcinoma cells might be more readily activated by ET-1, which is produced in abundance by carcinoma cells, leading to opioid secretion in the cancer microenvironment. Thus ET-A receptor antagonism indirectly leads to increase in endogenous opioid production through subsequent actions from the ET-B receptors.

In addition to ET-A receptor mediated opioid secretion from the carcinoma in the cancer microenvironment, ET-AR antagonism on the nerve terminal might directly modulate the opioid receptor. Previous studies have shown that ET-A receptor antagonism potentiates morphine analgesia and inhibits morphine tolerance [8,6,28,7]. ET-A receptor antagonism is thought to improve peripheral opioid receptor function by preventing G-protein uncoupling at the cytoplasmic tail.[7] Like the ET-A receptors, opioid receptors are also G-protein coupled receptors with seven transmembrane domains linked by alternating intracellular and extracellular loops.[38] There are four major opioid receptors cloned, namely μ-, δ-, κ-, and nociceptin/orphanin FQ receptors (opioid receptor-like 1 [ORL1]).[50] The G-proteins consist of three subunits – α, β, and γ, of which there are 17 genes encoding the α, 5 genes encoding the β, and 12 genes encoding the γ subunit.[47] Opioid receptor functions through G-proteins coupled on the

cytoplasmic tail of the receptor and uncoupling of the G-protein represents a state of tolerance.[17] By preventing G-protein uncoupling with ET-AR antagonists, opioid receptors might maintain active signaling at the nerve terminal to attenuate carcinoma nociception.

Endothelin-A receptor antagonists clearly demonstrate potentials for modulating cancer pain.[19,65,51,64,59,25,29] Given that peripheral administration of ET-A receptor antagonist produces antinociception through potentiation of endogenous opioids in the cancer model we studied, a drug targeting peripheral ET-A receptors could provide relief for cancer patients without complications related to opiate tolerance and withdrawal. These desirable effects of ET-A receptor antagonist show promise for management of cancer pain and may lead to improved analgesic therapy.

Acknowledgments

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FIGURES AND FIGURE LEGENDS

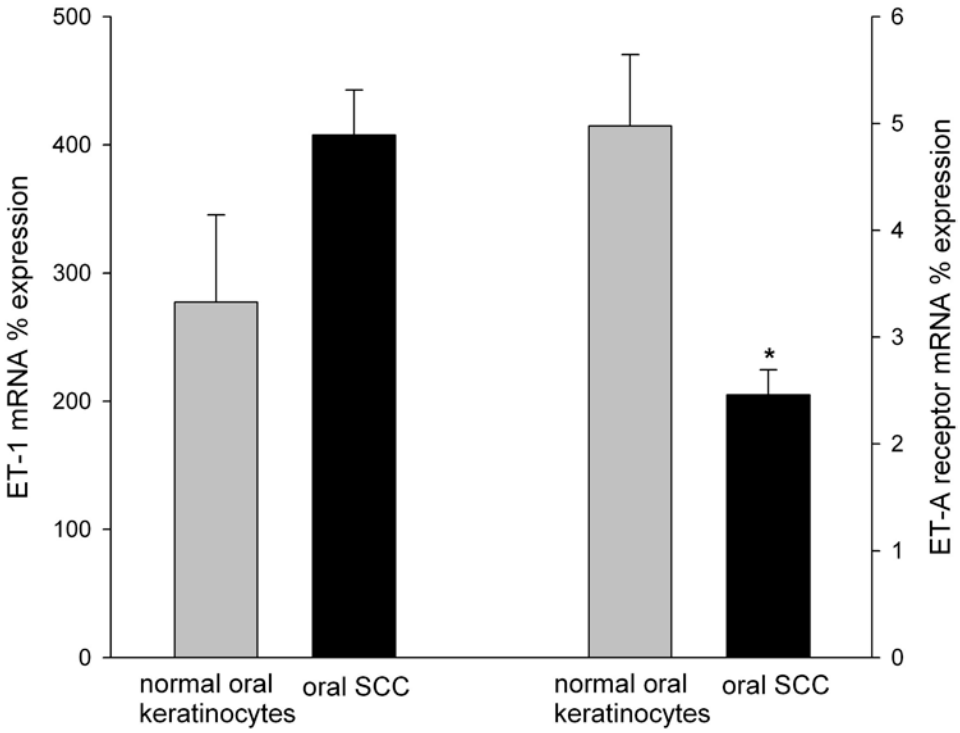
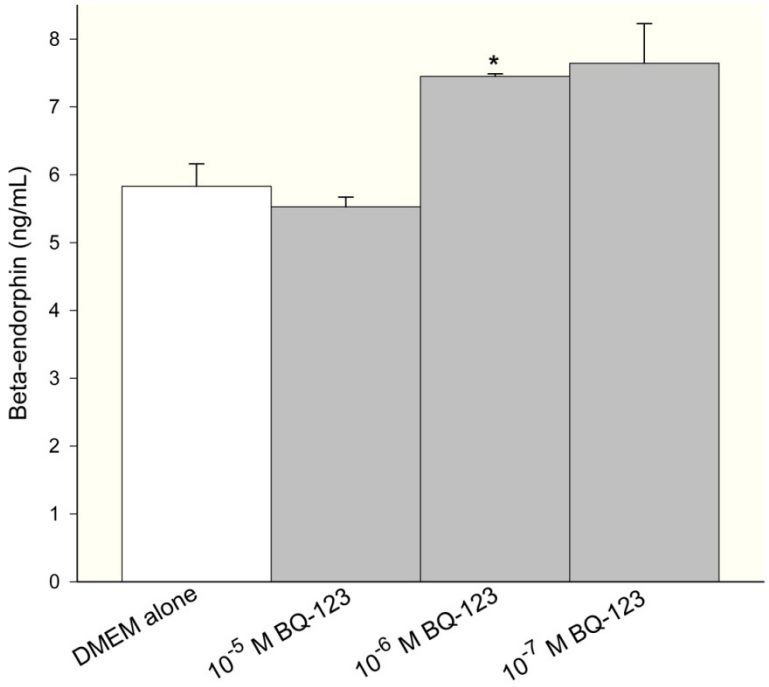


Figure 1.

Mean ET-1 and ET-A receptor mRNA percent expression in NOK (n = 3) and oral SCC (n = 3) cell cultures normalized to the respective β-GUS mRNA levels. ET-AR mRNA expression was significantly lower in oral SCC relative to NOK control ($p = 0.024$). [* indicates significance]

Figure 2.

a


Beta-endorphin (ng/mL)
0
1
2
3
4
5
6
7
8
*
DMEM alone
10-5 M BQ-123
10-6 M BQ-123
10-7 M BQ-123

b

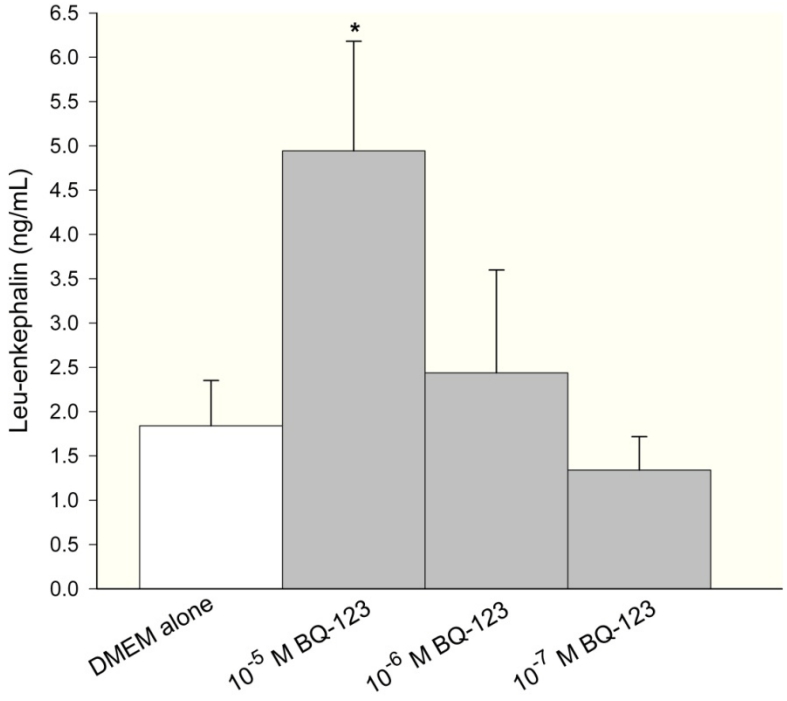

Leu-enkephalin (ng/mL)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
*
DMEM alone
10-5 M BQ-123
10-6 M BQ-123
10-7 M BQ-123

Figure 2.

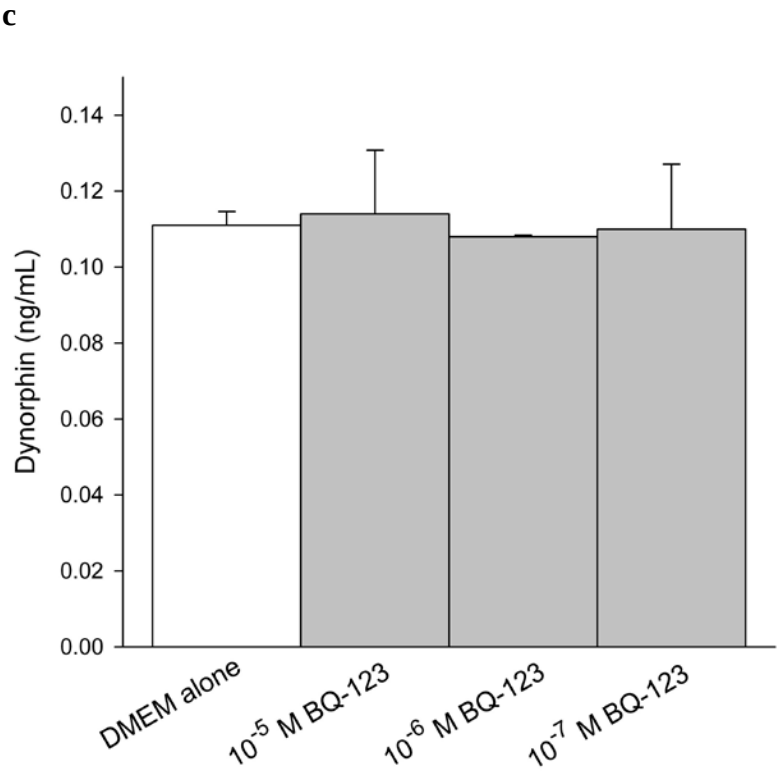


Figure 2.

ELISA quantification of endogenous opioid concentration in SCC conditioned media under different ET-A receptor antagonist (BQ-123) treatment. (A) β-endorphin level was significantly increased when treated with 10^{-6} M BQ-123 compared to DMEM control (*p* = 0.033) (n = 3). (B) Leu-enkephalin level responded in a dose-dependent manner to BQ-123 treatment, with a significant increase at 10^{-5} M BQ-123 (p = 0.035) and a significant decrease at 10^{-8} M BQ-123 (*p* = 0.036) compared to DMEM control (n = 3). (C) Dynorphin level was not affected by BQ-123 incubation compared to DMEM control (n = 3). [* indicates significance]

Figure 3.

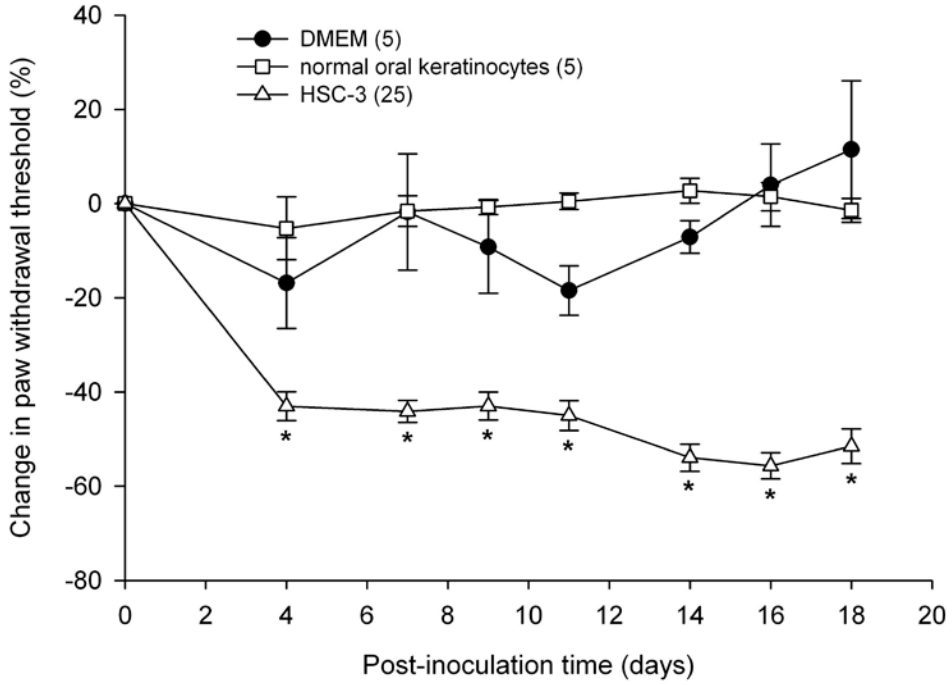


Figure 3.

Mean percent change in paw withdrawal threshold of the right hind paws of the HSC-3 group (n = 25), NOK group (n = 5) and sham-injected group (n = 5). Paw withdrawal thresholds in the oral SCC (HSC-3 cells) group significantly decreased starting at post-inoculation day 4 and maintained throughout until day 18 ($p < 0.05$) compared to both NOK and sham-injected groups. The NOK group was not significantly different from the sham-injected group. [* indicates significance]

Figure 4.

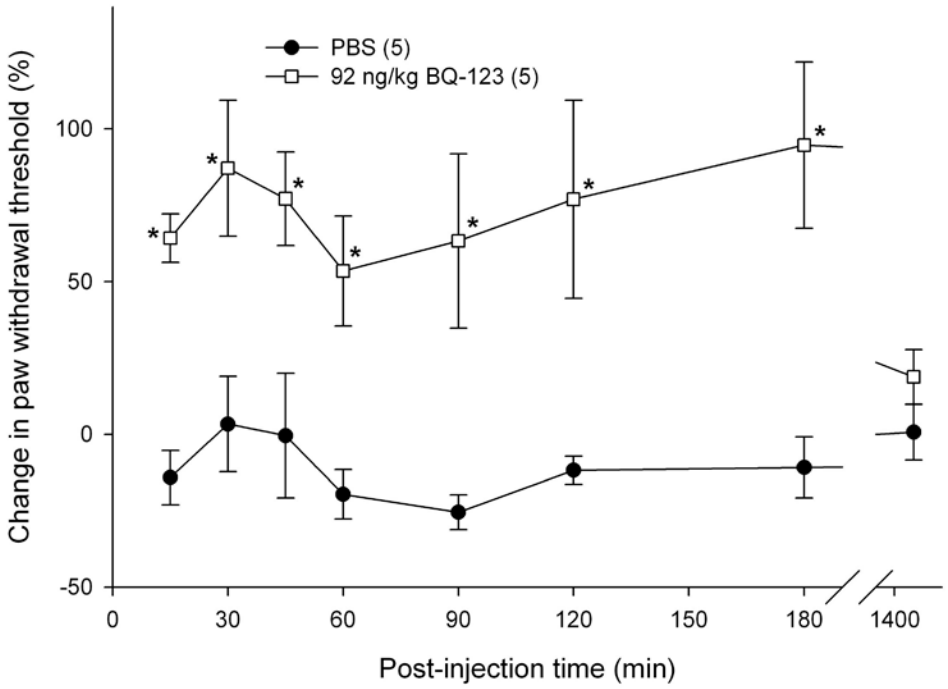
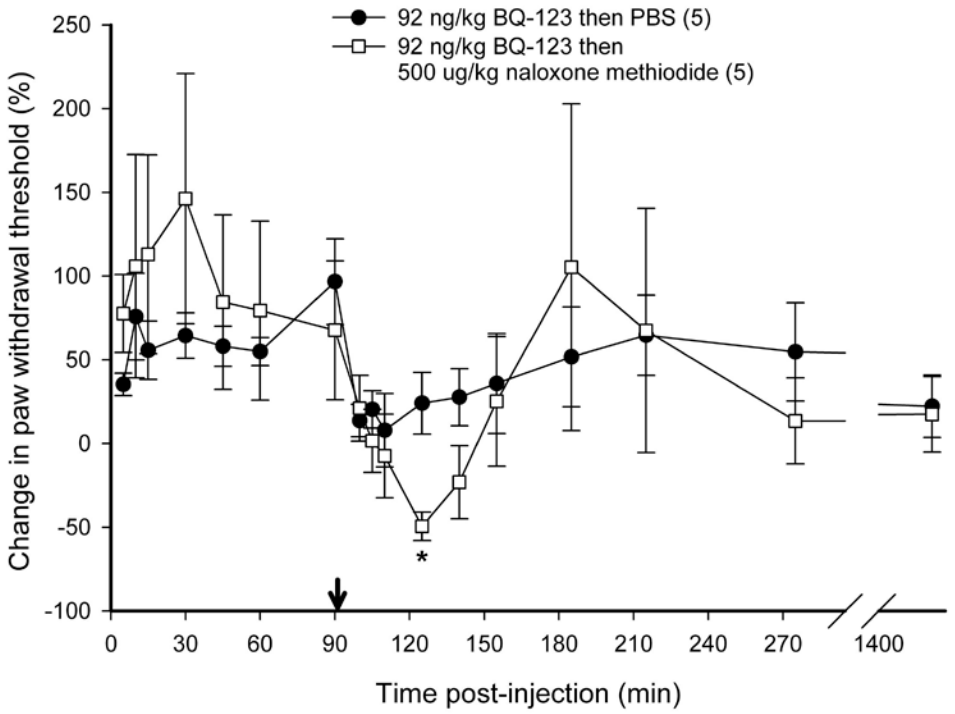


Figure 4.

Mean percent change in paw withdrawal threshold of the right hind paws of cancer-inoculated animals injected with either ET-AR antagonist (92 ng/kg BQ-123) or drug vehicle alone (PBS). BQ-123 attenuated carcinoma-induced nociception compared to PBS control in cancer-inoculated animals. Thresholds significantly increased at t = 15 min after drug injection and lasted up to 3 hrs post-injection ($p < 0.05$). [* indicates significance]

Figure 5.

a


92 ng/kg BQ-123 then PBS (5)
92 ng/kg BQ-123 then
500 ug/kg naloxone methiodide (5)
Change in paw withdrawal threshold (%)
250
200
150
100
50
0
-50
-100
0
30
60
90
120
150
180
210
240
270
1400
*
Time post-injection (min)

b

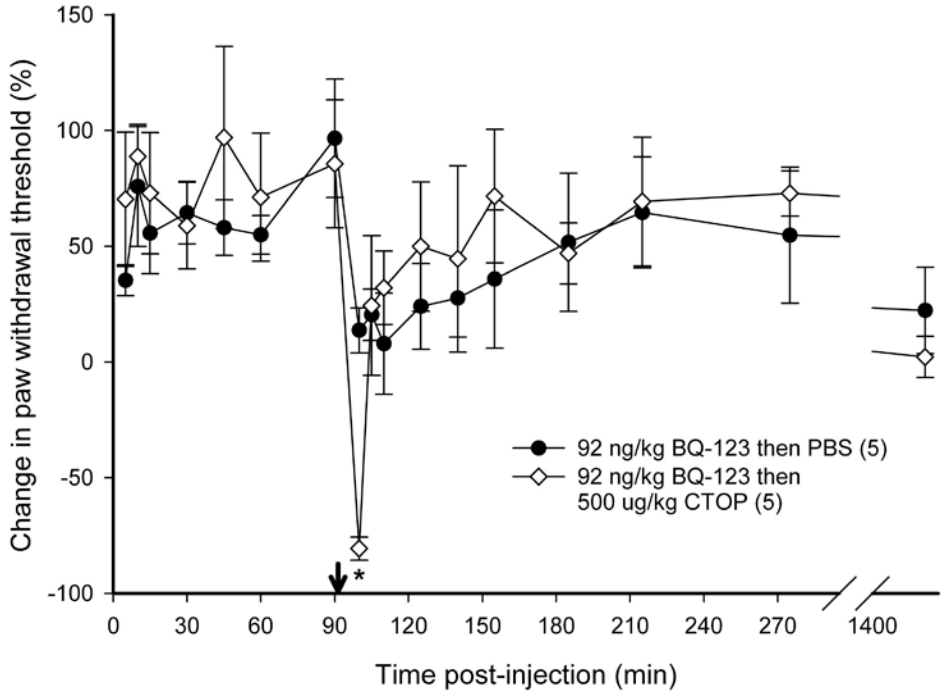
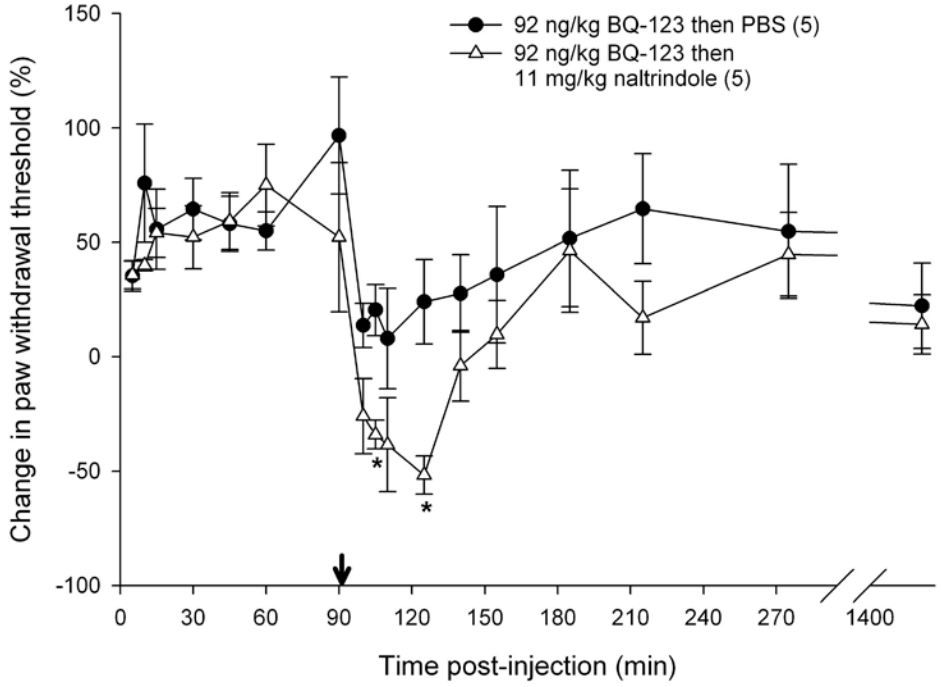

Change in paw withdrawal threshold (%)
150
100
50
0
-50
-100
92 ng/kg BQ-123 then PBS (5)
92 ng/kg BQ-123 then
500 ug/kg CTOP (5)
0
30
60
90
120
150
180
210
240
270
1400
*
Time post-injection (min)

Figure 5.

c


92 ng/kg BQ-123 then PBS (5)
92 ng/kg BQ-123 then
11 mg/kg naltrindole (5)
Change in paw withdrawal threshold (%)
Time post-injection (min)
150
100
50
0
-50
-100
0
30
60
90
120
150
180
210
240
270
1400
*
*

d

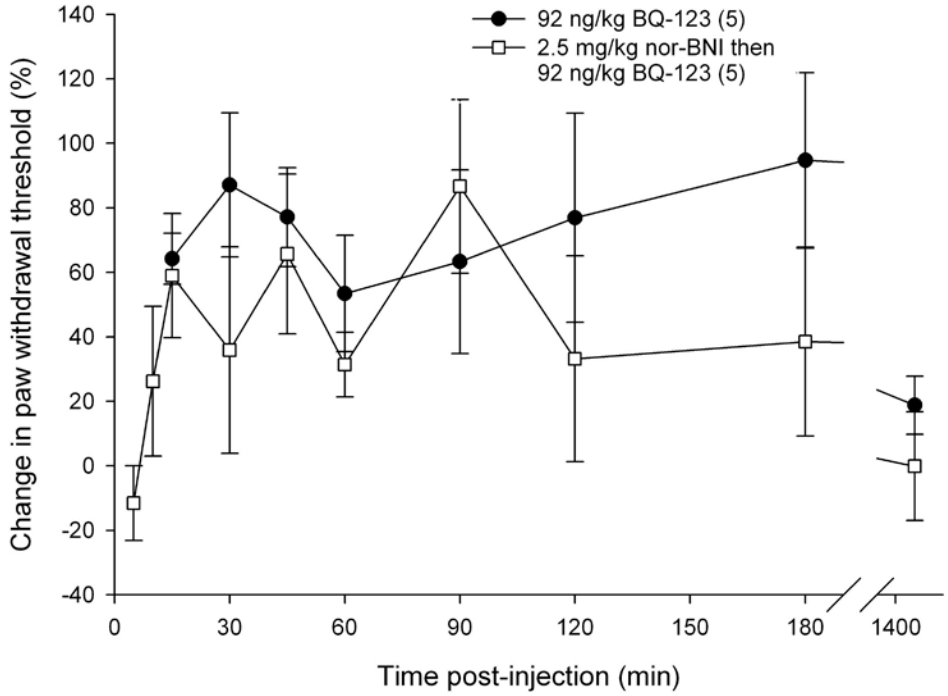

92 ng/kg BQ-123 (5)
2.5 mg/kg nor-BNI then
92 ng/kg BQ-123 (5)
Change in paw withdrawal threshold (%)
Time post-injection (min)
140
120
100
80
60
40
20
0
-20
-40
0
30
60
90
120
150
180
1400

Figure 5.

Mean percent change in paw withdrawal threshold of the right hind paws of cancer-inoculated animals injected with ET-AR antagonist (92 ng/kg BQ-123) followed by administration of different peripheral opioid antagonist drug or PBS alone (indicated by ↓). **(a)** Paw withdrawal for SCC group which received naloxone methiodide at 90 min after BQ-123 injection (n = 5) compared to PBS control group (n = 5). The mean withdrawal threshold for the naloxone methiodide group had a significant negative change at about 35 min following injection of the peripheral non-specific opioid receptor antagonist (t = 125 min), compared to the PBS control group ($p = 0.007$). **(b)** Paw withdrawal for SCC group which received CTOP at 90 min after BQ-123 injection (n = 5). CTOP injection resulted in an immediate but short-lived significant reduction of the mean paw withdrawal threshold ($p < 0.001$) at t = 100 min compared to the SCC group which received PBS control administration. **(c)** Paw withdrawal for SCC group which received naltrindole at 90 min after BQ-123 injection (n = 5). The mean paw withdrawal threshold for the naltrindole group had significant negative changes at t = 105 and 125 min compared to PBS control administration ($p = 0.003$ and 0.006, respectively). **(d)** Paw withdrawal for SCC group which received nor-binaltorphimine at 12 hrs prior to BQ-123 injection (n = 5). nor-BNI administration had no significant effect on the mean paw withdrawal threshold compared to the PBS control group. [* indicates significance]

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CHAPTER 4

PERIPHERAL ENDOTHELIN B RECEPTOR AGONIST-INDUCED ANTINOCICEPTION INVOLVES ENDOGENOUS OPIOIDS IN MICE

Abstract

Endothelin-1 (ET-1) produced by various cancers is known to be responsible for inducing pain. While ET-1 binding to ETAR on peripheral nerves clearly mediates nociception, effects from binding to ETBR are less clear. The present study assessed the effects of ETBR activation and the role of endogenous opioid analgesia in carcinoma pain using an orthotopic cancer pain mouse model. mRNA expression analysis showed that ET-1 was nearly doubled while ETBR was significantly down-regulated in a human oral SCC cell line compared to normal oral keratinocytes (NOK). Squamous cell carcinoma (SCC) cell culture treated with an ETBR agonist (10^{-4}M, 10^{-5} M, and 10^{-6} M BQ-3020) significantly increased production of β-endorphin without any effects on leu-enkephalin or dynorphin. Cancer inoculated in the hind paw of athymic mice with SCC induced significant pain, as indicated by reduction of paw withdrawal thresholds in response to mechanical stimulation, compared to sham-injected and NOK-injected groups. Intratumor administration of 3 mg/kg BQ-3020 attenuated cancer pain by approximately 50% up to 3 hours post-injection compared to PBS-vehicle and contralateral injection, while intratumor ETBR antagonist BQ-788 treatment (100 and 300 μg/kg and 3 mg/kg) had no effects. Local naloxone methiodide (500 μg/kg) or selective μ-opioid receptor antagonist (CTOP, 500 μg/kg) injection reversed ETBR agonist-induced antinociception in cancer animals. We propose that these results demonstrate that peripheral ETBR agonism attenuates carcinoma pain by modulating β-endorphins released from the SCC to act on peripheral opioid receptors found in the cancer microenvironment.

Introduction

Pain is a frequent and disabling consequence of many types of cancers in humans. It is a primary determinant of a poor quality of life, especially in head and neck cancer patients.[10] Eighty-five percent of cancer patients experience severe pain in their final days of life [55] and up to ninety percent of those in terminal stages must cope with opiate-resistant pain related to tumor progression.[37,38,48,51] The etiology of cancer pain is unknown, but may involve mediator-dependent signaling by cancer cells to primary afferent sensory neurons in the cancer microenvironment. One candidate mediator is endothelin-1 (ET-1) [44,54,45], a vasoactive 21-amino acid peptide first isolated from porcine aortic endothelial cells.[59] ET-1 is a member of the endothelin family, which includes ET-2, ET-3, and the sarafotoxins [29,33], and is synthesized from its precursor, pre-pro ET-1, through a proteolytic cleavage by endothelin converting enzyme (ECE).[25] The physiological actions of endothelin are mediated by two G-protein coupled receptors (GPCRs), endothelin A (ETAR) and endothelin B (ETBR), which have been successfully cloned in mammals.[3,52]

ET-1 is synthesized by neurons and glial cells in the central and peripheral nervous systems [23,34] and may serve as an algogen to induce nociception. Studies in animals have demonstrated that injection of ET-1 evokes tactile allodynia [6], hyperalgesia from thermal [17] and mechanical [20] stimulation, and overt inflammatory nociception [20,18,47]. ET-1 also causes pain in humans through activation and sensitization of C nociceptors.[26,40] In addition to mediating nociception in noncancerous conditions, ET-1 also plays a role in cancer pain. ET-1 is highly expressed in different cancers, including bone [57,44], lung [1], colorectal [4], breast [2], prostate

[41] and oral squamous cell carcinoma.[46] Furthermore, it has been demonstrated that treatment with selective ETAR antagonists attenuate ET-1 mediated pain associated with cancer [15,17,9,44,61,54,22,45], specifically by antagonizing ETARs present on primary afferent nociceptors.[16,24]

Whereas the role of ETAR in ET-1-induced pain is well characterized, the importance of ETBR for this pain is less clear. In rats, ETBR is believed to mediate mechanical hypernociception via cAMP formation and activation of a PKC-dependent phosphorylation cascade.[14] ETBR activation also elicits orofacial mechanical allodynia in rats with trigeminal neuralgia.[12] On the other hand, Khodorova and colleagues have demonstrated that selective activation of ETBR on normal skin keratinocytes stimulates release of β-endorphins [31] and further showed that ETBR agonism in rats inhibits ET-1-induced nociception in a naloxone-sensitive manner [30] involving an endogenous opioid-mediated analgesic cascade. Squamous cell carcinoma consists of malignant keratinocytes. Theoretically, the generation of peripheral opioids by carcinoma within the cancer microenvironment immediately adjacent to the sensitized afferent nociceptors is the ideal targeted approach for abrogating cancer pain. Since activation of ETBR on squamous cells modulates pain through endogenous opioid analgesia, we hypothesized that ETBR activation might modulate squamous cell carcinoma-induced pain. Therefore, in the present study, we investigated the effects of ETBR agonism on carcinoma-induced nociception, and whether these effects are dependent on opioid receptor functions.

Materials and Methods

2.1 Cell culture

HSC-3, an oral SCC cell line (ATCC, Manassas, VA) derived from a human tongue SCC, was cultivated in Dulbecco's Modification of Eagle's Medium (DMEM) with 4.5 g/L glucose, L-glutamine and sodium pyruvate, supplemented with 10% fetal bovine serum, 25 μg/mL fungizone, 100 μg/mL streptomycin sulfate, and 100 units/mL penicillin G. Primary normal oral keratinocytes (NOK) were harvested from normal gingival tissues using a modified technique described by Hybbinette et al.[28] Collection of oral epithelium was approved by the UCSF Committee on Human Research and consent was obtained from patients. Tissues were washed in 70% sterile ethanol, cut into 5 mm square sections, and incubated in 0.25 mg/mL dispase I at 37^{o}C for approximately 3 hrs. The separated epidermis was minced and transferred to 0.25% trypsin for 5 min incubation at 37^{o}C, then stopped with 0.25-0.5 mg/mL soybean trypsin inhibitor (Invitrogen, Carlsbad, CA) and centrifuged at 1300 rpm for 8 min. The sedimented cells were resuspended in Defined Keratinocyte Serum-free media (Invitrogen, Carlsbad, CA) supplemented with 50 μg/mL gentamicin and 25 μg/mL fungizone and cultivated at 37^{o}C in 5% CO_2.

2.2 Real-time quantitative RT-PCR in cell culture

Because ETBRs have not been previously quantified in oral SCC we used RT-PCR to quantify and compare transcript expression levels of ET-1 and ETBR in oral SCC and normal oral keratinocytes. ET-1 and ETBR mRNA expression levels were measured

in the HSC-3 cell line relative to the normal oral keratinocyte control (NOK). 10^4 cells were cultivated in 300 μL of DMEM supplemented with 10% fetal bovine serum, 25 μg/mL fungizone, 100 μg/mL streptomycin sulfate, and 100 units/mL penicillin G on 96-well cell culture plates until 75%-80% confluent. Cells were then harvested and lysed for quantitative PCR analysis using the TaqMan® Gene Expression Cells-to-CT Kit (Applied Biosystems/Ambion, Austin, TX), performed at the Genome Analysis Core (University of California, San Francisco). Samples were run on an ABI 7700 Prism (PE Biosystems, Foster City, CA). Relative expressions of ET-1 and ETBR mRNA were calculated using the comparative Ct method as previously described.[13,54] Analysis was carried out using the software supplied with the ABI 7700 Prism. Overexpression was defined as expression >2.0 relative to the reference gene β-*N*-acetyl-glucosaminidase (β-Gus).

2.3 Immunofluorescence

Immunofluorescence was performed to validate the presence of ETBR in oral SCC. HSC-3 cells were seeded onto glass cover slips in 6-well plates overnight at 37°C with 5% CO_2 in supplemented DMEM (see above). Cells were washed twice with PBS, fixed in ice-cold acetone for 5 min at room temperature (RT), permeabilized with 0.2% TritonX-100 for 15 min, washed three times with PBS then nonspecifically blocked with 3% bovine serum albumin (BSA) for 2 hrs. Incubation with primary rabbit polyclonal ETBR antibody (Abcam Inc., Cambridge, MA) diluted 1:100 in 3% BSA was performed at RT for 2 h followed by incubation with donkey anti-rabbit Texas Red-conjugated IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1:500 in 3% BSA for 1 h at RT. Nuclei were stained with 1:500 Hoechst stain

(Invitrogen, Carlsbad, CA). The cover slips were washed and mounted on slides in Gel/Mount^{TM} mounting medium (Biomeda Corp., Foster City, CA) and visualized on a Nikon Eclipse E600 microscope using epifluorescence. All images were captured and analyzed with RT Spot Software (Diagnostics Instruments, Inc., Sterling Heights, MI). Omission of the primary antibody was used as controls for the immunofluorescence.

2.4 ELISA measurement of endogenous opioids

To evaluate the effect of ETBR agonism on opioid production and secretion in oral SCC, we used enzyme-linked immunosorbent assay (ELISA) for opioid peptide measurement. 10^5 HSC-3 cells were seeded onto 6-well tissue culture plates with 3 mL of DMEM supplemented with 10% fetal bovine serum, 25 μg/mL fungizone, 100 μg/mL streptomycin sulfate, and 100 units/mL penicillin G. HSC-3 cells were cultured for 24 h until the wells reached 70-80% confluence. Each well was washed once with PBS then incubated for 12 h in 1 mL of one of the following media: 1) DMEM alone, 2) DMEM with 10 ng/mL synthetic beta-endorphin (Sigma, St. Louis, MO) or leu-enkephalin (Phoenix Pharmaceuticals, Burlingame, CA), or 3) DMEM with one of ten-fold concentrations of BQ-3020 (10^{-4}M to 10^{-9}M, American Peptide Co., Sunnyvale, CA). Culture media were collected and treated with 1x HALT Protease Inhibitor Cocktail (Pierce, Rockford, IL) before performing ELISA to detect levels of β-endorphin (MD Biosciences, St. Paul, MN), leucine-enkephalin (leu-enk) and dynorphin (Phoenix Pharmaceuticals, Burlingame, CA). Opioid concentrations were calculated based on a calibration curve, with 55% supernatant recovery for β-endorphin and 12% recovery for

leu-enk. Dynorphin concentrations were calculated with a 1:1 recovery ratio since no synthetic human dynorphin was readily available for recovery test.

2.5 SCC paw model

The cancer pain mouse model was produced as previously described.[54,45] Experiments were performed on 4 weeks-old adult female *Foxn1*^{nu}, athymic, immunocompromised mice weighing 16-20 g at the time of SCC inoculation. Mice were housed in a temperature-controlled room on a 12:12 h light cycle (0700–1900 h light), with *ad libitum* access to food and water; estrous cycles were not monitored. All procedures were approved by the University of California, San Francisco Committee on Animal Research. Researchers were trained under the Animal Welfare Assurance Program. Mice were divided into three experimental groups: those receiving an injection of squamous carcinoma cells (SCC group), those receiving an injection of normal oral keratinocytes (negative control), and those receiving an injection of DMEM (sham operated). All injections were into the right hind paw. All groups were anesthetized by inhalation with 1-3% isofluorane throughout the inoculation procedure. Cell injections consisted of either 10^6 HSC-3 cells (SCC group) or NOK cells (negative control) in a vehicle consisting of 50 μL of DMEM into the plantar surface of the right hind paw. The sham-operated group received 50 μL of DMEM alone.

2.6 Behavioral testing for the SCC paw model

Behavioral testing was performed as described previously.[54] Testing was

performed between 14:00 and 16:00 h (during the light phase). Mice were placed in a plastic cage with wire mesh floor which allowed access to the paws. Quantitative assay guidelines were used similar to a previously described technique.[10] 15 min were allowed for acclimation prior to testing. The probe was applied to the mid-plantar right hind paw, or the tumor-front on the hind paw toward the later stages of tumor development. Paw withdrawal thresholds were determined in response to pressure from an electronic von Frey anesthesiometer (2390 series, IITC Instruments, Woodland Hills, CA). The amount of pressure (g) needed to produce a paw withdrawal response was measured three times on each paw separated by 3 minute intervals to allow resolution of previous stimuli. The results of three tests were averaged for each paw for that day. The SCC, negative control and sham-injected groups were tested under this paradigm at 4, 7, 9, 11, 14, 16, and 18 days post-inoculation of SCC, NOK or vehicle.

2.7 Drug administration and pain behavioral testing

To determine whether agonism or antagonism of ETBR attenuates cancer-induced nociception, groups of tumor-inoculated animals were tested with either ETBR agonist (BQ-3020) or ETBR antagonist (BQ-788). Then to evaluate whether ETBR agonist-induced attenuation of carcinoma pain involves endogenous opioids, opioid receptor (OR) antagonists were administered following BQ-3020 injection. Drug testing was performed on days 18-25 following inoculation of oral SCC into the hind paw. Drugs were dissolved in a final volume of 20 μL PBS and injected subcutaneously into the mid-plantar hind paw at the site of greatest tumor development with a 30-gauge beveled needle. For single drug testing, either BQ-3020 (3mg/kg) or BQ-788 (100-3000 μg/kg)

was injected after a 15 min baseline period and paw withdrawal thresholds were recorded at 5, 10, 15, 30, 45, 60, 90, 120, 180 min, and 24 h post-injection. For dual drug testing, BQ-3020 (3 mg/kg) was injected after a 15 min pre-injection reading and paw withdrawal thresholds were recorded at post-injection times 5, 10, 15, 30, 45, 60, and 90 min. Following the 90 min post-ETBR agonist reading, a second drug (PBS or OR antagonist) was injected and paw withdrawal testing was performed at 5, 10, 15, 30, 45, 60, 90, 120, 180 min, and 24 h. Mice groups were injected with one of the following drug combinations: 1) BQ-3020 (3 mg/kg) followed by PBS control, 2) BQ-3020 followed by nonspecific OR antagonist (500 μg/kg naloxone methiodide, Sigma, St. Louis, MO), 3) BQ-3020 followed by selective μ-OR antagonist (500 μg/kg Cys^2-Tyr^3-Orn^5-Pen^7-amide [CTOP], Sigma, St. Louis, MO), or 4) BQ-3020 followed by selective δ-OR antagonist (11 mg/kg naltrindole [NTI], Sigma, St. Louis, MO). κ-OR antagonist (2.5 mg/kg nor-binaltorphimine [nor-BNI], Sigma, St. Louis, MO) was injected 12 hr prior to injection with PBS control or BQ-3020. The investigators performing the injections and behavioral testing were blinded to the drugs administered.

2.8 Statistical Analysis

A one-way Analysis of Variance (ANOVA) with a Tukey Multiple Comparisons post-test was used to compare the withdrawal threshold of the cancer inoculated mice and sham over 18 days. The same test was used to compare the percent change in withdrawal threshold of the SCC inoculated mice before and after drug or control injection. ELISA protein measurements and mRNA gene expressions were also analyzed with one-way ANOVA. When an Equal Variance Test failed, one-way ANOVA with Dunn's Method

for Multiple Comparison post-test was performed. For all tests a *p* value of less than 0.05 was considered significant. Statistical analysis was performed using SigmaPlot for Windows (Version 11.0).

Results

3.1 ET-1 and ETBR expression

ET-1 and ETBR mRNA expression levels in oral SCC were compared to NOK controls (Fig. 1a) and ETBR localization was visualized in oral SCC with immunofluorescence (Fig. 1b). Expression levels were normalized to the expression of the housekeeping gene β-Gus. ET-1 mRNA expression in SCC cell culture (407.69 ± 68.02%) was nearly two-fold higher than NOK control (277.28 ± 35.09%). ETBR mRNA expression in SCC (0%) was significantly lower ($p = 0.04$, Tukey test) than in NOK control (0.026 ± 0.009%). Although normalized ETBR mRNA expression level was not increased, immunofluorescent labeling revealed ETBR expression on both cell membranes and dispersed in the cytoplasm of SCC cells (Fig. 1b).

3.2 ELISA measurement of endogenous opioids

ELISA was performed on conditioned media of SCC cells to quantify production of endogenous opioids (β-endorphin, leu-enkephalin, and dynorphin). The concentration was calculated from a standard curve using a sigmoid logistics curve fitting program (Bio-Rad Laboratories, Inc., Hercules, CA) as appropriate for the opioid ELISA kits, followed by adjustments for percent recovery from synthetic peptide positive control treatments in culture. SCC cell culture treated with ETBR agonists significantly increased β-endorphin production compared to untreated SCC cultures at 5.38 ± 0.22 ng/mL (Fig. 2a). Treatment with 10^{-4} M BQ-3020 produced 8.02 ± 0.45 ng/mL of β-endorphin ($p = 0.002$, Tukey test); 10^{-5} M BQ-3020 produced 7.69 ± 0.53 ng/mL of β-endorphin ($p = 0.007$, Tukey test); and 10^{-6} M BQ-3020 produced 6.58 ± 0.31 ng/mL of β-endorphin (p =

0.019, Tukey test). ETBR agonist treatment had no effect on production of either leu-enkephalin or dynorphin (Fig. 2b-c).

3.3 ETBR agonist/antagonist effects using the SCC mouse model

To determine whether SCC inoculation induced mechanical hyperalgesia in the mouse cancer model, paw withdrawal thresholds for the SCC (30 mice), NOK (5 mice), and DMEM sham (4 mice) groups were compared. Paw withdrawal thresholds for SCC animals (Fig. 3a) significantly dropped starting on post-inoculation day (PID) 4 and lasted up to PID18 as compared to both NOK and the sham-injected groups ($p < 0.05$, Tukey test or Dunn's Method as appropriate). Either ETBR agonists or antagonists were administered to determine whether agonism or antagonism of the receptor affects carcinoma-induced nociception in the SCC mouse model. Intratumor injection of 3 mg/kg BQ-3020, an ETBR agonist, significantly increased paw withdrawal thresholds at 15 min post-injection and lasted up to 3 h compared to PBS-vehicle and contralateral drug injection ($p < 0.05$,Tukey test or Dunn's Method as appropriate), indicating an attenuation of carcinoma-induced nociception (Fig. 3b). Intratumor injection with ETBR antagonist BQ-788 (100-3000 μg/kg) had no effect on paw withdrawal thresholds (Fig. 3c).

3.4 Non-specific opioid receptor antagonist dosing effect on carcinoma pain

Since SCC is capable of producing endogenous opioids upon ETBR agonist treatment *in vitro*, the contribution of peripheral opioid receptors (OR) to attenuation of cancer pain was investigated *in vivo*. We previously reported that SCC produce elevated

ET-1 peptides [46], which indicate that inoculated SCC tumors are capable of activating ETBR in cancer animals without administration of exogenous ETBR agonists. Relying on endogenous ETBR activation levels, various doses of non-specific peripheral OR antagonist (naloxone methiodide; 5, 50, and 500 μg/kg) were injected either intratumor or in the contralateral paw to evaluate the dosing effect of OR antagonist on cancer-induced nociception. 500 μg/kg naloxone methiodide injected intratumor most effectively decreased paw withdrawal thresholds in cancer animals for up to 60 min post-injection ($p < 0.05$, Tukey test) compared to PBS-vehicle and lower doses of naloxone methiodide (Fig. 4a). All doses of naloxone methiodide treatment in the contralateral paw had no effect on paw withdrawal threshold (Fig. 4b).

3.5 Reversal of attenuation with opioid receptor antagonists

To determine if endogenous opioids were involved in the attenuation of carcinoma nociception with ETBR agonists, we evaluated whether opioid receptor (OR) antagonists could reverse the antinociceptive effect. Since ETBR agonism attenuated carcinoma-induced nociception for up to 3 hours post-injection (Fig. 3b), OR antagonists were administered at the midpoint time, immediately following the 90 min recording, to evaluate its nociceptive effect on paw withdrawal thresholds. 3 mg/kg BQ-3020 was first injected to establish antinociceptive behavioral response to an ETBR agonist (Fig. 5). Administration of non-selective opioid receptor antagonist naloxone methiodide (500 μg/kg) decreased paw withdrawal threshold (Fig. 5a) immediately after injection relative to vehicle control (PBS), lasting about one hour ($p < 0.05$, Tukey test or Dunn's Method as appropriate). Selective μ-opioid receptor antagonist CTOP (500 μg/kg) also reversed

antinociception upon administration (Fig. 5b, $p < 0.05$, Tukey test) and lasted for approximately one hour when compared to PBS control. Specific δ-opioid receptor antagonist naltrindole (11 mg/kg) did not reverse ETBR agonist-induced antinociception (Fig. 5c). In fact, naltrindole appeared to slightly enhance antinociception at 65 min post-injection ($p < 0.05$, Tukey test) but quickly resolved at the next measurement 30 min later. In order to achieve selective κ-opioid receptor inhibition, tumor animals were pretreated with κ-OR antagonist nor-binaltorphimine (2.5 mg/kg nor-BNI) 12 hours before injection with BQ-3020. nor-BNI had no effect on paw withdrawal thresholds compared to BQ-3020 injection (Fig. 5d).

Discussion

These results are evidence that endogenous opioids are important modulators of ETBR agonist-mediated antinociception in a mouse model of cancer pain. Administration of non-selective opioid receptor antagonist naloxone methiodide or selective μ-opioid receptor antagonist CTOP prevented analgesia induced by ETBR agonism. Also our *in vitro* immunoassay demonstrated that the ETBR agonist BQ-3020 induced secretion of β-endorphin in oral SCC cell culture. ETBR activation in carcinoma cells stimulates endogenous opioid production in the tumor microenvironment to act on neighboring primary afferents and modulate cancer pain. Our results in combination with available studies on endothelin suggest dual control of cancer-induced pain, whereby ETAR on nerve terminals mediate nociception[15,17,9,44,61,54,22,45] while ETBR on neighboring cancer cells promote modulation of the nociceptive signal. The binary function of endothelin in nociception is much like its divergent actions in the vasculature, where ETAR mediates vasoconstriction and ETBR mediates vasodilatation, depending on their expression on different vascular cells.[50]

ET-B receptors are G-coupled protein receptors with seven transmembrane domain.[3] Unlike the G proteins associated with ETAR, ETBR predominantly interacts with $G_{\alpha i}1$, $G_{\alpha i}2$, and $G_{\alpha q}/11$.[56] ETBR is expressed by various normal cell types [43], including dorsal root ganglion satellite cells, nonmyelinating ensheathing Schwann cells[47], skin keratinocytes[60,31] , and human gingival keratinocytes.[21] ETBR is also found on different cancer cells, such as melanoma[32], breast carcinoma[2,58], and oral squamous carcinoma cell line SCC25[5]. In the current study, we localized ETBR proteins in cell culture with immunofluorescence but found that ETBR mRNA expression

in our oral SCC cell line, HSC-3, was significantly lower than normal oral keratinocytes after normalizing to the housekeeping gene β-*N*-acetyl-glucosaminidase (β-Gus). *EDNRB* expression in SCC was undetected compared to normal oral keratinocytes. Note that expression levels were normalized to the expression of the housekeeping gene β-Gus to allow direct comparison between the cancer and normal cells. *EDNRB* is still expressed at the basal level similar to that of β-Gus. This may explain why ETBR proteins were detected in our SCC cells under immunofluorescent imaging. Downregulation of the *EDNRB* gene in cancer is not uncommon, for previous findings in melanoma[32], hepatocellular carcinoma[27], and small cell lung carcinoma[11] have similarly reported *EDNRB* downregulation due to promoter hypermethylation. The epigenetic alteration of the tumor suppressor *EDNRB* gene plays an important role in cancer pathogenesis.

Regulation of *EDNRB* expression may also serve as a mechanism for modulating peripheral nociception. Mice with ETBR knockdown specifically in the sciatic nerves have been shown to have increased mechanical hyperalgesia and tactile allodynia.[7] Here, we demonstrated that mechanical allodynia is increased in mice inoculated with an oral SCC cell line (HSC-3) that is characterized with downregulated ETBR mRNA expression and increased ET-1 protein transcripts.[45] Paw withdrawal thresholds significantly dropped starting at PID4 and lasting to PID18. Furthermore, ETBR agonist BQ-3020 treatment in oral SCC increased secretion of β-endorphin peptides. A range of ten-fold concentrations of BQ-3020 were tested and higher doses at 10^{-4} to 10^{-6} M significantly induced β-endorphin levels compared to untreated cells. Endogenous opioids are commonly produced by peripheral neuronal cells to promote opioid analgesia, but other cell types are also capable of making opioids, such as leukocytes[8], visceral

lining epithelial cells[19], skin keratinocytes[31], and even a number of cancers. Malignant melanoma[39], ovarian tumors[42], small cell lung carcinoma[36], and epidermoid carcinoma cells[53] have been reported to produce opioids, but our data indicating opioid production and secretion by an oral SCC is a novel finding. Opioids secreted by non-neuronal cells mediate analgesia in the same manner as their neural-derived counterparts. Studies have shown that β-endorphins produced by leukocytes are responsible for inhibiting inflammatory pain in both humans and animals.[35,49] Our *in vitro* and *in vivo* findings using an orthotopic cancer pain mouse model demonstrate that endogenous opioids are implicated in cancer pain attenuation induced by an ETBR agonist (BQ-3020). These findings also raise the question of whether peripherally-restricted opiate agonists or agents that increase the peripheral concentration of opiates can be used to treat cancer pain. Activation of peripheral μ-opioid receptors produces an antinociceptive effect in a rat neuropathic pain model (Obara et al., *Neuroscience Letters* 2004; 360:85). In patients with inflammatory knee pain selective activation of peripheral opioid receptors produces analgesia (Stein et al., Lancet 1993; 342:321). Selective peripheral receptor activation is controlled by administering low, systemically inactive doses that are unable to cross the blood-brain barrier. These results with peripherally acting opioid agonists have been confirmed in a number of different randomized, controlled studies (Ness TJ, Pain Pract 2001; 1:243). When low dose morphine is administered into an inflamed, sequestered anatomic site which can limit systemic redistribution (e.g. a joint) it is more effective (both magnitude and duration) than when a similar amount of the opioid is administered systemically (Ness TJ, Pain Pract 2001; 1:243).

If peripherally-restricted opioid agonists are capable of producing analgesia there is the question of why peripherally restricted opiate receptor antagonists do not increase pain or decrease the analgesia produced by systemic opioid receptor agonists. Most studies that have looked at the effect of peripherally-restricted opiate receptor antagonists have evaluated the effect of these peripherally-restricted opiate receptor antagonists on gastrointestinal motility. Peripherally-restricted μ opioid receptor antagonists, including alvimopan and methylnaltrexone, have been developed for the treatment of opioid-induced bowel dysfunction, a very common consequence of opioid analgesics in cancer pain (Foss JF (2001) Am J Surg 182:19S–26S,; Yuan CS (2004) J Support Oncol 2:111–122). Clinical data suggest that these peripherally-restricted opioid antagonists successfully treat opioid-induced bowel dysfunction but do not reduce the analgesia produced by the opioid agonists (Foss JF (2001) Am J Surg 182:19S–26S; Schmidt WK (2001) Am J Surg 182:27S–38S; Wolff BG, Michelassi F, Gerkin T, Techner L, Gabriel K, Du W, Wallin BA (2004) Ann Surg 240:728–735; Chamberlain BH (2009), J Pain Symptom Management). These drugs act at the gastrointestinal tract and likely have very low penetration into the diseased tissue causing pain. Moreover, systemic opiate receptor agonists continue to produce analgesia through central mechanisms.

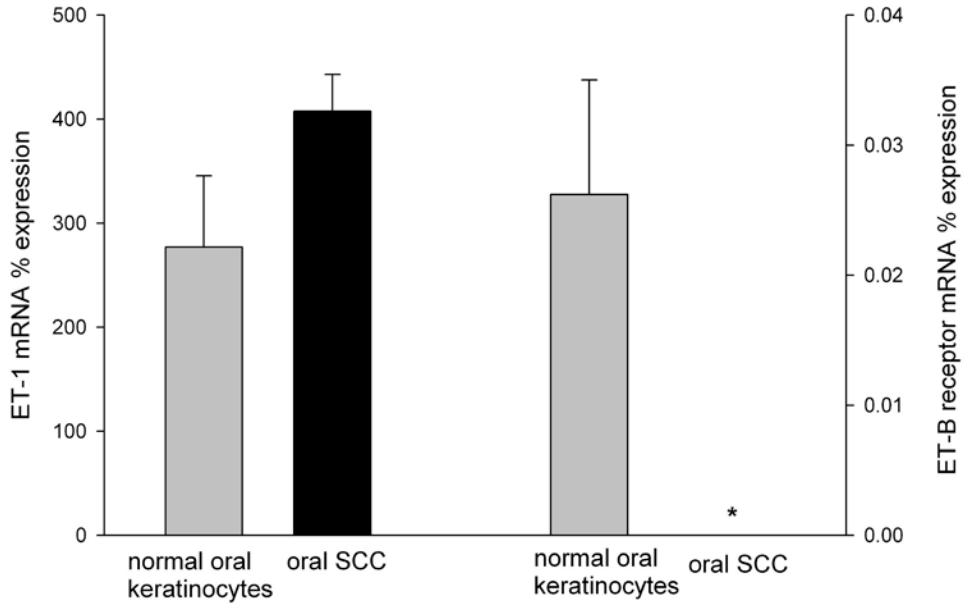
Previous reports have indicated that β-endorphin is involved in ETBR agonist-mediated inhibition of ET-1-induced nociception in both rats and mice.[30,31] Our data further connects the mechanism to cancer-induced nociception since oral SCC is characterized with significantly elevated ET-1 peptides.[54,45] The analgesic effect of ETBR agonism is unlikely to be through sensory fibers, where ETBRs have not been detected[47], but instead is more likely the result of an indirect action mediated by β-

endorphins secreted by oral SCC cells that are stimulated by increased ET-1 in the cancer microenvironment. *In vitro* ELISA data demonstrates that oral SCC is the source for the opioids. It is difficult to determine whether the opioid levels we measured *in vitro* occur *in vivo*. Regardless, the levels of secreted opiates are high enough to induce analgesia as demonstrated by our behavioral studies. Given that carcinoma pain is likely due to hypersensitivity of the nociceptive afferents within the cancer microenvironment the critical result of our study in terms of functional significance is that the carcinoma secretes opioids precisely where they are most likely to have a direct analgesic effect. Currently, there are no clinical trials evaluating the analgesic efficacy of ETBR agonists in patients. The development of drugs which both target peripheral ETBRs within the cancer microenvironment and are free from the complications associated with systemic opioids have significant potential for improved pain management in cancer patients.

FIGURES AND FIGURE LEGENDS

Figure 1.

a



b

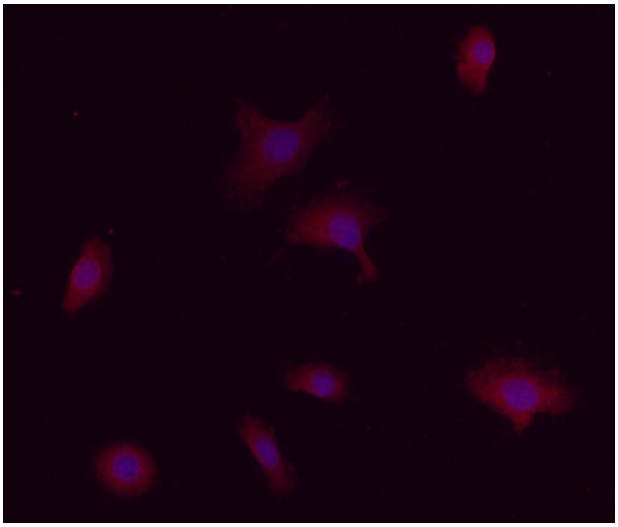
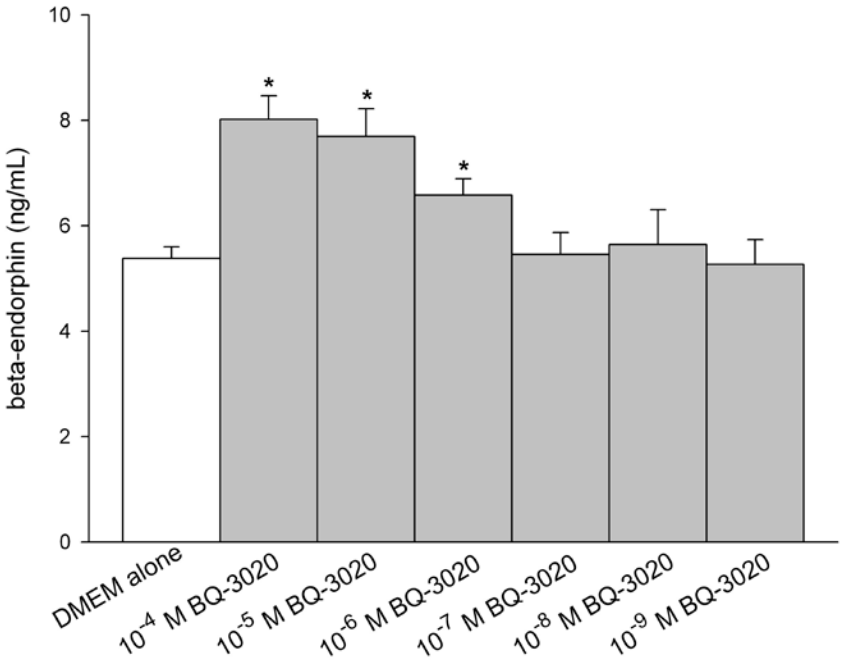


Figure 1.

(a) Mean ET-1 and ETBR mRNA relative percent expression in NOK (n = 3) and oral SCC (n = 3) cell cultures normalized to expression of β-GUS mRNA levels. ETBR mRNA expression was undetectable in oral SCC compared to NOK control ($p = 0.040$, indicated with *). **(b)** Immunofluorescence staining of ETBR in oral SCC cell line (HSC-3). Rabbit polyclonal antibody against ETBR (1:100, Texas Red) shows dispersed staining in the cells. Nuclei are stained with Hoechst dye (1:500, blue).

Figure 2.

a


beta-endorphin (ng/mL)
10
8
6
4
2
0
*
*
*
DMEM alone
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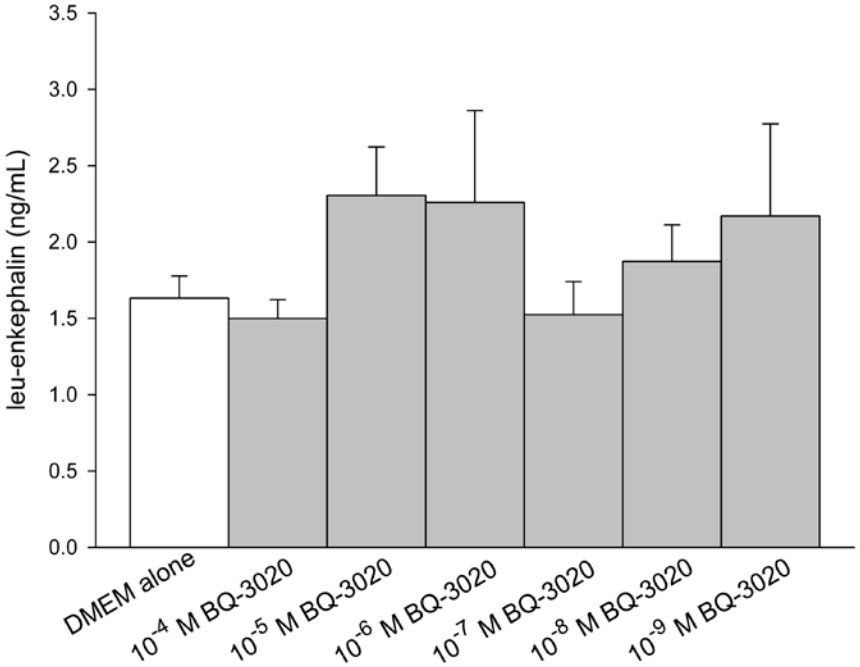
10-5 M BQ-3020
10-6 M BQ-3020
10-7 M BQ-3020
10-8 M BQ-3020
10-9 M BQ-3020

b


leu-enkephalin (ng/mL)
3.5
3.0
2.5
2.0
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1.0
0.5
0.0
DMEM alone
10-4 M BQ-3020
10-5 M BQ-3020
10-6 M BQ-3020
10-7 M BQ-3020
10-8 M BQ-3020
10-9 M BQ-3020

Figure 2.

c

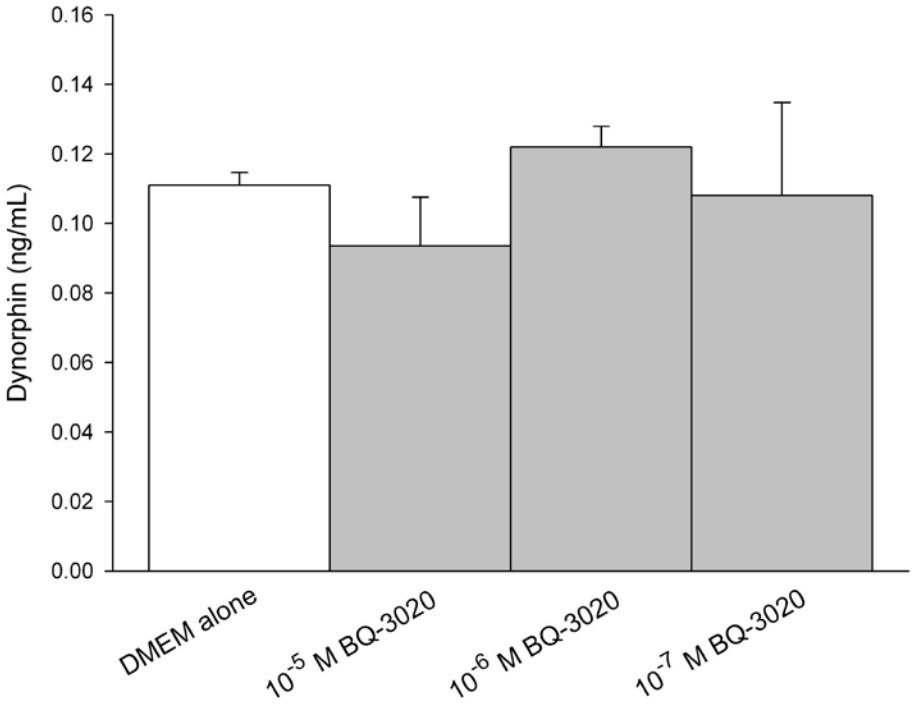
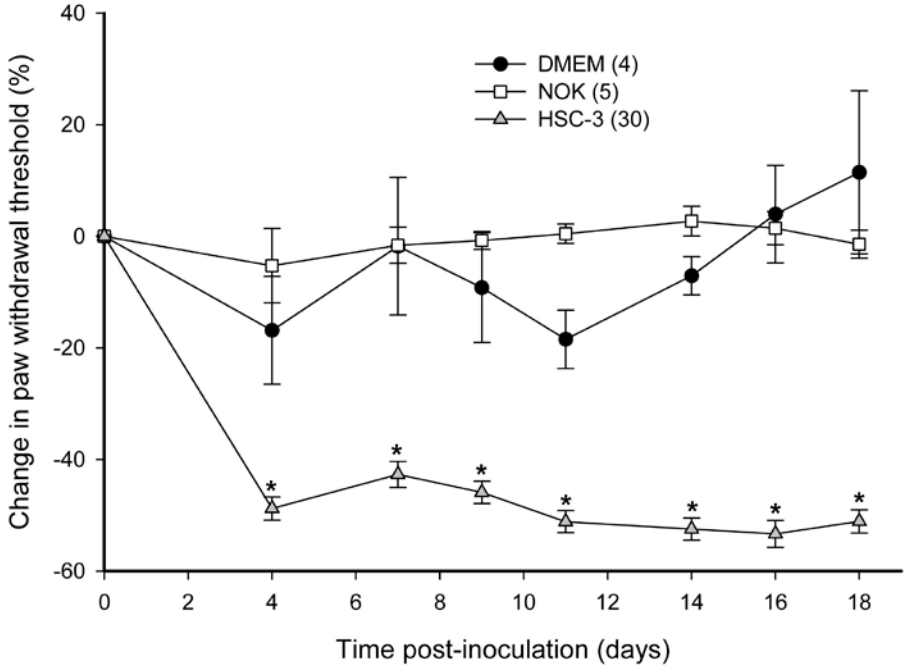


Figure 2.

ELISA quantification of endogenous opioid concentrations in oral SCC conditioned media under different ETBR agonist (BQ-3020) treatment. **(a)** β-endorphin level is significantly increased when treated with 10^{-4} M, 10^{-5} M, and 10^{-6} M BQ-3020 compared to DMEM control ($p = 0.002$, 0.007, and 0.019, respectively), as indicated by an *. **(b-c)** Both leu-enkephalin and dynorphin levels are not affected by BQ-3020 incubation compared to DMEM control.

Figure 3.

a


DMEM (4)
NOK (5)
HSC-3 (30)
Change in paw withdrawal threshold (%)
Time post-inoculation (days)

c

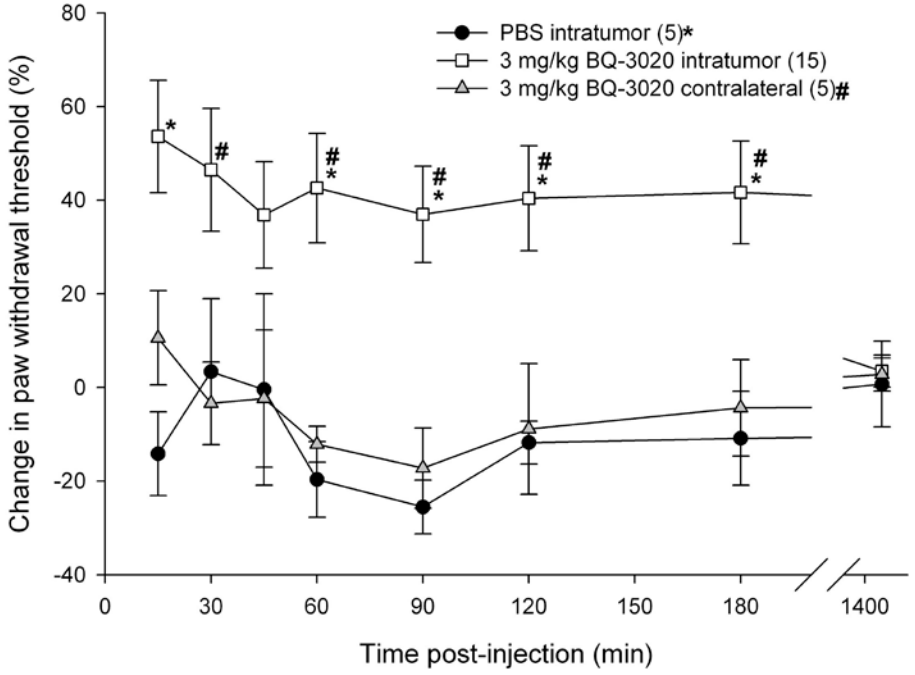

PBS intratumor (5)*
3 mg/kg BQ-3020 intratumor (15)
3 mg/kg BQ-3020 contralateral (5)#
Change in paw withdrawal threshold (%)
Time post-injection (min)

Figure 3.

c

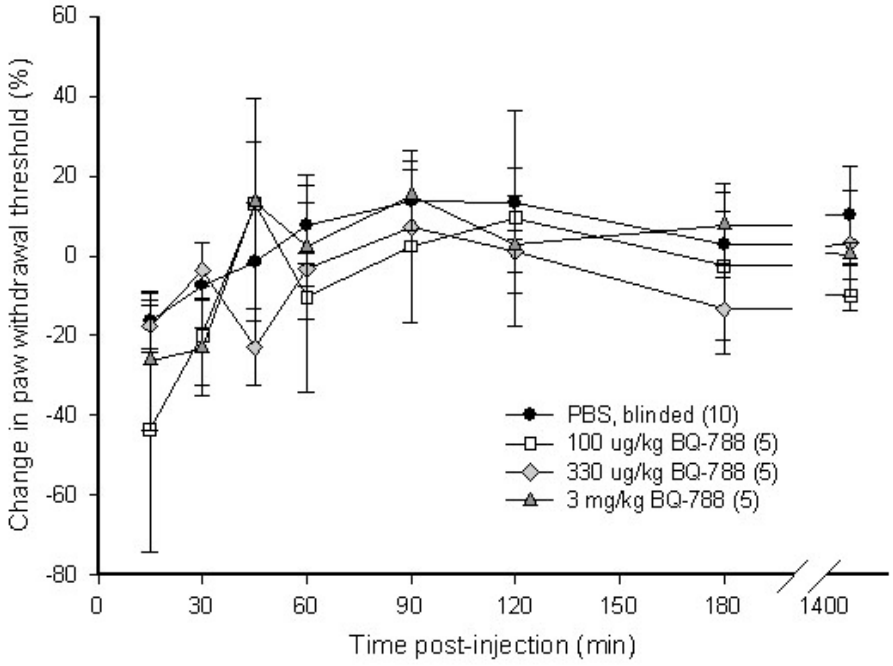


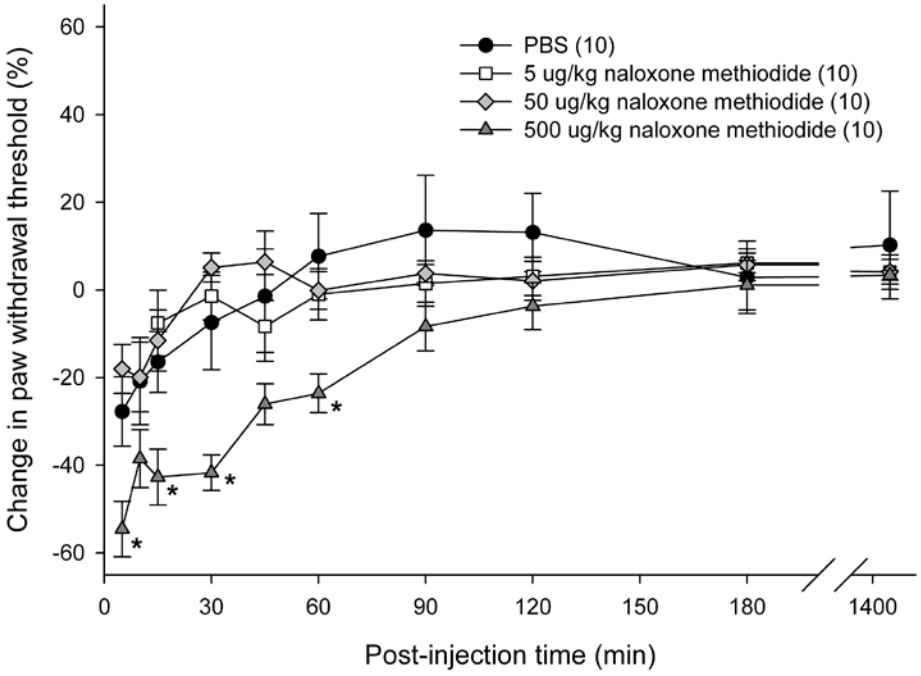
Figure 3.

(a) Mean percent change in paw withdrawal threshold (PWT) of the right hind paws of the HSC-3 group (n = 30), NOK group (n = 5), and DMEM sham-injected group (n = 4). Mean PWT in the cancer group significantly decreases starting at PID4 and maintains throughout until PID18 ($p < 0.05$) compared to both NOK and DMEM groups. The NOK group is not significantly different from the DMEM group. **(b)** Mean PWT of cancer animals injected with PBS vehicle (n = 5) or BQ-3020 (3 mg/kg, n = 15) in the cancer, or BQ-3020 (3 mg/kg, n = 5) in the contralateral paw. Overall, BQ-3020 injection in the cancer significantly increases PWT compared to both PBS and contralateral BQ-3020

administrations ($p < 0.05$) for a minimum of 3 hrs, indicating the presence of antinociception with localized ETBR agonism. **(c)** Mean PWT of cancer animals injected with PBS vehicle (n = 10) or BQ-788 (100 µg, 330 µg, or 3 mg/kg, n = 5). BQ-788 has no significant effect on mean PWT. [* and # denote statistical significance compared to PBS vehicle and contralateral BQ-3020, respectively]

Figure 4.

a


PBS (10)
5 ug/kg naloxone methiodide (10)
50 ug/kg naloxone methiodide (10)
500 ug/kg naloxone methiodide (10)
Change in paw withdrawal threshold (%)
Post-injection time (min)
60
40
20
0
-20
-40
-60
0
30
60
90
120
150
180
1400
*

b

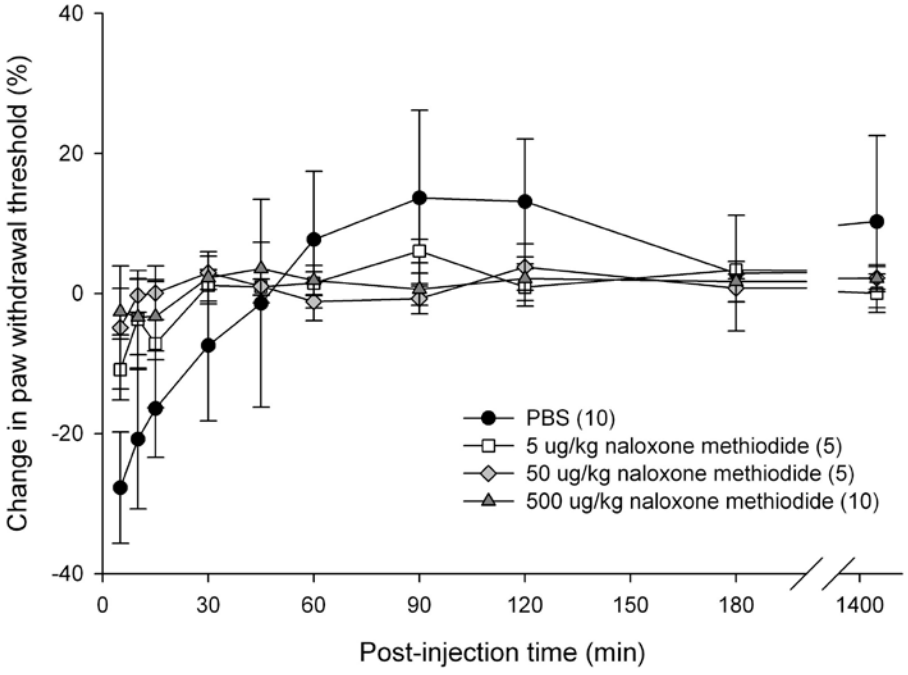
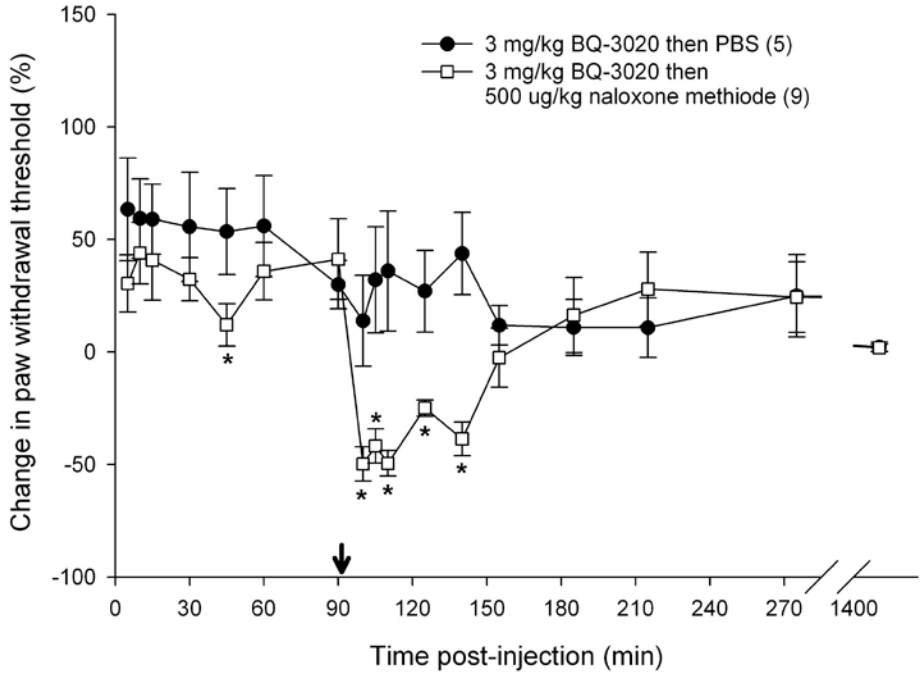

PBS (10)
5 ug/kg naloxone methiodide (5)
50 ug/kg naloxone methiodide (5)
500 ug/kg naloxone methiodide (10)
Change in paw withdrawal threshold (%)
Post-injection time (min)
40
20
0
-20
-40
0
30
60
90
120
150
180
1400

Figure 4.

Mean percent change in PWT of cancer-inoculated animals injected with a ten-fold magnitude range of nonspecific opioid receptor (OR) antagonist, naloxone methiodide, either in the cancer or into the contralateral paw. **(a)** 500 µg/kg naloxone methiodide (n = 10) administered into the cancer paw of animals significantly reduced mean PWT compared to PBS (n = 10) and lower concentrations at 5 and 50 µg/kg (n = 10, each group) at t = 15 min after drug injection, lasting up to 1 hr post-injection ($p < 0.05$). [* denotes statistical significance] **(b)** 5 µg/kg (n = 5), 50 µ/kg (n = 5), and 500 µg/kg (n = 10) of naloxone methiodide administered into the contralateral hind paw has no effect on mean PWT compared to PBS (n = 10) injected into the cancer paw of animals.

Figure 5.

a


3 mg/kg BQ-3020 then PBS (5)
3 mg/kg BQ-3020 then
500 ug/kg naloxone methiode (9)
Change in paw withdrawal threshold (%)
150
100
50
0
-50
-100
0
30
60
90
120
150
180
210
240
270
1400
Time post-injection (min)

b

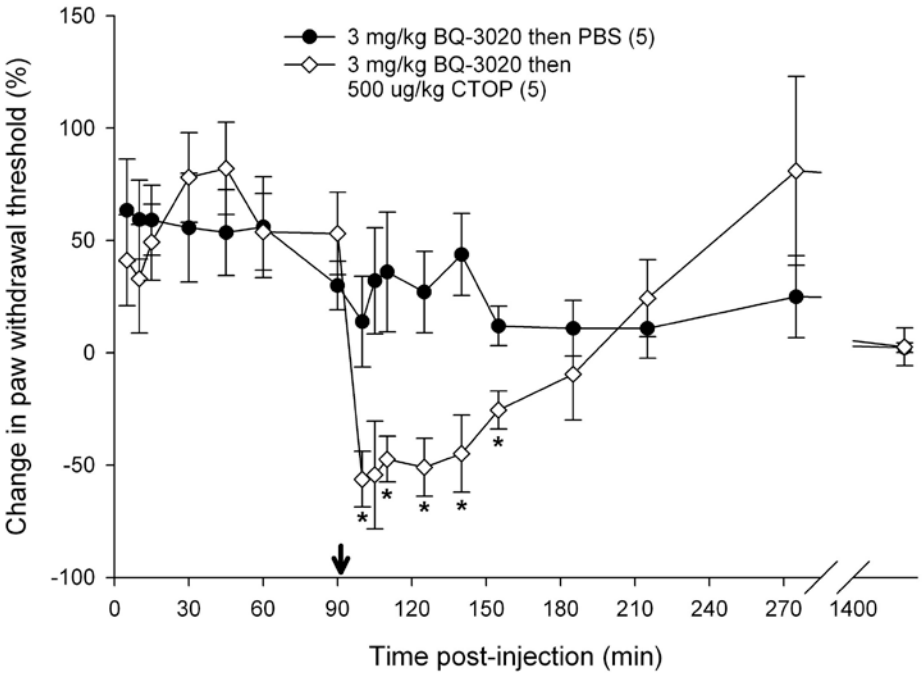
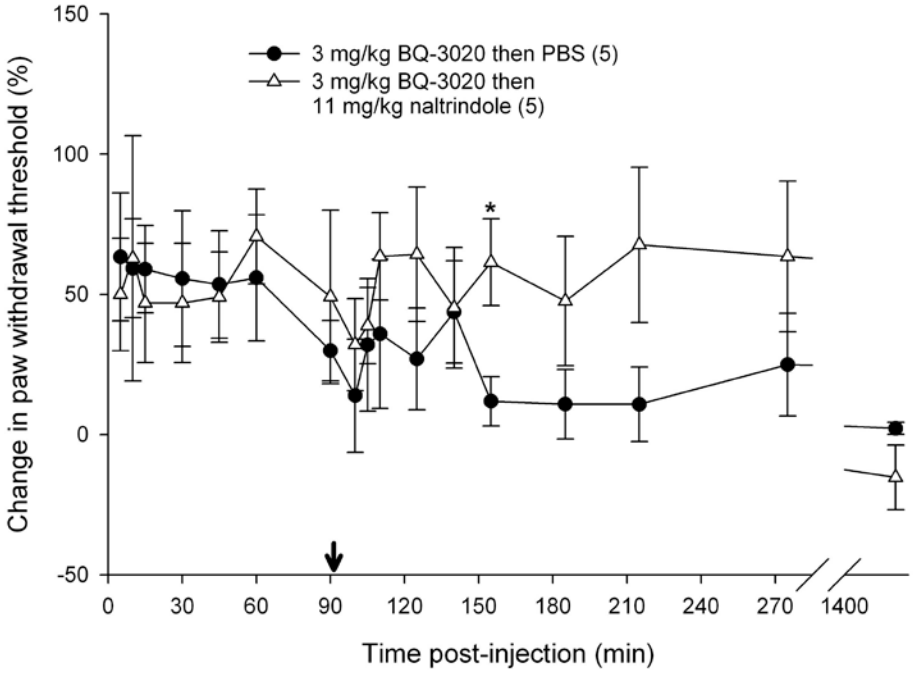

3 mg/kg BQ-3020 then PBS (5)
3 mg/kg BQ-3020 then
500 ug/kg CTOP (5)
Change in paw withdrawal threshold (%)
150
100
50
0
-50
-100
0
30
60
90
120
150
180
210
240
270
1400
Time post-injection (min)

Figure 5.

c


3 mg/kg BQ-3020 then PBS (5)
3 mg/kg BQ-3020 then
11 mg/kg naltrindole (5)
Change in paw withdrawal threshold (%)
Time post-injection (min)
*
150
100
50
0
-50
0
30
60
90
120
150
180
210
240
270
1400

d

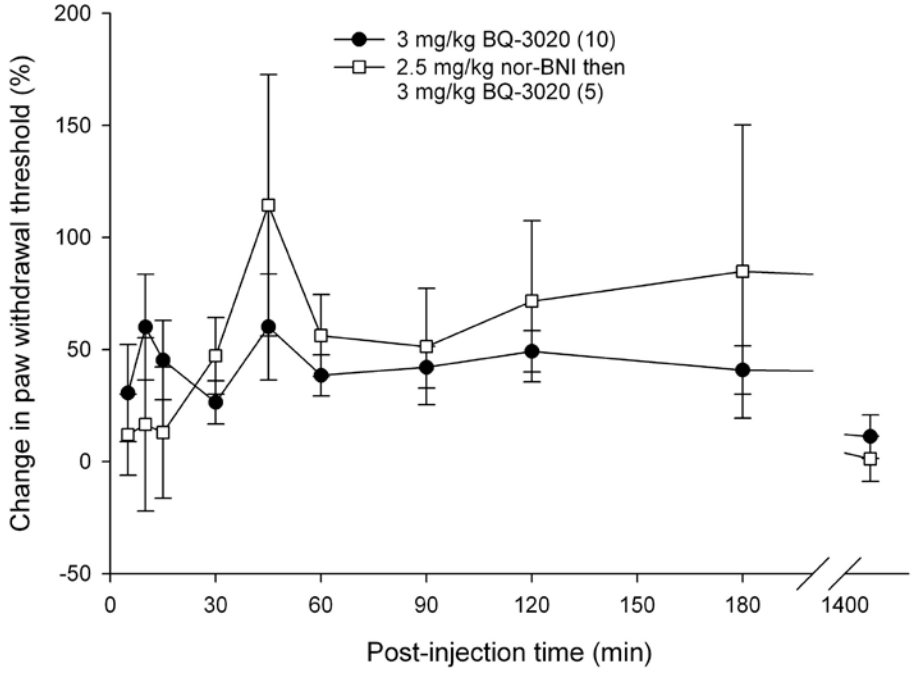

3 mg/kg BQ-3020 (10)
2.5 mg/kg nor-BNI then
3 mg/kg BQ-3020 (5)
Change in paw withdrawal threshold (%)
Post-injection time (min)
200
150
100
50
0
-50
0
30
60
90
120
150
180
1400

Figure 5.

Mean percent change in PWT of the right hind paws of cancer-inoculated animals injected with ETBR agonist (3 mg/kg BQ-3020) followed by administration of different peripheral opioid receptor (OR) antagonist drugs or PBS alone. (OR antagonist injection indicated by ↓**.**) **(a)** Paw withdrawal for SCC group which received naloxone methiodide (500 µg/kg) at 90 min after BQ-3020 injection (n = 9) compared to PBS control group (n = 5). Mean PWT for the naloxone methiodide group has a significant negative change starting at 10 min following injection of the peripheral OR antagonist, with about 50 min duration (t = 100, 105, 110, 125, and 140 min), compared to the PBS control group ($p < 0.05$). **(b)** Mean PWT for SCC group which received µ-OR antagonist CTOP (500 µg/kg) at 90 min after BQ-3020 injection (n = 5). CTOP injection results in an immediate reduction of the mean PWT compared to the PBS control group ($p < 0.05$), starting at t = 100 min and lasting to t = 155 min. **(c)** Mean PWT for SCC group which received δ-OR receptor antagonist naltrindole (NTI, 11 mg/kg) at 90 min after BQ-3020 injection (n = 5). NTI injection has no overall effect on mean PWT compared to PBS controls, except for a single aberration at t = 155 min, where PWT is higher than the control group ($p = 0.03$). **(d)** Paw withdrawal for SCC group which received nor-binaltorphimine (2.5 mg/kg) 12 hrs prior to BQ-3020 treatment (n = 5). nor-BNI administration has no significant effect on mean PWT compared to the PBS control group. [* denotes statistical significance]

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CHAPTER 5

Conclusion

The results of this thesis further support existing data implicating endothelin in both pain induction and its modulation. Animal and human cancer studies have clearly documented the nociceptive role of endothelin-A receptor (ETAR) activation and attenuation of the pain with ETAR antagonism; however the current study provides evidence that endogenous opioid peptides are responsible for the attenuation of pain with ETAR antagonists. Furthermore, we demonstrate that ETBR agonism also attenuates carcinoma-induced nociception through modulation of endogenous opioids. The combined *in vitro* and behavioral data suggest a role for innate opioid analgesia in modulation of carcinoma-induced nociception through manipulation of endothelin receptor signaling. Our conclusions can be applied to current understanding of cancer pain mechanisms to help improve cancer pain management in patients at terminal disease states.

Different types of normal as well as cancer cells producing endogenous opioids have been reported [1,2,6,8-11,13,14,16,19], but our data demonstrating production of opioids by an oral squamous cell carcinoma is a novel finding. The *in vitro* study in Chapter 2 established for the first time the role of endothelin as a direct regulator of endogenous opioid production, primarily β-endorphin and leu-enkephalin, in an oral SCC cell line. The oral SCC cell line expresses both ETAR and ETBR, as confirmed by immunofluorescence staining, however mRNA expression for both receptors are significantly less when compared to normal oral keratinocytes (Chapters 3-4). Decreased expression of the two receptors is potentially a result of feedback inhibition from abundant ET-1 peptides in cell culture. Similar feedback inhibition has been described in the regulation of glucocorticoid receptors by glucocorticoid-induced peroxisome

proliferator-activated receptor alpha (PPARα).[3] Treatment with ETAR antagonist significantly increased production of β-endorphin and leu-enkephalin. Contrary to antagonism of ETAR, ETBR antagonism resulted in decreased β-endorphin peptide production without affecting leu-enkephin levels. However, ETBR agonism stimulated production of β-endorphins. Taken together it appears that ETAR antagonists have more effects on oral SCC cells by inducing production of both types of endogenous opioids, as compared to ETBR agonists which induced only β-endorphins.

The function of endogenous opioid released through ETAR antagonism was investigated in a behavioral animal model in Chapter 3. Existing published studiess have established the antinociceptive effects of ETAR antagonism in animals [4,5,7,12,15,17,18], however the mechanism underlying the antinociception remains poorly understood. Our results demonstrating that ETAR antagonism directly affects endogenous opioid production by oral SCC *in vitro* and its consequent *in vivo* effects provide evidence to elucidate the mechanism of cancer pain. Conforming to our previously reported data, ETAR antagonist treatment in cancer-inoculated mice attenuated carcinoma-induced nociception [15]. When peripheral opioid receptors were antagonized with either naloxone methiodide or specific μ- or δ-opioid receptor blocking peptides (CTOP or naltrindole, respectively), the observed antinociception from ETAR antagonism was effectively reversed, which demonstrates that opioids are responsible for ETAR antagonist-induced attenuation of cancer-associated pain. Inhibition of the κ-opioid receptor had no effect on paw withdrawal threshold, which is consistent with *in vitro* results demonstrating no changes in dynorphin levels upon BQ-123 treatment.

The effects of ETBR activity on carcinoma-induced nociception was investigated in Chapter 4. Similar to the results for ETAR mRNA expression analysis, ETBR expression was significantly reduced in oral SCC compared to normal oral keratinocytes. However ETBR polypeptides are present as they were detected by immunofluorescence in cell culture. The *in vitro* studies demonstrated unlike ETAR antagonism ETBR antagonism resulted in decreased levels of β-endorphin. This result was consistent with and justified the lack of effect on carcinoma-induced nociception in animals after treatment with an ETBR antagonist BQ-788. ETBR agonism significantly increased production of β-endorphin but had no effect on leu-enkephalin or dynorphin. Conforming with the *in vitro* finding, animals treated with a nonspecific opioid receptor inhibitor naloxone methiodide or a μ-opioid receptor inhibitor CTOP reversed the antinociception induced by ETBR agonists, whereas δ- or κ-opioid receptor antagonists (naltridole and nor-binaltorphimine, respectively) had no effect. Our combined *in vitro* and *in vivo* findings suggest that β-endorphin is responsible for the attenuation of carcinoma-induced nociception with ETBR agonists.

In conclusion, this study has provided evidence for the role of endogenous opioids in the attenuation of cancer pain with endothelin receptor-targeted drugs. Our results suggest that innate opioid analgesia is responsible for antinociception observed from ETAR antagonist or ETBR agonist treatment in an animal cancer pain model (Fig. 1). These findings will help to elucidate the mechanism of cancer pain in order to improve pain management of cancer patients.

FIGURES AND LEGENDS

Figure 1.

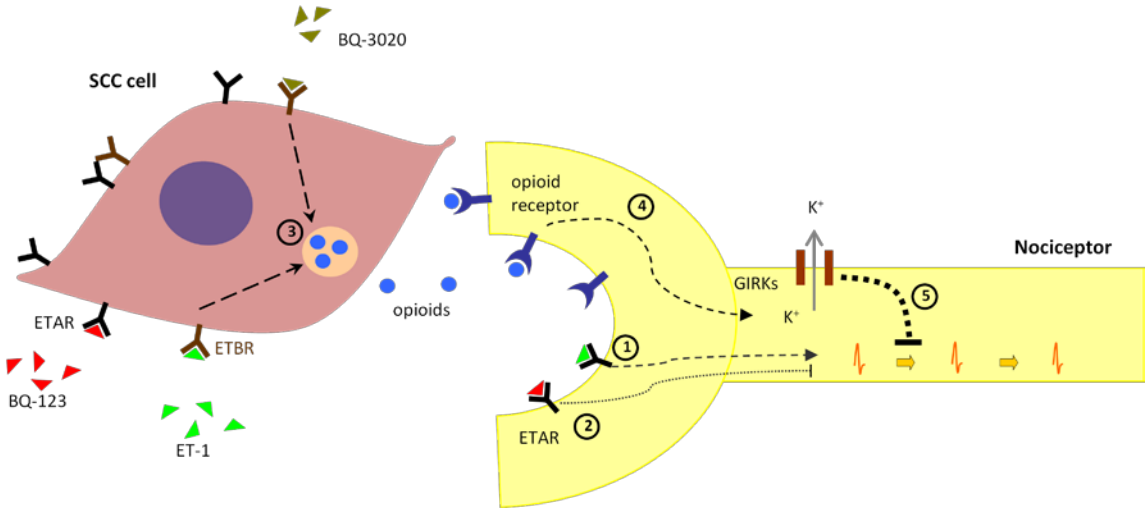

Figure 1.

Proposed endothelin and cancer pain pathway. **1)** ETAR expressed on nerve endings mediate nociceptive signaling. **2)** Its antagonism results in inhibition of nociception. **3)** ETAR and ETBR exist as single or dimerized receptors on the membrane surface of OSCC. ETAR antagonists dissociate the dimers to allow ETBR to bind ET-1 in the tumor microenvironment. ETBR activation leads to increased opioid (endorphins and enkephalins) released by OSCC. **4)** Released opioids activate peripheral opioid receptors to turn on GIRK channels and allow outward flow of K^+. **5)** K^+ outflow hyperpolarizes neural membranes and thereby decreases excitability of the neuron.

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