Demethylating Drugs as Novel Analgesics for Cancer Pain

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ABSTRACT

Purpose: In this study we evaluated the analgesic potential of demethylating drugs on oral cancer pain. While demethylating drugs could affect expression many genes, we focused on the mu-opioid receptor (OPRM1) gene pathway, because of its role in pain processing. We determined the antinociceptive effect of OPRM1 re-expression in a mouse oral cancer model.

Experimental Design: Using a mouse oral cancer model we determined whether demethylating drugs produced antinociception through re-expression of OPRM1. We then re-expressed OPRM1 with adenoviral transduction and determined if, and by what mechanism, OPRM1 re-expression produced antinociception. To determine the clinical significance of OPRM1 on cancer pain, we quantified OPRM1 methylation in painful cancer tissues and non-painful contralateral normal tissues of oral cancer patients, and non-painful dysplastic tissues of oral dysplasia patients.

Results: We demonstrated that OPRM1 was methylated in cancer tissue, but not normal tissue, of oral cancer patients, and not in dysplastic tissues from oral dysplasia patients. Treatment with demethylating drugs resulted in mechanical and thermal antinociception in the mouse cancer model. This behavioral change correlated with OPRM1 re-expression in the cancer and associated neurons. Similarly, adenoviral-mediated OPRM1 re-expression on cancer cells resulted in naloxone-reversible antinociception. OPRM1 re-expression on oral cancer cells in vitro increased beta-endorphin secretion from the cancer, and decreased activation of neurons that were treated with cancer supernatant.

Conclusion: Our study establishes the regulatory role of methylation in cancer pain. OPRM1 re-expression in cancer cells produces antinociception through cancer-mediated endogenous opioid secretion. Demethylating drugs have an analgesic effect that involves OPRM1.
**Translational Relevance:** In this study we evaluated the analgesic potential of demethylating drugs for oral cancer pain. We showed that systemic demethylating drugs produced mechanical and thermal antinociception in the mouse cancer model. We determined that the analgesic effect of demethylating drugs correlated with re-expression of *OPRM1* in the cancer and associated neurons, and beta-endorphin (endogenous opioid receptor ligand) secretion from cancer cells. To determine the clinical significance of *OPRM1* expression on cancer pain, we quantified *OPRM1* methylation in painful cancer tissues and non-painful dysplastic or normal tissues. Finally, we performed focused re-expression of *OPRM1* with adenoviral transduction and demonstrated that *OPRM1* re-expression produced analgesia in a mouse cancer model, increased beta-endorphin secretion from the cancer, and decreased activation of neurons in culture. Our results established demethylating drugs as novel analgesics, and the role of methylation in regulating cancer pain.
INTRODUCTION

For most cancer patients, uncontrollable pain creates a poor quality of life (1, 2). Eighty percent of cancer patients evaluated by the Pain Management Index report ineffective relief with prescribed analgesics (3). Although opioids can be initially effective, escalating doses are required to maintain pain relief due to opioid tolerance.

Effective cancer pain treatment will require an understanding of cancer pain mechanisms. One area of research that has gone unnoticed is the role of DNA methylation in cancer pain. We have demonstrated in a previous study that methylation of EDNRB, the endothelin B receptor gene that is involved in pain processing, contributes to cancer pain, and that reversal of methylation with adenoviral transduction produces analgesia (4). While gene re-expression with adenoviruses is not clinically feasible, drugs such as decitabine and zebularine, which demethylate genes, are immediately available and potentially offer an attractive analgesic approach (5-7).

In this translational study we hypothesized that downregulation of genes mediating endogenous analgesia results in cancer pain. To test our hypothesis we administered demethylating drugs and measured the antinociceptive effects in an oral cancer xenograft mouse model; oral cancer patients have a higher prevalence and higher pain intensity than other cancer patients (4). We then focused on in vitro and in vivo effects of targeted demethylation of OPRM1, the mu-opioid receptor gene. Finally, to determine whether our results were clinically relevant we determined whether OPRM1 was methylated in painful oral cancer tissue of patients compared to non-painful normal or dysplasia tissues.

MATERIALS AND METHODS

Patient recruitment and tissue collection

All procedures were approved by the New York University, Committee on Human Research. We enrolled oral squamous cell carcinoma (SCC) or oral dysplasia patients with the following inclusion
criteria: 1) biopsy-proven oral cavity SCC or oral dysplasia and 2) no history of prior treatment for oral SCC. We collected tissue at time of surgery from the primary tumor site and contralateral normal epithelium. Samples were flash frozen in liquid nitrogen and stored in -80°C. Oral pain was assessed using the UCSF Oral Cancer Pain Questionnaire (UOCPQ).

Cell culture

Cancer cells: The human tongue squamous cell carcinoma cell line, HSC-3, was obtained from JCRB Cell Bank and authenticated by isoenzymology. The human melanoma cell line, WM164, was purchased from ATCC and authenticated by short tandem repeat profiling. Cells were cultivated in Dulbecco’s Modification of Eagle’s Medium (DMEM) with 4.5 g/L glucose, L-glutamine and sodium pyruvate, 10% fetal bovine serum (FBS), at 37 °C in 5% CO2.

Neurons: Mouse trigeminal ganglia were harvested and cultured as previously described (8). Trigeminal ganglia were removed and enzyme-digested by incubation with papain (Worthington), collagenase type II (Worthington), and dispase type II (MB). Dissociated neurons were plated on glass coverslips coated with poly-d-lysine and laminin and maintained at 37°C at 5% CO2/95% air in F12 media (Life Technologies) with 5% FBS.

Transduction of OPRM1

Human cDNA of OPRM1 containing a C-terminal GFP tag (OriGene) was subcloned into a pVQAd CMV K-NpA shuttle plasmid. Subcloning and viral particle purification were completed through Viraquest. HSC-3 or WM164 was transduced with recombinant adenovirus (Ad-OPRM1 or Ad-GFP) at increasing multiplicities of infection (MOI) to determine transduction efficiency. Transduction was performed in DMEM with 2% fetal bovine serum (FBS) and the aforementioned supplements.

Xenograft mouse model
The cancer pain mouse model was produced as previously described (9) on BALB/c, athymic mice (see Supplementary Methods). 24 hours prior to inoculation, HSC-3 or WM-164 cells were transduced with Ad-OPRM1 or Ad-GFP at 200 MOI. The mice were divided into three groups and inoculated with the respective cell types: (1) non-transduced, (2) Ad-OPRM1, and (3) Ad-GFP. From our preliminary immunofluorescence experiments we had determined that HSC-3 and WM-164 cells expressed low levels of mu-opioid receptor.

**Demethylating drug treatments**

Decitabine dosage was based on a previous study on BALB/c mice (7). Mice received daily intraperitoneal (IP) injections of either decitabine (2µg/g body weight) or drug vehicle (phosphate-buffered saline; PBS) starting PID 4. Mice were treated with either 3% sucrose water mixed with 1 mg/ml zebularine or vehicle given *ad libitum* starting PID 4.

**Paw volume measurement**

Paw volume measurements were performed with a plethysmometer (IITC Life Sciences) as previously described (4).

**Mechanical allodynia measurement**

Paw withdrawal thresholds were determined as described (9) in response to pressure from an electronic von Frey anesthesiometer (2390 series, IITC Life Sciences). The paw withdrawal response was measured six times on each paw separated by 3 minute intervals.

**Thermal hyperalgesia measurement**

Thermal hyperalgesia was assessed as previously described (10) using a focused projection bulb to deliver a thermal stimulus to the right footpad of each mouse with a cutoff of 20 seconds. Paw withdrawal to heat was calculated as a mean of six measurements.
Naloxone administration prior to behavioral testing

Naloxone (500 µg/kg) dissolved in 20 µl PBS or vehicle was injected into the right hind paw at the site of cancer growth. Behavioral testing was performed at intervals up to 24 hours post-injection.

Quantitative methylation analysis

The target region on the OPRM1 promoter was -304 to +71 relative to the transcription start site (TSS) in the mouse gene and -232 to +109 in the human gene. Primers were designed using EpiDesigner. Quantitative methylation analysis was performed using the MassARRAY system (Sequenom). Fully methylated DNA was used as positive control and water as negative control.

Quantitative reverse transcription PCR analysis

30 mg of each fresh frozen tissue sample was processed with the AllPrep DNA/RNA Kit (Qiagen). mRNA was reverse transcribed with Random Hexamers (Applied Biosystems), then amplified with the Taqman gene expression assays for OPRM1. Human GUSB and mouse ACTB were used as the endogenous control. The delta-delta CT method was used to quantify relative expression.

Immunohistochemistry

Paw tissues and dorsal root ganglia (DRG) were harvested, fixed in 4% paraformaldehyde and embedded in paraffin. Sections were heated, de-paraffinized, then pretreated with Target Retrieval Solution (Dako). Endogenous peroxidases were quenched by immersing sections in 3% hydrogen peroxide and Tris-Buffered Saline (TBS) for 5 minutes, then placed in a TBS bath for 5 minutes. Sections were incubated for 2 hours with monoclonal mu-opioid receptor antibody (1:500, Epitomics). The primary antibody was replaced with non-specific antibody (Epitomics) for the negative control. Following 45 minute incubation with a goat anti-rabbit secondary antibody, DAB substrate (Abcam) was placed on the sections for 2 minutes. The slides were then dehydrated and cover slipped.
**In situ hybridization**

Paraffin-embedded tumor tissue sections (5 μm) were processed as detailed in supplementary methods. Hybridization was carried out overnight at 57 °C with 30nM custom-designed OPRM1 digoxigenin (DIG)-labeled LNA probes (Exiqon).

**Beta-endorphin quantification by ELISA**

Collected supernatant was treated with 10 µl HALT Protease Inhibitor Cocktail (Pierce) and protein concentration was normalized with a BCA protein assay (Pierce) before performing ELISA to detect beta-endorphin (MD Biosciences). Treatment conditions are detailed in supplementary methods.

**Calcium imaging**

HSC-3 cells or dissociated neurons were seeded onto glass coverslips, loaded with 1µM of the cell permeable calcium sensitive dye, Fura 2AM (Molecular Probes) for 30 minutes and washed with HBSS before use. Coverslips containing cells were placed in a chamber with constant infusion of HBSS at room temperature. Fluorescence was detected by a Nikon Eclipse TI microscope at 340 and 380 excitation wavelengths and analyzed with the TI Element Software (Nikon). HSC-3 cells were counted as responsive to 10µM [D-Ala², N-MePhe⁴, Gly-ol]-enkephalin (DAMGO) infusion and neurons were counted as responsive to cancer supernatant infusion if the 340/380 ratio was ≥0.2 from baseline.

**Statistical analysis**

Statistical analysis was performed using Sigma Plot, v11.0. Data were analyzed using Student’s t-test, Two-way RM ANOVA, One-way ANOVA and Holm-Sidak post hoc test, or ANOVA on Ranks and Dunn’s post hoc test as appropriate. Results were presented as mean ± standard error of the mean (SEM).

**RESULTS**
The anti-tumor effect of zebularine and decitabine was greater than either drug alone

While demethylating drugs are used in treatment of other cancers, their anti-tumor effect on oral SCC is unknown. We showed in earlier studies that tumor growth correlates with pain, pain is not dependent on tumor size alone (9). Therefore prior to evaluating the analgesic effect of demethylating drugs we first determined whether these drugs had an anti-tumor effect on oral SCC. The five drug treatment groups in our preclinical model were naïve, cancer control vehicle, cancer treated with decitabine only, cancer treated with zebularine only, and cancer treated with combination treatment. Naïve mice were treated with the same drug regimen as the combination group and did not display any weight loss, changes in sensory thresholds, or systemic effects by PID 21 (data not shown). Mice from the four cancer groups developed visible tumors by PID 4. Control mice displayed a 97% increase (from 0.185 ml to 0.364 ml) in paw volume by PID 21. The decitabine group only produced a modest anti-tumor effect; tumor volume in this group increased from 0.177 ml on day 0 to 0.290 ml on PID 21. Zebularine treatment significantly reduced tumor growth starting on PID 9. Combination treatment of zebularine and decitabine showed the strongest anti-tumor effect, with a reduction in paw volume back to baseline on PID 7. The paw volume average was 0.17 ml on day 0, increased to 0.27 ml on PID 4, and decreased back to 0.19 ml by PID 7 (Figure 1). Mice with drug treatments discontinued on PID15 showed an increase in tumor growth, such that paw volumes were not significantly different between the control vehicle group and drug treatment groups after PID18 (Figure 1).

The antinociceptive effect of zebularine and decitabine was greater than either drug alone

We determined whether demethylating drugs had mechanical and thermal analgesic effects in the oral SCC model. Our earlier studies had shown that while oral SCC growth contributes to nociception, cancer-induced nociception is not entirely explained by tumor burden. By PID 4, all four cancer groups demonstrated a decrease in paw withdrawal threshold, consistent with cancer-induced mechanical allodynia. Drug treatment was initiated on PID 4. Control mice showed a progressive decrease in paw
withdrawal, indicating increasing mechanical allodynia, during the entire trial. The average withdrawal threshold for the control group by PID 21 was 1.0 g. Mice treated with decitabine showed a similar decrease in paw withdrawal, but on PID 21 this group has significantly less mechanical allodynia than the control group. While the zebularine group gradually showed an increase back to baseline by PID 13, the combination treatment group showed an immediate increase to baseline by PID 7. The average paw withdrawal threshold for the combination group remained at baseline for the remainder of the experiment. The average withdrawal threshold was 2.9 g on PID 7 and 3.9 g on PID 21. This result indicated that the combination treatment had a stronger antinociceptive effect than either drug alone (Figure 1).

**Tumors from mice treated with demethylating drugs showed lower methylation of the OPRM1 gene, higher mRNA expression, and increased mu-opioid receptor expression**

While the mechanical nociceptive assays suggested that demethylating drugs had antinociceptive properties, to differentiate between their anti-tumor and antinociceptive properties we chose to analyze the effect of demethylating drugs on OPRM1 expression in the tissues. OPRM1 is classically involved in pain processing and has an equivocal role in cancer proliferation (11, 12). We first showed that treatment with zebularine for nineteen days (i.e., up until PID 25) produced demethylation in the OPRM1 promoter in the cancer tissue (Figure 2). We chose zebularine-treated tissue because in our drug treatment schedule zebularine treatment was intended to maintain demethylation. Oprm1 mRNA expression levels were correspondingly 51 times higher in zebularine-treated tumors than non-treated tumors (p = 0.008, Student’s t test). We performed immunohistochemistry and in situ hybridization to detect the mu-opioid receptor in the tumors of mice treated with zebularine and those of control mice. Our results demonstrated that mu-opioid receptor expression was relatively absent on control, non-treated SCC paw tumors. Tumors that were treated with zebularine, either in combination with decitabine or alone, showed re-expression of the mu-opioid receptor. Tumors that had received combination treatment showed significantly higher expression of mu-opioid receptor (Figure 2A). We
then analyzed the neural tissue providing sensory innervation to the hind paw. We showed that DRGs (L4-L5) that innervated the inoculated hind paw also had increased mu-opioid receptor expression in the combination treatment group, compared to DRGs from the control cancer group (Figure 2B). Oprm1 transcript levels were correspondingly higher in the drug treatment groups compared to the control cancer group, with the combination group DRG showing the highest Oprm1 mRNA expression (Figure 2C).

**OPRM1 promoter was hypermethylated in oral SCC tissue compared to contralateral normal tissue and oral dysplasia tissue**

With the finding that demethylating drugs induced Oprm1 demethylation and expression in cancer and cancer-associated neurons prompted us to determine the clinical significance of OPRM1 methylation in oral SCC. We quantified OPRM1 methylation in oral SCC and contralateral normal tissue taken from our existing cohort of oral SCC patients, and dysplasia tissue of oral dysplasia patients (Table S1). The mean age of the patients was 66 years (range 50-93). Oral SCC patients in the study cohort had significantly more spontaneous and functional pain than oral dysplasia patients (13). The average UOCPQ score in the oral SCC cohort was 340.94 (± SEM 31.28) out of a maximum possible score of 800. The average UOCPQ score in the oral dysplasia cohort was 34.76 (± SEM 11.19) (13). We quantified OPRM1 methylation in cancer tissue, contralateral normal tissue, and dysplasia tissue. A heat map of methylation values obtained for each CpG unit was generated (Figure 3). Normal contralateral tissue had relatively low baseline methylation. Methylation levels for all CpG units were significantly higher in cancer tissue than contralateral normal tissue (Table S2). Dysplasia tissue had methylation values in between those values of normal tissue and cancer tissue. When compared to cancer tissue, the dysplasia tissue had lower methylation values at all but one CpG site (Table S2).

**Hind paw tumors transduced with Ad-OPRM1 demonstrated mu-opioid receptor re-expression**
Based on our result that OPRM1 was methylated in painful cancer tissues, but not in non-painful normal and dysplasia tissues, we created a cancer mouse model that specifically re-expressed OPRM1 in the tumor. We transduced oral SCC cells with an adenovirus expressing OPRM1 (Ad-OPRM1) and used Ad-GFP as a transduction control. After establishing the xenograft cancer model we harvested the tumors from the site of inoculation (hind paw) and evaluated mu-opioid receptor expression. Ad-OPRM1 transduced tumors showed markedly high expression of mu-opioid receptor compared to Ad-GFP-transduced or non-transduced cancer tissues. A hind paw section from a naïve mouse showed mu-opioid receptor expression in the epidermal layer, an expected staining pattern as keratinocytes express mu-opioid receptor (14) (Figure S1).

SCC paw tumors influenced mu-opioid receptor expression on corresponding DRGs

We also quantified mu-opioid receptor expression in DRGs (i.e., L4-L5) innervating the hind paw tumors. Oral SCC growth in the hind paw correlated with suppression of mu-opioid receptor expression in the associated DRG relative to the contralateral DRG (Figure 4). Given this finding, we wanted to determine whether mice in the Ad-OPRM1 group had increased mu-opioid receptor expression in DRGs. Receptor re-expression occurred mainly in small-diameter neurons, which transmitted nociceptive signals. Mu-opioid receptor expression in the DRGs associated with Ad-ORPM1-transduced SCC tumors was similar to the levels seen in the contralateral, normal DRG. mRNA quantification also demonstrated significantly higher transcript levels of Oprm1 in the ipsilateral DRG of the Ad-OPRM1 group. These findings of restored Oprm1 expression paralleled the findings in the demethylating drug treatments. Transcript levels of Oprm1 in the contralateral DRG were similar between all groups.

Mice with Ad-OPRM1 tumors did not have reduced tumor size, but demonstrated less mechanical allodynia than control cancer mice
We showed that re-expressing OPRM1 in oral SCC tumors did not affect cancer growth. The average paw volumes of the Ad-GFP, Ad-OPRM1, and non-transduced cancer groups were 0.17 ml, 0.17 ml, and 0.18 ml, respectively, at baseline and 0.29 ml, 0.29 ml, and 0.29 ml on PID 21. This finding eliminates tumor growth as a direct cause of cancer-induced nociception in our xenograft model of Ad-OPRM1-transduced cancer. We therefore focused on quantifying the effects of OPRM1 expression on mechanical and thermal nociception. Since oral cancer patients most frequently complain of mechanical sensitivity and functional restriction due to pain (1), we quantified the effect of treatment on mechanical allodynia in our preclinical model. The gold standard assay for mechanical allodynia is the paw withdrawal assay; for this reason we inoculated oral SCC cells into the hind paw. Mice in the Ad-OPRM1 group had mechanical antinociception relative to control (Ad-GFP and non-transduced) groups. Mice with from the Ad-OPRM1 group had mechanical thresholds that stabilized at 36% below baseline (PID 0 = 3.9 g; PID 21 = 2.5 g), despite a paw volume increase that was comparable to the other two groups (Figure 5A; Table S3). Mice with control tumors, on the other hand, had significantly more mechanical alldynia. Mice with Ad-GFP-transduced tumors showed a 60% decrease (4.0 g to 1.6 g) and mice with non-transduced tumors demonstrated a 69% decrease (4.2 g to 1.3 g). Mechanical thresholds of mice with Ad-GFP-transduced tumors were not significantly different from those of the non-transduced control group. To translate our findings to another cancer model we also produced a xenograft model using melanoma (WM-164) cells to determine the effect of Ad-OPRM1 transduction on melanoma pain. We had shown previously that WM-164 cells produce significantly less nociception than HSC-3 cells (9). We showed that WM-164 tumors did not produce mechanical nociception until PID 25, which is late onset nociception compared to the HSC-3 model. The Ad-OPRM1 WM-164 group had lower mechanical alldynia than the Ad-GFP WM-164 group (Figure S2A). The WM-164 tumor volumes were not different between the two groups (data not shown).

**Mice with Ad-OPRM1 tumors demonstrated less thermal hyperalgesia than control mice**
Mice with HSC-3 tumors re-expressing mu-opioid receptor demonstrated lower thermal hyperalgesia than the control groups. Mice with HSC-3 tumors re-expressing mu-opioid receptor had an average latency to a thermal stimulus of 8.2 seconds, whereas mice with Ad-GFP tumors had an average latency of 5.3 seconds and mice with non-transduced tumors had a latency of 4.0 seconds (Figure 5B). The same antinociceptive trend was seen in mice with WM-164 tumors that re-expressed mu-opioid receptor. For the melanoma (WM-164) model, the thermal thresholds at baseline on day 0 for the Ad-GFP and Ad-OPRM1 groups were 4.3 and 4.4 seconds, respectively (Figure S2B). At the onset of nociception on PID 25, WM-164 tumors that were transduced with Ad-OPRM1 had higher thermal thresholds, signifying antinociception, and this effect lasted for the duration of the experiment until PID 39. The Ad-OPRM1 treatment group had significantly lower thermal nociception than the Ad-GFP group.

**Mechanical and thermal antinociception in mice with Ad-OPRM1-transduced oral SCC tumors were reversed by local naloxone administration**

To determine whether the antinociceptive effect that resulted from mu-opioid receptor expression was driven by the endogenous opioid system, we tested the effect of local naloxone administration to the hind paws of mice with tumors on PID 14 (Figure 5C). The baseline mechanical paw withdrawal threshold was 4.3 g for both the Ad-OPRM1 and Ad-GFP groups. Mice in the Ad-OPRM1 group had a lower mechanical allodynia than the Ad-GFP group; mean mechanical threshold change from day 0 was -41% for the Ad-OPRM1 group and -73% for the Ad-GFP group. However, the antinociceptive effect present in the Ad-OPRM1 group was reversed with naloxone. Naloxone did not cause the same change in mechanical withdrawal threshold in the Ad-GFP group. The Ad-OPRM1 group also demonstrated thermal antinociception compared to the Ad-GFP group, with mean thermal threshold change of -33% compared to the -50% in the Ad-GFP group. This thermal antinociceptive effect was reversed in the Ad-OPRM1 group after local naloxone administration. Naloxone had no significant effect on thermal latency in the Ad-GFP group (Figure 5D).
Supernatant from oral SCC cells transduced with Ad-OPRM1 suppressed neuronal activation in a naloxone-dependent manner

The naloxone-reversible antinociceptive behavior in the xenograft models in response to OPRM1 re-expression in oral SCC cells suggested that the endogenous analgesic system was involved. This finding was consistent with our previous publication that oral SCC secreted endogenous opioids to modulate pain relief (4). However the in vivo environment contained many cell types that could have secreted endogenous opioids. To completely isolate the effect of oral SCC cells on neurons, we performed calcium imaging on dissociated mouse neurons that were treated with oral SCC supernatant. We showed that oral SCC supernatant applied to neurons caused neuronal activation through an increase in intracellular calcium (Figure 6A). Supernatant from oral SCC cells transduced with Ad-OPRM1 activated significantly fewer neurons than control supernatant from oral SCC cells transduced with Ad-GFP. When neurons were pre-treated with an infusion of 10µM naloxone for three minutes prior to supernatant application, the inhibitory effect on neurons observed with Ad-OPRM1 supernatant was reversed. Ad-OPRM1 with 10µM naloxone activated the same proportion of neurons as Ad-GFP supernatant. Naloxone addition had no significant effect on the neuronal activation potential of Ad-GFP supernatant (Figure 6B).

Secretion of beta-endorphin was increased in Ad-OPRM1 oral SCC cells and suppressed by naloxone

To search for the mediator in the Ad-OPRM1 supernatant that could have produced an inhibitory effect on neurons in a naloxone-reversible manner, we quantified levels of beta-endorphin, an endogenous opioid that we had previously shown to be secreted from oral SCC (4), in the supernatant of transduced cancer cells. We showed a MOI dose-dependent increase in beta-endorphin secretion in WM-164 (Figure S2C) and HSC-3 (Figure S3A) cells. While control HSC-3 supernatant contained almost no beta-endorphin, HSC-3 cells transduced with Ad-OPRM1 at 200 MOI secreted an average of 400pg/ml
beta-endorphin. The Ad-GFP cells showed low beta-endorphin secretion. Beta-endorphin secretion in HSC-3 cells transduced with Ad-OPRM1 was suppressed by naloxone, in a dose-dependent manner from $10^{-6}$ to $10^{-12}$ mol/L. HSC-3 cells transduced with Ad-GFP did not show dose-dependent suppression of beta-endorphin secretion following naloxone treatment (Figure -S3B). Beta-endorphin secretion was also higher in HSC-3 cells treated with decitabine and zebularine (Figure S3C).

**Beta-endorphin secretion from oral SCC cells was mediated by calcium signaling**

We hypothesized that beta-endorphin secretion from oral SCC cells was mediated by activation of the mu-opioid receptor and downstream calcium signaling. We first determined whether activation of the transduced mu-opioid receptor by the agonist DAMGO (10µM) resulted in cancer cell excitation by measuring intracellular calcium. We showed that DAMGO application to HSC-3 cells that were transduced with Ad-OPRM1 resulted in an increase in intracellular calcium (Figure 6C). Significantly more Ad-OPRM1 HSC-3 cells had a positive calcium response compared to Ad-GFP HSC-3 cells (10.1% vs 3.1%, p<0.05, Fisher’s exact test). We then determined the effect of depleting extracellular and intracellular calcium stores on beta-endorphin secretion. Treatment of HSC-3 cells with 1µM thapsigargin, which depleted intracellular calcium, along with incubation in calcium-free DMEM, resulted in a significant decrease in beta-endorphin secretion (Figure 6D).

**DISCUSSION**

**Demethylating agents are novel therapies for cancer pain**

In this study we showed that global and targeted demethylation alleviates cancer pain. We tested zebularine and decitabine, two currently available demethylating drugs that could be repurposed to treat cancer pain. These drugs produced significant antinociception to mechanical stimuli in the mouse oral SCC model. Moreover, combination treatment with decitabine and zebularine produced a more rapid and sustained reduction of tumor volume and tumor-induced nociception than either drug alone. While these drugs have clear antineoplastic effects in hematological and solid malignancies (5, 6, 15-
19), their analgesic role has not been explored. Decitabine and zebularine act synergistically to initiate and maintain demethylation of genes, respectively. Zebularine inhibits the degradation of decitabine by cytidine deaminase (16, 20) and potentiates the effects of decitabine (21). Zebularine is available orally, which makes it an attractive drug for maintenance demethylation. The drugs have good safety profiles (5, 6).

**OPRM1 methylation in cancer cells mediates cancer pain**

The antinociceptive mechanism of zebularine and decitabine was likely multifactorial—systemic treatment with these drugs induced demethylation of a multitude of genes in different tissues. The drugs reduced tumor volume, which likely contributed to reduction in cancer-induced pain. However our previous studies show that cancer pain is not entirely controlled by tumor volume (4, 9). We therefore chose to focus on one gene to demonstrate proof of principle that demethylation of pain-mediating genes could produce antinociception. We chose OPRM1 because it is critically involved in pain processing. Furthermore, as we were looking at OPRM1 expression on cancer cells, it was important to confirm that OPRM1 did not play a role in cancer proliferation (11, 12). Our evidence for the role of OPRM1 methylation in cancer pain in patients was that the OPRM1 promoter was hypermethylated in patient oral SCC tumors, which are painful, but not their matched normal oral tissue. Tissues of oral dysplasia, which are not painful (13), had lower levels of methylation relative to oral SCC tumors. To confirm the role of OPRM1 promoter methylation in cancer pain we created a mouse cancer pain model using a human oral SCC cell line that re-expressed OPRM1 through adenoviral transduction. Targeted re-expression of OPRM1 on oral SCC cells with adenoviruses or demethylating drugs had significant mechanical and thermal antinociceptive effects in xenograft oral SCC and melanoma models without affecting tumor proliferation. Furthermore these antinociceptive effects in mice with Ad-OPRM1 oral SCC were reversed with local naloxone. Local naloxone injection had no effect on nociceptive thresholds of control Ad-GFP oral SCC mice. It is well established that mu-opioid receptor expression
on peripheral neurons mediates analgesia (22-27), but the finding that mu-opioid receptor expression on the cancer cells reduces pain in a cancer model is novel.

To isolate the effect of mediators secreted from cancer cells on neurons, we quantified neuronal activation in response to cancer supernatant using calcium imaging. We demonstrated that supernatant from oral SCC cells re-expressing OPRM1 activated significantly fewer neurons compared to supernatant from oral SCC cells not expressing OPRM1. We further demonstrated that the relative inhibitory effect of Ad-OPRM1 oral SCC supernatant was reversed with naloxone. These findings represent the in vitro correlate to our in vivo findings in mice with Ad-OPRM1 oral SCC tumors.

**Beta-endorphin secretion in the cancer microenvironment contributes to the antinociceptive effect of OPRM1 re-expression**

To determine whether cancer cells were the source of endogenous opioids in our cancer pain model, we measured beta-endorphin levels in the supernatant of Ad-OPRM1 oral SCC cells. Keratinocytes, from which carcinomas are derived, secrete beta-endorphin and express the associated receptor (14, 28, 29). Secretion of beta-endorphin from keratinocytes elicits antinociception in vivo (30, 31). Another source of beta-endorphin, aside from keratinocytes, is lymphocytes. Beta-endorphin secretion from lymphocytes is mediated by intracellular calcium release (32). We therefore used calcium imaging and ELISA following depletion of intra- and extracellular calcium to determine whether beta-endorphin secretion from carcinoma cells is calcium dependent. Our in vitro results showed that beta-endorphin secretion in Ad-OPRM1 cells was mediated by an excitatory mechanism that led to an increase in intracellular calcium. Furthermore, antagonizing the mu-opioid receptor with naloxone suppressed beta-endorphin secretion in a dose-dependent manner. The suppression of mu-opioid receptor mediated beta-endorphin secretion likely explains the mechanical and thermal nociception seen in vivo after naloxone administration.
From these results we propose that mu-opioid receptor expression and activation on the carcinoma cell causes beta-endorphin secretion into the cancer microenvironment, which produces an antinociceptive effect. A similar feed-forward endogenous opioid effect occurs in the neurointermediate lobe of the pituitary where activation of mu-opioid receptors leads to beta-endorphin secretion (33), and this effect is reversed with a mu-opioid receptor antagonist. Endogenous analgesic mechanisms have been described with other cancer models and in other human cancers. In a pancreatic cancer mouse model endogenous opioid mechanisms suppress visceral pain-related behaviors in early-stage disease, and are reversible by naloxone and naltrexone (34). In patients with either intractable bone or abdominal visceral cancer pain there is a significant increase in serum beta-endorphin following treatment with opioid analgesics (35-37).

**Mu-opioid receptor expression on associated neurons correlates with cancer-induced nociception**

We were also interested in mu-opioid receptor expression on the DRG innervating the cancer microenvironment (*i.e.*, L4-L5) and how mu-opioid re-expression on the cancer might affect the DRG. Mu-opioid receptor expression on DRGs in the control (*i.e.*, Ad-GFP and non-transduced cancer) groups was reduced. We propose that reduced expression of mu-opioid receptor on the DRG in the cancer pain model was secondary to pain. In contrast the expression of mu-opioid receptor on the associated DRGs in animals treated with Ad-OPRM1 or demethylating drugs (*i.e.*, animals with reduced pain) was similar to naive DRGs. The main behavioral difference between the control groups (*i.e.*, Ad-GFP and non-transduced) and the treatment groups (*i.e.*, Ad-OPRM1 or demethylating drugs) was that the control groups had significantly more pain throughout the course of the experiment. Our finding that sustained pain reduces mu-opioid receptor expression in associated DRGs is consistent with a chronic neuropathic pain model wherein mu-opioid receptor expression on the associated DRG is reduced (38). Similarly, mu-opioid receptor expression is reduced in the DRG of a mouse model of abdominal carcinomatosis pain (39).
The thrust of our study was to determine whether, and by what mechanisms, demethylating drugs produce antinociception in a cancer pain model. While demethylating drug treatment alters expression of many genes in different tissues, our adenoviral transduction approach isolated OPRM1 as a candidate gene; silencing of OPRM1 in cancer cells contributes to cancer pain. We demonstrated the utility of adenoviral gene transduction in pain treatment. Virus-mediated gene delivery has previously been explored to treat chronic pain in patients. An HSV-based vector expressing inhibitory neurotransmitters or anti-inflammatory mediators were delivered to peripheral sensory neurons, and reduced pain-related responses in rodent models of inflammatory pain, neuropathic pain, and bone cancer-induced pain (40-49). The main difference between our technique and HSV-based gene transfer lies in the target cell. While HSV preferentially acts on peripheral neurons, we transduced cancer cells. Our result that re-expression of the mu-opioid receptor in the cancer cell produces analgesia is consistent with our hypothesis of cancer-mediated endogenous analgesia. We showed that cancer cells can secrete opioids that reduce cancer pain, and that methylation plays an important role in this process.

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References


Figure Legends

**Figure 1. Combination therapy with decitabine and zebularine had an antitumor and antinociceptive effect in the cancer mouse model.** (A and B) The graphs show that combination treatment of zebularine and decitabine produced stronger antitumor and antinociceptive effects in an oral SCC mouse model than either drug alone (*p<0.05, **p<0.01, ***p<0.001, compared to control vehicle group (1), with treatment as the main effect, see Table S3 for statistical analysis). (C and D) The graphs show paw volume and mechanical threshold changes of groups in which zebularine and decitabine treatments were discontinued on PID 15 (vertical dashed line). Significant differences in paw volume and mechanical threshold changes existed between the groups and control group between PID 7 and PID 18 (**p<0.01, ***p<0.001, treatment as the main effect).

**Figure 2. Mice treated with demethylating drugs showed increased expression of mu-opioid receptor relative to control.** (A) Immunohistochemistry (IHC) and in situ hybridization (ISH) images of tumor tissue (from hind paw) of control vehicle, decitabine only, zebularine only, and combination treatment groups. The epidermal layer expressed mu-opioid receptor, as expected. The combination treatment group (right-most image) had high expression of mu-opioid receptor. (B) IHC and ISH images of DRGs (L4-L5) that provided sensory innervation to the cancer inoculation area in the hind paw. DRG mu-opioid expression correlated with expression pattern in the cancer, such that the combination treatment group yielded qualitatively higher expression of mu-opioid receptor compared to control. Mu-opioid receptor was expressed on small diameter neurons in the DRGs, which are activated by noxious stimuli. Black bar = 100µm. (C) Control cancer DRG had significantly lower Oprm1 mRNA expression than ipsilateral naïve DRG, combination treatment DRG, and contralateral naïve DRG (*p<.05, **p<.01, ***p<.001, One-way ANOVA, Holm-Sidak test). (D) Long-term zebularine treatment groups had hypomethylation of the OPRM1 promoter compared to control (*p<.05, **p<.01, Student’s t test).
Figure 3. Heat map of OPRM1 promoter region in the cancer and anatomically-matched tissue of oral cancer patients and in the dysplasia of oral pre-cancer patients. **(A)** The analyzed region is -232 to +109 relative to transcription start site (dotted line). Quantified CpG units are depicted as green circles and omitted CpG units are depicted as grey circles. **(B)** The heat map compares methylation of the OPRM1 promoter in oral SCC tumor versus contralateral normal tissue of 19 patients with biopsy-proven oral SCC. **(C)** The heat map depicts the methylation of the OPRM1 promoter region in DNA extracted from dysplastic tissue of 5 patients with biopsy-proven oral dysplasia.

Figure 4. Mu-opioid receptor expression in DRGs correlated with expression in tumors. **(A)** Immunohistochemistry (IHC) and in situ hybridization (ISH) of DRGs (L4-L5) with mu-opioid receptor antibody are shown at 20X objective (black bar = 100µm). The left-most panel shows contralateral (relative to paw with cancer) DRGs in non-transduced, Ad-GFP, and Ad-OPRM1 groups, with high expression of the mu-opioid receptor. The middle and right-most panels show ipsilateral DRGs of the corresponding groups stained for mu-opioid receptor with IHC and ISH, respectively. **(B)** Graph of Oprm1 mRNA expression in DRGs shows that transcript levels were significantly suppressed in control (non-transduced or Ad-GFP) mice with tumors. However in mice with Ad-OPRM1 tumors, transcript levels were restored to normal levels as seen in the contralateral DRGs. (*p<0.05, One-way ANOVA, Holm-Sidak test).

Figure 5. Mice with xenograft oral SCC (HSC-3) transduced with Ad-OPRM1 demonstrated mechanical and thermal antinociception that was mediated by endogenous opioid release and was naloxone-reversible. **(A)** The graph shows percent change in mechanical threshold from baseline for each treatment group. Mechanical threshold changes of the Ad-OPRM1 group, but not the Ad-GFP group, were significantly higher than the non-transduced HSC-3 control group (see Table S3 for statistical analysis). Significant differences existed at the time points indicated (**p<0.01, ***p<0.001, Holm-Sidak test, treatment as the main effect). **(B)** The box plot represents the median, 10th, 25th, 75th, and 90th percentiles (black dots represent outliers) of thermal withdrawal thresholds on PID 20. Mice in
the Ad-OPRM1 group had significantly longer heat latency than non-transduced HSC-3 control mice (*p < 0.05, ANOVA on Ranks, Dunn’s test). (C) The graph shows percent change from baseline mean paw withdrawal threshold, before and after naloxone administration. Prior to administration mice in the Ad-OPRM1 group had mechanical antinociception relative to control mice, but this effect was reversed with naloxone administration. The Ad-GFP and Ad-OPRM1 groups had significantly different responses to naloxone (p=0.002, see Table S3). The Ad-OPRM1 group had significantly lower mechanical threshold up to 60 minutes post-injection, compared to pre-injection (***p<0.001, Holm-Sidak test, time as main effect). (D) Mice in the Ad-OPRM1 group developed thermal hyperalgesia in response to naloxone, whereas the Ad-GFP group did not. The Ad-OPRM1 group had significantly lower thermal thresholds up to 60 minutes after naloxone injection, compared to pre-injection (**p<0.01, ***p<0.001, Holm-Sidak test, time as main effect).

Figure 6. Supernatant from Ad-OPRM1 oral SCC cells secreted beta-endorphin through a calcium-dependent mechanism; supernatant suppressed neuronal activation in a naloxone-dependent manner. (A) The graph plots the ratio of F340 to F80 versus time, with an uptick in the trace representing calcium influx. Representative calcium traces are shown. Infusion conditions are represented by the colored lines. Potassium chloride (KCl) is used as a positive control for viable neurons. Infusion with cancer supernatant produced calcium influx in neurons. (B) Ad-OPRM1 oral SCC supernatant activated significantly fewer neurons that control Ad-GFP oral SCC supernatant. This relative inhibitory effect on neurons was reversed with naloxone pre-application (*p<.05, Chi square test). (C) We showed that activation of mu-opioid receptor on HSC-3 cells (by 10µM DAMGO) led to calcium influx. (D) To determine if calcium influx was responsible for beta-endorphin secretion, we quantified beta-endorphin secretion in HSC-3 cells (transduced with either Ad-GFP or Ad-OPRM1) that were depleted of either extracellular calcium (incubated in calcium-free media) or both extracellular and intracellular calcium (incubated in calcium-free media and 1µM thapsigargin). We showed that beta-
endorphin secretion was significantly reduced in the absence of extracellular and intracellular calcium
(*p<0.05, **p<0.01, ***p<0.001, One-way ANOVA, Holm-Sidak test).
Figure 3
Figure 5

A

Mean Mechanical Threshold Change (% of baseline ± SEM)

Days

-5 0 5 10 15 20 25

1. Non-transduced HSC-3 (n=6)
2. HSC-3 transduced with Ad-GFP (n=6)
3. HSC-3 transduced with Ad-OPRM1 (n=6)

B

Time to paw withdrawal (Seconds)

HSC-3 Ad-GFP Ad-OPRM1

C

Mean Mechanical Threshold Change (% of baseline ± SEM)

Post-injection time (minutes)

-30 0 30 60 90 120 150 180 210 240 270 300 330 360 390 420 450 480 510 540 570 600 630 660 690 720 750 780 810 840 870 900 930 960 990 1020 1050 1080 1110 1140 1170 1200 1230 1260 1290 1320 1350 1380 1410 1440 1470 1500

1. HSC-3 transduced with Ad-GFP (n=6)
2. HSC-3 transduced with Ad-OPRM1 (n=6)

D

Mean Thermal Threshold Change (% of baseline ± SEM)

Post-injection time (minutes)

-30 0 30 60 90 120 150 180 1400 1500

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Figure 6

A  

Time (seconds)

F340/380

0  1  2  3  4

cancer supernatant  KCl

B  

HSC-3 supernatant
Ad-OPRM1 HSC-3 supernatant
HSC-3 supernatant with 10μM naloxone
Ad-OPRM1 HSC-3 supernatant with 10μM naloxone

Percentage of trigeminal neurons responding to treatment

34/99
21/103
20/65
22/63

C  

HBSS  10μM DAMGO

F340/380

0  0.2  0.4  0.6  0.8  1.0

D  

Control media
Calcium free media
1 μM thapsigargin

[β-endorphin] (pg/ml)

0  200  400  600  800  1000  1200  1400  1600

HSC-3 transduced with Ad-GFP  HSC-3 transduced with Ad-OPRM1

*  **  ***

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