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. Although demethylating drugs could affect expression of 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 nonpainful contralateral normal tissues of patients with oral cancer, and nonpainful dysplastic tissues of patients with oral dysplasia.

Results: We demonstrated that OPRM1 was methylated in cancer tissue, but not normal tissue, of patients with oral cancer, and not in dysplastic tissues from patients with oral dysplasia. 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 β-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. Clin Cancer Res; 20(18); 4882–93. ©2014 AACR.

Introduction

For most patients with cancer, uncontrollable pain creates a poor quality of life (1, 2). Eighty percent of patients with cancer 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 because of 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. To test our hypothesis, we administered demethylating drugs and measured the antinociceptive effects in an oral cancer xenograft mouse model. Patients with oral cancer have a higher prevalence and higher pain intensity than other patients with cancer (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 tissues of patients compared with nonpainful 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: (i) biopsy-proven oral...
Demethylating Drugs and Cancer Pain

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 β-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 β-endorphin secretion from the cancer, and decreased activation of neurons in culture. Our results showed demethylating drugs as novel analgesics, and the role of methylation in regulating cancer pain.

cavity SCC or oral dysplasia and (ii) 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 (UCOPQ).

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, WM-164, was purchased from ATCC and authenticated by isoenzymology. The human melanoma cell line, HSC-3, was obtained from JCRB Cell Bank and authenticated by isoenzymology. Cells were cultivated in Dulbecco’s Modified Eagle 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 in 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% 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). Twenty-four hours before inoculation, HSC-3 or WM-164 cells were transduced with Ad-OPRM1 or Ad-GFP at 200 MOI. The mice were divided into 3 groups and inoculated with the respective cell types: (i) nontransduced, (ii) Ad-OPRM1, and (iii) 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 (i.p.) injections of either decitabine (2 µg/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 zebrulin 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 before 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 after 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

Thirty milligrams 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 ΔΔCt 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, deparaffinized, 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 nonspecific 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 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 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 30 nmol/L custom-designed OPRM1 digoxigenin (DIG)-labeled LNA probes (Exiqon).

β-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 β-endorphin (MD Biosciences). Treatment conditions are detailed in the Supplementary Methods.

Calcium imaging

HSC-3 cells or dissociated neurons were seeded onto glass coverslips, loaded with 1 μmol/L 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 nm. Excitation wavelengths were set at 340 and 380 nm.

Statistical analysis

Statistical analysis was performed using Sigma Plot, v11.0. Data were analyzed using Student t test, Two-way RM ANOVA, one-way ANOVA, and Holm–Sidak post hoc test, or ANOVA on Ranks and Dunn post hoc test as appropriate. Results were presented as mean ± standard error of the mean (SEM).

Results

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

Although demethylating drugs are used in treatment of other cancers, their antitumor 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, before evaluating the analgesic effect of demethylating drugs, we first determined whether these drugs had an antitumor effect on oral SCC. The five drug treatment groups in our preclinical model were naive, 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 0.185–0.364 mL) in paw volume by PID 21. The decitabine group only produced a modest antitumor 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 antitumor 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 (Fig. 1). Mice with drug treatments discontinued on PID 15 showed an increase in tumor growth, such that paw volumes were not significantly different between the control vehicle group and drug treatment groups after PID 18 (Fig. 1).

The antinociceptive effect of zebularine and decitabine was greater than that of 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 4 cancer groups demonstrated a decrease in paw withdrawal threshold, consistent with cancer-induced mechanical alldynia. Drug treatment was initiated on PID 4. Control mice showed a progressive decrease in paw withdrawal, indicating increasing mechanical alldynia, 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 alldynia than the control group. Although the zebularine group gradually showed an increase back to baseline by PID 13, the
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 (Fig. 1).

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

Although the mechanical nociceptive assays suggested that demethylating drugs had antinociceptive properties, to differentiate between their antitumor 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 19 days (i.e., up until PID 25) produced demethylation in the OPRM1 promoter in the cancer tissue (Fig. 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 nontreated tumors ($P = 0.008$, Student $t$ 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, nontreated 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 (Fig. 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 with DRGs from the control cancer group (Fig. 2B).

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

With the finding that demethylating drugs induced Oprd1 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 patients with oral SCC, and dysplasia tissue of patients with oral dysplasia (Supplementary Table S1). The mean age of the patients was 66 years (range 50–93). Patients with oral SCC in the study cohort had significantly more spontaneous and functional pain than patients with oral dysplasia (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; ref. 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 (Fig. 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 (Supplementary Table S2). Dysplasia tissue had methylation values in between those values of normal tissue and cancer tissue. When compared with cancer tissue, the dysplasia tissue had lower methylation values at all but 1 CpG site (Supplementary 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 nonpainful 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 with Ad-GFP-transduced or nontransduced 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 (ref. 14; Supplementary Fig. 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 (Fig. 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-OPRM1-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.

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 nontransduced cancer groups were 0.17, 0.17, and 0.18 ml, respectively, at baseline and 0.29, 0.29, 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. Because patients with oral cancer most frequently complain of mechanical sensitivity and functional restriction because of pain (1), we quantified the effect of

Transcript levels of Oprm1 in the contralateral DRG were similar between all groups.

Figure 3. Heat map of OPRM1 promoter region in the cancer and anatomically matched tissue of patients with oral cancer and in the dysplasia of oral precancer 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 gray 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.
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 nontransduced) groups. Mice with control tumors, on the other hand, had significantly more mechanical allodynia. Mice with Ad-GFP–transduced tumors showed a 60% decrease (4.0–1.6 g) and mice with nontransduced tumors demonstrated a 69% decrease (4.2–1.3 g). Mechanical thresholds of mice with Ad-GFP-transduced tumors were not significantly different from those of the nontransduced control group.

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 (Fig. 5C). The baseline mechanical nociception at baseline on day 0 for the Ad-GFP and Ad-OPRM1 groups was 4.3 and 4.4 seconds, respectively (Supplementary Fig. 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.

**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 nontransduced tumors had a latency of 4.0 seconds (Fig. 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 (Supplementary Fig. 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 was 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 (Fig. 5C). The baseline mechanical

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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 ×20 objective (black bar = 100 μm). The left-most panel shows contralateral (relative to paw with cancer) DRGs in nontransduced, 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 (nontransduced 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).
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 with the Ad-GFP group, with mean thermal threshold change of −33% compared with 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 (Fig. 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
representing calcium in graph plots the ratio of F340 to F80 versus time, with an uptick in the trace. Supernatant from Ad-OPRM1 oral SCC cells secreted -endorphin through a calcium-dependent mechanism. 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 pretreated with an infusion of 10 µmol/L naloxone for 3 minutes before supernatant application, the inhibitory effect on neurons observed with Ad-OPRM1 supernatant was reversed. Ad-OPRM1 with 10 µmol/L 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 (Fig. 6B).

**Secretion of -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 -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 -endorphin secretion in WM-164 (Supplementary Fig. S2C) and HSC-3 (Supplementary Fig. S3A) cells. Although control HSC-3 supernatant contained almost no -endorphin, HSC-3 cells transduced with Ad-OPRM1 at 200 MOI secreted an average of 400 pg/mL -endorphin. The Ad-GFP cells showed low -endorphin secretion. -Endorphin secretion in HSC-3 cells transduced with Ad-OPRM1 was suppressed by naloxone, in a dose-dependent manner from 10 to 10 mol/L. HSC-3 cells transduced with Ad-GFP did not show dose-dependent suppression of -endorphin secretion following naloxone treatment (Supplementary Fig. S3B). -Endorphin secretion was also higher in HSC-3 cells treated with decitabine and zebularine (Supplementary Fig. S3C).

![Figure 6. Supernatant from Ad-OPRM1 oral SCC cells secreted -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.](image)

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 than control Ad-GFP oral SCC supernatant. This relative inhibitory effect on neurons was reversed with naloxone pre-application (*P < 0.05, y-test). C, we showed that activation of mu-opioid receptor on HSC-3 cells (by 10 µmol/L DAMGO) led to calcium influx. D, to determine if calcium influx was responsible for -endorphin secretion, we quantified -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 1 µmol/L thapsigargin). We showed that -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).
β-Endorphin secretion from oral SCC cells was mediated by calcium signaling

We hypothesized that β-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 (10 μmol/L) resulted in cancer cell excitation by measuring intracellular calcium. We showed that DAMGO application to HSC-3 cells had a positive calcium response compared with Ad-GFP HSC-3 cells (10.1% vs. 3.1%, P < 0.05, Fisher exact test). We then determined the effect of depleting extracellular and intracellular calcium stores on β-endorphin secretion. Treatment of HSC-3 cells with 1 μmol/L thapsigargin, which depleted intracellular calcium, along with incubation in calcium-free DMEM, resulted in a significant decrease in β-endorphin secretion (Fig. 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, 2 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. Although these drugs have clear antinociceptive effects in hematologic 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 with 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.

β-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 β-endorphin levels in the supernatant of Ad-OPRM1 oral SCC cells. Keratinocytes, from which carcinomas are derived, secrete β-endorphin and express the associated receptor (14, 28, 29). Secretion of β-endorphin from keratinocytes elicits antinociception in vivo (30, 31). Another source of β-endorphin, aside from keratinocytes, is lymphocytes. β-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 β-endorphin secretion from carcinoma cells is calcium dependent. Our in vitro results showed that β-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 β-endorphin secretion in a dose-dependent manner. The suppression of mu-opioid receptor mediated β-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 β-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 β-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 β-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 nontransduced 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 naïve DRGs. The main behavioral difference between the control groups (i.e., Ad-GFP and nontransduced) 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 where mu-opioid receptor expression on the carcinoma 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.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.T. Viet, D. Dang

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Administrative, technical, or material support (i.e., reporting and organizing data, constructing databases): C.T. Viet

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**Acknowledgments**

The authors thank Victor E. Marquez, from the National Cancer Institute, for providing zebularine.

**Grant Support**

This work was supported by NIH R21 DE018561, NIH R01 DE19796, and an Oral and Maxillofacial Surgery Foundation Research Support Grant. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 15, 2014; revised June 13, 2014; accepted June 19, 2014; published OnlineFirst June 24, 2014.

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Demethylating Drugs as Novel Analgesics for Cancer Pain

Chi T. Viet, Dongmin Dang, Yi Ye, et al.

*Clin Cancer Res* 2014;20:4882-4893. Published OnlineFirst June 24, 2014.

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