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Fufang Kushen injection inhibits sarcoma growth and tumor-induced hyperalgesia via TRPV1 signaling pathways

Zhizheng Zhao^{a,b}, Huiting Fan^{a,b}, Tim Higgins^a, Jia Qi^c, Diana Haines^d, Anna Trivett^a, Joost J. Oppenheim^a, Hou Wei^b, Jie Li^b, Hongsheng Lin^b, and O.M. Zack Howard^{a,*}

^aLaboratory of Molecular Immunoregulation, Cancer and Inflammation Program, Center for Cancer Research, National Cancer Institute-Frederick, Frederick, MD 21702, USA

^bGuang An Men Hospital of China Academy of Chinese Medical Sciences, Beijing, China

^cNeuronal Networks Section, Intramural Research Program, National Institute on Drug Abuse, NIH, 251 Bayview Blvd, Baltimore, MD 21224, USA

^dLeidos Biomedical, Frederick National Laboratory for Cancer Research, Frederick, MD 21702, USA

Abstract

Cancer pain is a deleterious consequence of tumor growth and related inflammation. Opioids and antiinflammatory drugs provide first line treatment for cancer pain, but both are limited by side effects. Fufang Kushen injection (FKI) is GMP produced, traditional Chinese medicine used alone or with chemotherapy to reduce cancer-associated pain. FKI limited mouse sarcoma growth both *in vivo* and *in vitro*, in part, by reducing the phosphorylation of ERK and AKT kinases and BAD. FKI inhibited TRPV1 mediated capsaicin-induced ERK phosphorylation and reduced tumor-induced proinflammatory cytokine production. Thus, FKI limited cancer pain both directly by blocking TRPV1 signaling and indirectly by reducing tumor growth.

Keywords

Sarcoma; Hyperalgesia; TRPV1; ERK; Fufang Kushen injection

Introduction

Cancer pain is a frequent symptom in late stage cancer patients, but the molecular factors contributing to tumor-induced, increased sensitivity to nociceptive stimuli, known as tumor-associated hyperalgesia, remain unclear. Further, chemotherapy induces peripheral neuropathy giving rise to cancer related pain [1]. No effective way to deal with cancer

*Corresponding author. Tel.: +1 301 846 1348; fax: +1 301 846 6789. howardz@mail.nih.gov (O.M.Z. Howard).

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Conflict of interest

None.

related hyperalgesia has been developed [2–5]. Therefore, understanding the molecular basis for tumor-associated hyperalgesia and development of new therapies are urgently needed.

Cancer pain shares common features with neuropathic [6] and inflammatory pain [7]. Tumor invasion of tissue causes pressure or direct neuronal tissue damage resulting in neuropathic pain. We have therefore studied pain induced by injecting transplantable pleiotropic sarcoma (S-180) cells near the sciatic nerve as previously reported by Qi et al. and others [8–11]. Pain signals are transmitted by the transient receptor potential channel receptors; the vanilloid subfamily member 1 (TRPV1) is a member of this family of receptors [12]. Studies of TRPV1 deficient mice showed that this receptor plays a crucial role in responding to and conveying thermal, chemical and mechanical stimuli, which are associated with peripheral inflammation and neuropathies [12]. Deletion of this receptor results in inhibiting experimental inflammatory hyperalgesia and neurogenic inflammation pain [13]. The ERK signal pathway has recently been identified as central to pain sensitivity in inflammatory and neuropathic pain models [14–16]. A noxious stimulus or tissue injury rapidly leads to phosphorylation of ERK within a few minutes, which correlates well with the rate of development of pain hypersensitivity [17]. While pERK induction is well accepted as a marker of pain induced by thermal, chemical and mechanical stimulation, the role of TRPV1 induced pERK [18] has not been reported in a murine model of cancer-induced pain. Further, clinical pain studies [19] and rodent cancer-pain models [20,21] have shown increases in inflammatory cytokine expression (IL1 β , CCL2, TNF α and IL6). In experimental pain models, inflammatory chemokines have been shown to increase sensitivity to pain and promote TRPV1 response to capsaicin [22]. The role of these cytokines in maintenance of TRPV1 and pERK expression and function needs to be evaluated.

Traditional Chinese medicines (TCM) are being employed globally to treat cancer. Fufang Kushen injection (FKI) is a TCM extracted from the roots of two herbs, *Sophora flavescens* and *Heterosmilaxis Japonica*. This injectable formula is a patented (Chinese patent:ZL: 97112532.5.) and standardized Good Manufacturing Process (GMP) entity. FKI is used alone or combined with conventional chemotherapy to treat lung cancer patients in China improving anticancer efficacy and reducing the side effects of chemotherapy [23,24]. Evaluation of anti-inflammatory compounds contained in *Sophora flavescens* and *Heterosmilaxis Japonica* root extracts have identified several discrete alkaloids. For example, matrine and oxymatrine combined are the most abundant chemicals in FKI [23] that inhibit cytokine production, oxygen radical production and tumor cell growth in vitro [25–31]. However, they do not account for all the effects of FKI and a more complete mechanism(s) of FKI action and optimal dosing in combination with chemotherapy still needs to be determined.

Sarcomas are malignant tumors arising from connective tissue that have been shown to cause hyperalgesia due to pressure on nerves or destruction of nervous tissue by the tumor [32]. We have therefore evaluated the molecular effects of FKI in a murine model of sarcoma-induced hyperalgesia. Here, we report that FKI not only decreased the size of the tumor, but also decreased hyperalgesia induced by sarcoma as well as TRPV1 and ERK phosphorylation.

Materials and methods

Reagents

Anti-mouse antibodies to p-ERK (4370), T-ERK (9102), p-38 (4511p), p-NF κ b (3033p), p-MEK (9154), T-AKT (9272), p-AKT (9275), p-BAD (4366) and GAPDH (2118) were purchased from Cell Signaling (Boston, MA). Anti-mouse antibody TRPV1 (NB100-1617) was purchased from Novus Biologicals (Littleton, CO) and anti-mouse P-TRPV1 from Abnova (PAB8499) (Walnut, CA). Cytokines were purchased from PeproTech (Rocky Hill, NJ). Chemicals, including capsaicin and matrine were obtained from Sigma/Aldrich (St. Louis, MO). FuFang Kushen injection was obtained from Shanxi Zhendong Pharmaceutical Co.Ltd (Shanxi, China).

Cell lines and proliferation assay

S-180 sarcoma cell line (ATCC, Manassas, VA) was maintained in MEM media containing 10% heat-inactivated fetal calf serum (Hyclone; Thermo) with nonessential amino acids (Gibco), 2 mM L-Glutamine and 100 U/ml penicillin and 100 μ g/ml streptomycin at 37 °C in 5% CO₂ and saturated humidity. M3-9M sarcoma cells were a kind gift from Crystal Mackall, Bethesda, MD. 4T1 and Hepa1-6 were also obtained from ATCC. TRPV1-HEK cells were generated as previously described [22]. TRPV1-HEK cells were cultured in DMEM containing 10% heat-inactivated fetal calf serum with 2 mM L-Glutamine plus 100 U/ml penicillin and 100 μ g/ml streptomycin at 37 °C in 5% CO₂, with saturated humidity. Tumor cells were seeded at a density of 3×10^3 cells/well in a 96-well plate and subsequently incubated with FKI. After 24, 48 and 72 h of incubation, cells were pulsed with [3H]dT for 4 h (Amersham Pharmacia) at 1 μ Ci per well [33].

Mice

C57BL/6 mice, Swiss Webster and NIH Webster mice were provided by the Animal Production Area of the NCI (Frederick, MD). Frederick National Laboratory for Cancer Research is accredited by AAALAC International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the "Guide for Care and Use of Laboratory Animals" (National Research Council; 1996; National Academy Press; Washington, D.C.). These studies were performed under approved protocol ASP 13–218.

Tumor-induced temperature sensitivity (hyperalgesia)

Our laboratory adapted a tumor-induced temperature sensitivity model from earlier studies [8–11]. Two million S-180 cancer cells cultured in vitro, washed and resuspended in buffered saline were inoculated into the muscular tissue of female Swiss Webster mice in the immediate vicinity of the sciatic nerve near the trochanter, immediately distal to where the posterior biceps semitendinosus branches off the common sciatic nerve. A negative control group was injected with PBS. Paw withdrawal latencies to radiant heat stimulation at 55 °C were measured before any procedure and on days 2, 4, 6, 8, 10, 12 after tumor inoculation. Treatment groups consisted of 10 animals. FKI was delivered i.p., in a dose

range from 0 to 100 mg/ kg/mouse once or twice in 24 h; the tumor control groups received PBS i.p.

Embryonic liver cells harvest and proliferation assay

Embryonic liver cells were harvested from newborn Swiss Webster mice. Liver tissue was mechanically disrupted followed by filtration through 70 and 30 μ m cell strainers. The resulting single cells were resuspended in DMEM (Life Technologies) supplemented with 10% heat-inactivated fetal calf serum, 50 μ M 2-mercaptoethanol, 2 mM L-Glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin and plated at 0.5×10^5 cell/well into 96-well flat bottom plates. After 24 h of culture in 37 °C in 5% CO₂ and saturated humidity increasing amounts of FKI were added to culture, after an additional 48 h the cells were subjected to ³H Thymidine uptake assay for 4 h.

Spinal cord cell harvest and in vitro stimulation

Spinal cords were harvested from Swiss Webster mice and single cells prepared as described by Berghmans et al. [34]. Individual spinal cords were mechanically disrupted followed by filtering through 70 and 30 μ m cell strainers. Single cells (1×10^6 /well) were then plated into 24-well plates, in neurobasal medium (Life Technologies) supplemented with 10% heat-inactivated horse serum (Sigma-Aldrich). After 30 min of incubation at 37 °C in 5% CO₂ and humidified air, to eliminate the influence of mechanical stimulation, specific stimuli were added for varying amounts of time. Following stimulation, cells were subjected to Western blotting.

Western blot analysis

Cells or tissues from spinal cord were lysed in ice-cold lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT), briefly sonicated, and centrifuged at 14,000 $\times$ g at 4 °C for 30 min. Total protein in the supernatant was determined using the BCA Protein assay (Pierce, Thermo Scientific). Equal amounts of protein were subjected to electrophoresis on NuPAGE Bis-Tris (Invitrogen, Carlsbad, CA) in 4–12% gradient gels in MES buffer and then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA). The membranes were blocked in buffer (TBST: 0.25 M Tris base, 1.37 M NaCl, 0.03 M KCl, and 0.1% Tween 20, pH 7.4, with 5% nonfat milk) at room temperature for 1 h. The membranes were then incubated with rabbit polyclonal antibody against p-ERK, t-ERK, p-AKT, t-AKT, p-P38, p-NF κ b (1:1000; Cell Signaling), rabbit anti-TRPV1 (1:1000), rabbit anti-phospho TRPV1 (1:500) and rabbit anti-GAPDH (1:1000) overnight at 4 °C. The blots were washed three times in TBST and incubated with HRP-conjugated anti-rabbit secondary Ab (1:2000) for 1 h at room temperature. Blots were then visualized with ECL reagent (GE Healthcare, Pittsburgh, PA). All Western blot band quantification steps were performed using custom ImageJ (National Institutes of Health; <http://rsb.info.nih.gov/ij/>) script.

Immunohistochemistry analysis

Mouse tissues were fixed in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. Tissues were sectioned at 5 μ m and stained with hematoxylin and eosin for histopathological examination. For immunohistochemistry, 5 μ m sections were

deparaffinized into ethanol and endogenous peroxidase activity was blocked using 0.6% H₂O₂ in methanol. Antigen retrieval was carried out by microwaving in citrate buffer and slides were stained with biotin-conjugated anti-CD45R/B220 (BD Biosciences #553086) 1:200 for B-cell lymphocytes. Detection of CD45R signal was performed using the avidin-biotinylated enzyme complex (Vector Laboratories) with 3,3'-diaminobenzidine (Sigma) as a chromagen. Slides were counterstained with hematoxylin. Slides were photographed using an Olympus BX40 microscope, Q-image RGB camera using Bioquant (Nashville, TN) software to capture and achieve the images.

Multiplex cytokine quantification

Following the last hypersensitivity study, mice were anesthetized and serum samples taken. The serum was analyzed by Leidos Clinical Services Program using a Meso Scale Discovery mouse proinflammatory 7-plex kit.

Statistical methods

Statistical analysis was performed using two-sided paired Student *t* test comparison with control. The *p* values <0.05 were considered statistically significant. Linear regression, least squares and Deming comparison were used to determine the slope and correlation within treatment groups and one-way ANOVA with post tests was used to determine the probability and dosing trend (GraphPad Prism, version 6.0c; GraphPad).

Results

FKI inhibited S-180 sarcoma cell proliferation: ERK and AKT phosphorylation

The proliferation of S-180 sarcoma cells was markedly inhibited by FKI as compared with the other cell lines (Fig. 1A and data not shown). The IC₅₀ of FKI on S-180 cells at 48 h was 0.54 mg/ml. In contrast, the growth of embryonic liver cells was not inhibited by FKI (Fig. 1B). Further, we investigated short-term signal transduction pathways in S-180 cells activated by the IC₅₀ dose of FKI. FKI in a time dependent manner inhibited ERK and AKT phosphorylation (Fig. 2A and 2C), but there was no effect on P38 phosphorylation (data not shown). The signal transduction pathway in tumor cells that includes ERK and AKT phosphorylation also includes BAD phosphorylation, a likely component of the pathway leading to S-180 death [35]. We therefore compared the ability of FKI and matrine, the chemically defined active metabolite of oxymatrine found in high abundance in FKI, to inhibit MEK, ERK, AKT and BAD phosphorylation. Although FKI more potently inhibited MEK2 and ERK phosphorylation, matrine also inhibited the phosphorylation of these two kinases. Unlike FKI, matrine did not inhibit AKT or BAD phosphorylation at tested concentrations. Thus FKI was more effective at inhibiting these signaling molecules than matrine (Fig. 2B and 2D).

Effect of FKI on capsaicin-induced ERK phosphorylation in TRPV1 transfected HEK cells and spinal cord cells

We utilized a TRPV1 transfected HEK cell line to show that capsaicin, which activates TRPV1 in a dose (0–120 μmol/l) and time (0–5 min) dependent manner, induced TRPV1 and ERK phosphorylation (Fig. 3A). Capsaicin at 30 μmol/l (maximally effective dose in

HEK cells see Supplementary Fig. S1) also induced ERK phosphorylation in spinal cord cells (Fig. 3C), indicating that TRPV1 is functional in such cells. We next investigated the effect of FKI on capsaicin-induced ERK phosphorylation. FKI (0.27–1.08 mg/ml) in a dose dependent manner inhibited capsaicin induced ERK phosphorylation in TRPV1-HEK cells (Fig. 3B) and also in spinal cord cells (Fig. 3D). This suggests that FKI directly inhibited TRPV1-mediated signals associated with tumor-induced hyperalgesia.

Tumor induced inflammation and neuropathic pain

When mice were sacrificed following maximum growth of S-180 near the sciatic nerve, we observed that S-180 growth displaced muscle fibers (Fig. 4a, c, e, g and i) in tumor bearing mice as compared with naïve mice (Fig. 4b, d, f, h and j). Tumor (Fig. 4Ac versus d) and immune cells (Fig. 4Ae versus f) accumulated near the sciatic nerve of tumor bearing animals. We observed massive accumulation of CD45+ cells around the sciatic nerve as well as in the tumor (Fig. 3Ae). The nerve appears to be undergoing necrosis at the later stages of S-180 growth (Fig. 3Ac). However, we observed hyperalgesia as early as day 2 following S-180 sarcoma cell implantation near the sciatic nerve (Fig. 5A), which suggests that implanting the tumor induced the production of soluble inflammatory cytokines. We observed that IFN γ , IL-10, IL-6, IL-12p70, and CXCL1 (KC) were markedly increased in the serum of tumor bearing mice (Fig. 5B). Further, single spinal cord cells isolated from mice injected with S-180 cells showed increased pERK and TRPV1 expression (Fig. 5C). Taken together, these data suggest that the S-180 model of cancer pain maybe attributable to all three possible causes of pain; mechanical, neuropathic and inflammatory.

FKI inhibited tumor-induced hyperalgesia in S-180 tumor bearing mice

We investigated the dose and kinetics of FKI required to reduce S-180 tumor-induced pain. As shown in Fig. 6A, FKI treatment was maximally effective at or above 25 mg/kg over the 12 days of therapy in reducing tumor-induced hyperalgesia. Based on the pharmacokinetics of matrine, which showed a bioavailable half-life of 9 h [36–38], we attempted to maximize the therapeutic effect by using twice daily treatments. FKI injected twice daily appears to be more effective than once a day (Fig. 6B and C), at limiting both hyperalgesia as measured by mouse paw withdrawal and reducing tumor growth as measured by tumor weight. No significant side effects were observed by gross necropsy or behavioral changes in mice given 50 mg/kg twice daily. However, a once daily 100 mg/kg dose had a sedative effect.

FKI inhibited inflammatory cytokine expression *in vivo*

In addition to the analgesic and anti-tumor effects of FKI, we evaluated the effects of FKI on tumor-induced cytokine production. The 50 mg/kg dose delivered once or twice daily markedly reduced IFN- γ , IL-6, and KC levels in the serum of tumor-bearing mice, but did not reduce IL-12p70 or IL10 levels (Fig. 7).

FKI inhibited tumor growth and TRPV1-ERK phosphorylation *in vivo*

FKI delivered intraperitoneally to S-180 bearing mice reduced the growth of tumor implanted in the thigh muscle near the sciatic nerve in a dose dependent manner (Fig. 8A).

Increasing the dose to twice daily further decreased the tumor growth by at least 50% compared with PBS treated tumor bearing mice.

Western blotting demonstrated that FKI inhibited both tumor-induced TRPV1 and ERK phosphorylation in spinal cord cells. Cells were isolated following *in vivo* FKI treatment. In contrast to the increase in phosphorylation, total TRPV1 levels were not changed by FKI (Fig. 8B). Therefore, in the S-180 model of cancer-induced pain, FKI limited tumor growth and tumor-induced hyperalgesia based on several possible mechanisms including; limiting inflammatory cytokine production; directly reducing tumor growth and inhibiting TRPV1 mediated ERK activation.

Discussion

Late stage tumor patients can experience increased hyperalgesia which can be further enhanced by both chemotherapy and opioid treatment [5]. Therefore, we have investigated several mechanisms of action for a TCM therapy, FKI, based on its known clinical efficacy in reducing tumor growth and tumor-associated hyperalgesia. We utilized the pleiotropic mouse sarcoma S-180 model, which is characterized by both neuropathic and inflammation-induced hyperalgesia (Figs. 4 and 5B), for our studies. The S-180 tumor, like many tumors, exhibits elevated levels of phosphorylated ERK, AKT and BAD (Fig. 2), which are often induced by growth factor-dependent signals [39]. The activation of the ERK signaling pathway has recently also been associated with neuropathic pain through activation of TRPV-1 and other ion channel receptor signaling cascades [40,41]. Here we show that ERK is phosphorylated in spinal cord cells following tumor implantation, and FKI reduces both this phosphorylation and tumor-induced hyperalgesia (Figs. 6 and 8B).

Further, FKI inhibits inflammatory cytokine production in mice. To further evaluate the mechanisms of action for FKI, we demonstrated a direct effect of FKI on tumor cell growth and TRPV1-mediated signal transduction. FKI also inhibited capsaicin-TRPV1-induced ERK phosphorylation in both TRPV1 transfected HEK cells and cultured spinal cord cells. Thus, FKI inhibited both tumor growth and tumor-induced hyperalgesia in part by inhibiting tumor-induced inflammation and the TRPV1-ERK pathway.

Anti-inflammatory and cytotoxic components of *Sophora flavescens* and *Heterosmilaxis Japonica* have previously been identified [25,26,28,30,42–44]. The inhibitory concentrations for these individual compounds depends on the cell type being targeted, but tend to require greater than 1.2 mg/ml, which is at least twice the concentration of FKI required to limit S-180 cell growth *in vitro*. This observation suggests that chemical mixtures from plant extracts have an advantage over single agent in therapies.

Quinolizidine alkaloids, like matrine, appear to inhibit tumor cell growth by driving apoptosis through mitochondrial dependent signal cascades [45]. However, in our studies matrine at equal dosing was not as effective as FKI at inhibiting S-180 growth or signal cascades including pBAD, suggesting that the formulation of FKI results in an enhanced response over the effect of individual chemicals. Recently, Guenther et al. reported that combining inhibitors of PI3K/ Akt/mTor and Ras/MEK/ERK pathways synergistically

induced apoptosis in sarcoma cell lines [46]. They observed reduced phosphorylation of both Akt and ERK when cell lines were given U0126, a MEK inhibitor, and PI103 a PI3K/mTOR inhibitor. This observation is similar to our observation that FKI inhibits ERK and Akt phosphorylation (Fig. 2) and we have extended this *in vitro* observation with the *in vivo* observation that FKI treatment reduced tumor growth (Fig. 8).

While the potent effects of FKI in clinical settings are beginning to undergo quantitative statistical analysis [47], the quality and reproducibility of FKI preparations remains in question. These are common concerns with many traditional Chinese medicinals. China's State Food and Drug Administration (SFDA) is currently revising the quality control standards for FKI. The new standards include testing the quality of the plant roots grown under good agricultural practice for the presence and concentration of reference chemicals. Furthermore the final good manufacturing practice product is tested for pyrogenic activity, quantification of reference components by MS-HPLC that are further compared with a reference standard [48]. These added quality control standards should reduce concerns related to safety and reproducibility for any pharmaceutical and thereby broaden the clinical use of FKI as a cancer therapy.

Chemotherapies targeting mTOR, e.g., rapamycin, sirolimus, ridaforolimus, are used to treat sarcomas and have been reported to enhance neuronal excitability resulting in hyperalgesia when delivered systemically [49]. However, when delivered by intrathecal injection to the spine these same chemotherapies inhibit neuropathic pain [50]. Melemedjian et al. [50] report that mTOR inhibitors activate ERK pathways leading to spontaneous pain, however, combining rapamycin treatment with metformin, which blocks metabolic disease in part by blocking the S6 kinase cascade, blocked ERK phosphorylation and development of spontaneous pain. Sorafenib is a chemotherapy broadly used to treat sarcomas [49] that appears to act in part by inhibiting ERK phosphorylation [51]. Taken together, these studies suggest that our future studies should evaluate the effect of FKI on rapamycin and sorafenib mediated anti-cancer effects considering the chemotherapy delivery site, possible synergistic effects and overall effects on cancer-associated pain.

Extensive investigation into the cause and elevation of cancer pain has identified several partially effective therapies, each with their own limitations. Pharmaceutical inhibitors of TRPV1 resulted in hyperthermia [52–54]. Opioids by cross talking with both the TRPV1 signal cascade and cytokine or chemokine receptors signal cascades reduce pain sensation at the cost of immune competence [55]. Thus FKI represents a novel, complementary therapy for the control of tumor growth and tumor associated pain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix: Supplementary material

Supplementary data to this article can be found online at [doi:10.1016/j.canlet.2014.08.037](https://doi.org/10.1016/j.canlet.2014.08.037).

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Fig. 1.

FKI inhibited S-180 sarcoma cell proliferation. (A) FK1 inhibited S-180 cells proliferation in a dose depended manner. (B) FK1 promoted proliferation of newborn mouse liver cells. Relative viability of newborn mouse liver cells after incubation with increasing amounts of FK1 for 48 h is shown. Cell proliferation activity was determined using tritiated thymidine uptake assay. Untreated control cells were assigned 100% and all increases or decreases are relative to the control culture. Mean and standard error of the mean are reported. $N = 6$.

Fig. 2.

(A) Effect of FK506 (FKI) on ERK and AKT signaling pathway. FK506 0.54 mg/ml (48 h IC_{50}) was used to stimulate S-180 cells for increasing amounts of time. Total (t) and phospho- (p) ERK and AKT were determined by Western blotting. Both ERK and AKT phosphorylation was inhibited within 5 min of adding FK506. (B) Relative levels of ERK, and AKT signal for Fig. 2A. All Western blot bands were quantified using custom ImageJ (National Institutes of Health; <http://rsb.info.nih.gov/ij/>) script. The control or time zero time point was assigned a value of 1.0. (C) Comparing FK506 and matrine effects on

ERK/AKT and BAD signaling pathway. Western blotting was used to evaluate the amount of MEK2, ERK, AKT and BAD phosphorylation following stimulation of S-180 cells with equal concentrations of FKI or a chemical component, matrine. FKI inhibited phosphorylation of all four proteins; however, matrine only inhibited ERK phosphorylation. $N = 3$ representative Western result is shown. (D) Relative levels of ERK, AKT and BAD signal for Fig. 1C. All Western blot bands were quantified using custom ImageJ (National Institutes of Health; <http://rsb.info.nih.gov/ij/>) script. The control or time zero time point was assigned a value of 1.0.

Fig. 3.

Western blot analysis of capsaicin-induced ERK phosphorylation in TRPV1-HEK cells (A) or ex vivo spinal cord cells (C) shows FK506 inhibits ERK phosphorylation. Capsaicin-induced ERK phosphorylation in TRPV1-HEK Cells in a dose dependent manner see Supplementary Fig. S1. Dose response was evaluated after 5-min stimulation. (A) Capsaicin-induced ERK phosphorylation in TRPV1-HEK cells is time dependent. Western blotting shows that ERK and TRPV1 phosphorylation increases from untreated control through 5 min. Relative expression of pTRPV1 is shown to the left below the Western blot, while relative expression

of p-ERK is shown to the right. All Western blot bands were quantified using custom ImageJ (National Institutes of Health; <http://rsb.info.nih.gov/ij/>) script. The control or time zero time point was assigned a value of 1.0. (B) Capsaicin-induced TRPV1 and ERK phosphorylation in TRPV1-HEK cells is inhibited by FKI. Following 30 μ M capsaicin stimulation for 5 min, Western blotting for phosphorylated ERK and TRPV1 shows a three-fold increase in ERK phosphorylation and a two-fold increase in TRPV1 phosphorylation. Adding FKI to stimulated cells reduces the phosphorylation to control levels. Relative expression of pTRPV1 is shown to the left below the Western blot, while relative expression of p-ERK is shown to the right. All Western blot bands were quantified using custom ImageJ (National Institutes of Health; <http://rsb.info.nih.gov/ij/>) script. The control or time zero time point was assigned a value of 1.0. (C) Capsaicin-induced ERK phosphorylation in *ex vivo* cultured spinal cord cells is time dependent. Western blotting shows that ERK phosphorylation increases from untreated control through 5 (300 s) min following 30 μ M capsaicin stimulation of *ex vivo* cultured spinal cord cells. Relative expression levels are shown below the blot. (D) Capsaicin-induced ERK phosphorylation in cultured spinal cord cells is inhibited by FKI. Western blotting shows that ERK phosphorylation is increased following 30 μ M capsaicin stimulation for 5 min of *ex vivo* cultured spinal cord cells. The capsaicin induced ERK phosphorylation is decreased when FKI is added to the culture. $N = 3$ representative Westerns shown. Relative expression levels are reported in graphs below blots.

T:tumor M:muscle B:bone S:sciatic nerve BM:bone marrow
N:No tissue present due to sectioning artifact

T:tumor V: blood vessel M:muscle B:bone S:sciatic nerve

Fig. 4.

Histopathological analysis of S-180 tumor cells transplanted near the sciatic nerve of Swiss-Webster mice. (A) S-180 tumor cells grow around and into nervous tissue. (a) (200× HE staining) Cross section of S-180 sarcoma cells invading muscle and bone tissue. The arrows show the edge between sarcoma cells, muscle and bone. (b) (200× HE staining) A cross section of normal muscle and bone tissue. The arrows show the normal boundary of muscle, bone and bone marrow. (c) (200× HE staining) Cross section of sarcoma surrounding and invading nerve tissue. The arrows indicate the edge of tumor and nerve. (d)(200× HE

staining) Section of normal muscle and nerve tissue. The arrow indicates sciatic nerve. (e) (200× IHC staining CD45) CD45+ immune cells (brown staining) surround sciatic nerve. The arrows indicate the edges of the nerve. (f) (200× IHC staining CD45) Section of control naïve sciatic nerve. The arrow indicates the sciatic nerve. $N = 3$ for each histopathology study. (B) S-180 tumor implantation increases CD45+ immune cell infiltration. (g)(200× HE staining): A cross section of thigh muscle and tumor cells surrounded by the immune cells in S-180 bearing mouse. The arrows indicate the location of mononuclear immune cells. (h) (200× HE staining) A cross section of naïve thigh muscle with normal vessel. The arrows indicate the location of a normal vessel, (i) (200× IHC staining) CD45+ immune cells (brown) surrounding tumor and blood vessels. The arrows mark the location of blood vessels and tumor cells (pale blue staining) surrounded by immune cells (brown), (j) (200× IHC staining) Normal vessel in naïve muscle. The arrow marks the location of a blood vessel. $N = 3$ for each histopathology study.

Fig. 5.

Tumor-induced hyperalgesia correlates with increased inflammatory cytokine and phosphorylated ERK and TRPV1 levels. (A) S-180 induces hypersensitivity to temperature (hyperalgesia). Paw withdrawal latency time measured in seconds was quantified over 17 days in response to 55 °C stimulation. The mean time and standard error of the mean for the tumor-free naïve mice ($N = 10$) is shown with circles, while that of the S-180 bearing mice is shown with squares. Deming Model II analysis of linear regression indicates that S-180 transplantation induces a significant change for the horizontal ($p < 0.05$). (B) S180 sarcoma

induces inflammatory cytokine expression *in vivo*. Serum samples were subjected to Meso Scale Discovery analysis of murine inflammatory cytokines. Cytokine levels are reported as mean $\pm$ SEM in picograms/ml with the naïve control levels reported with black bars and the S-180 tumor samples being reported with gray bars. $N = 5$. (C) S-180 sarcoma induces ERK phosphorylation along with TRPV1 expression and phosphorylation in spinal cord cells. Spinal cords were isolated and quick frozen on day 12 following tumor implantation. Tissues were lysed and subjected to Western blotting for total ERK, phosphorylated ERK and TRPV1 protein levels. $N = 3$ representative Westerns shown. (D) Relative amounts of expression are reported. All Western blot bands were quantified using custom ImageJ (National Institutes of Health; <http://rsb.info.nih.gov/ij/>) script. The control or time zero time point was assigned a value of 1.0.

Fig. 6.

FKI inhibited tumor-induced hyperalgesia in a dose dependent manner. (A) Dose response to FKI in the hyperalgesia model. Mean paw withdrawal latency time in seconds $\pm$ standard error of the mean is reported for naïve mice, untreated tumor control mice and mice receiving 25 mg/kg of FKI for 12 days. The day 12 times are reported. The 50 mg/kg dose resulted in significant increase in latency between tumor control and treatment $p < 0.05$. However the 100 mg/kg dose induced additional behavioral side effects. $N = 10$. (B) Comparison of once daily and twice daily 25 mg/kg treatment on tumor-induced

hyperalgesia. Mean paw withdrawal latency times in seconds $\pm$ standard error of the mean for naïve mice, untreated tumor control mice and mice receiving one or two 25 mg/kg treatments of FK1 for 12 days are reported. $N = 10$. (C) Comparison of once daily and twice daily 50 mg/kg treatment on tumor-induced hyperalgesia. Mean paw withdrawal latency times in seconds $\pm$ standard error of the mean are reported for naïve mice, untreated tumor control mice and mice receiving one or two 50 mg/kg treatments of FK1 for 12 days. $N = 10$. Deming analysis of linear regression was used to demonstrate difference from naïve control.

Fig. 7.

FKI inhibited tumor-induced inflammatory cytokine expression *in vivo*. Serum samples were subjected to Meso Scale Discovery analysis for murine inflammatory cytokines. Cytokine levels are reported as mean $\pm$ SEM in pg/ml. The individual cytokines or chemokines (KC also known as CXCL1) are indicated at the top of each graph. Compared on each graph are the serum cytokines levels detected from naïve, untreated tumor and those from 25 or 50 mg/kg FK1 given once (1 $\times$) or twice (2 $\times$) daily for 12 days. Statistical differences are indicated between tumor control and naïve mice and tumor control and 50 mg/kg $\times$ 2 treatments. $N = 5$.

Fig. 8.

FKI treatment inhibited S180 tumor growth, which correlates with inhibited phosphorylation of ERK and TRPV1 *in vivo*. (A) FKI, in dose dependent manner, inhibited S180 tumor growth *in vivo*. After 12 days of *in vivo* treatment, mice were sacrificed. The weight of the non-tumor bearing left leg was subtracted from the weight of the tumor bearing right leg. Mean tumor weights in grams $\pm$ SEM are reported for untreated tumor control vs. once (1 $\times$) or twice (2 $\times$) daily treated at 25 mg/kg or 50 mg/kg tumors. Statistical difference is observed at 50 mg/kg treatment. $N = 10$ per group. (B) FKI inhibited ERK and TRPV1

Phosphorylation *in vivo*. Spinal cords were isolated and quick frozen at day 12 following tumor implantation. Tissues were lysed and subjected to Western blotting for total ERK, phosphorylated ERK and TRPV1 protein levels. $N = 3$ representative Westerns shown. (C) Relative amounts of expression are reported FK1 inhibited ERK and TRPV1

Phosphorylation *in vivo*. All Western blot bands were quantified using custom ImageJ (National Institutes of Health; <http://rsb.info.nih.gov/ij/>) script. The control or time zero time point was assigned a value of 1.0. Mice treated twice daily (2×) with 50 mg/kg FK1 had phosphorylated ERK and TRPV1 levels similar to tumor naïve (0) mice while untreated tumor bearing mice had 2-fold higher phosphorylation levels.