



Bindarit Attenuates Pain and Cancer-Related Inflammation by Influencing Myeloid Cells in a Model of Bone Cancer

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Abstract

C–C motif chemokine ligand 2 (CCL2) is a small cytokine that functions in inflammation and cancer development. Bindarit, a CCL2 inhibitor, is a small anti-inflammatory molecule proven safe by phase II trials in type 2 diabetic nephropathy patients. As cancer-related inflammation is a cause of pain, we investigated whether Bindarit suppresses cancer-related inflammation and pain. We established a bone-cancer mouse model by inoculating cancer cells. After applying Bindarit, we performed pain sensitivity tests and checked cancer development by X-ray. Using flow cytometry and qRT-PCR assays, we assessed the effect of Bindarit on peripheral blood monocyte mobilization and M2 macrophage polarization. We also investigated the targets of Bindarit using western blotting and luciferase assay. Bindarit-treated mice performed better in pain sensitivity tests compare to control mice. X-ray imaging showed that Bindarit-treated mice had fewer cancer cell colonies and smaller overall tumor burden. Bindarit reduced the number of monocytes in peripheral blood and down-regulated the expression of M2 macrophage polarization makers. Bindarit also inhibited IKK β phosphorylation. Bindarit efficiently relieves cancer-related pain and suppresses cancer development. Bindarit inhibits monocyte mobilization in peripheral blood as well as M2 macrophage polarization. IKK β is identified as a target of Bindarit.

Keywords Bindarit · CCL2 · Cancer · Inflammation · Pain

Introduction

C–C motif chemokine ligand 2 (CCL2), also referred to as monocyte chemoattractant protein 1 or small inducible cytokine A2, is a small cytokine which belongs to the CC chemokine (or β -chemokine) family. The CC chemokine proteins contain two tandem cysteines located at their amino terminus. More than 20 distinct members of this family have been identified, named as CCL1–CCL28. CCL2 induces monocytes to leave the bloodstream and migrate into the

surrounding tissues turning to tissue macrophages. CCL2 also functions in inflammation by recruiting monocytes memory T cells and dendritic cells to the inflammation sites which are caused by tissue injury or infection (Carr et al. 1994; Xu et al. 1996). CCL2 is highly expressed and secreted by monocytes, macrophages and dendritic cells and located at the plasma membrane of endothelial cells.

CCL2 has been implicated in pathogeneses of various diseases such as psoriasis, rheumatoid arthritis, atherosclerosis epilepsy, brain ischemia, Alzheimer's disease, traumatic brain injury and cancer (Fabene et al. 2010; Foresti et al. 2009; Gerard and Rollins 2001; Hickman and El Khoury 2010; Kim et al. 1995; Ransohoff et al. 1993; Semple et al. 2010a, b; Xia and Sui 2009). CCL2 was identified as a tumor-derived chemotactic factor which recruits monocytes/macrophages to tumor tissues (Caronni et al. 2015; Mantovani et al. 1986). Specifically, it has been extensively reported that CCL2 is related to cancer-related inflammation (Caronni et al. 2015) and pain (Lu et al. 2014). The major cell type that is affected by CCL2 is the myeloid cells, including inflammatory monocytes, macrophages (tumor-associated macrophages: TAM), neutrophils

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(tumor-associated neutrophils), dendritic cells, and myeloid-derived suppressor cells. CCL2 is highly expressed in multiple tumors, and the high expression of CCL2 increases monocyte mobilization, which decreases the survival rate of patients. Meanwhile, in some types of cancers, e.g. pancreatic cancer, down-regulation of monocytes in peripheral blood significantly increases the survival rate of patients (Sanford et al. 2013). In the addition of its role in regulating monocyte mobilization, CCLs also play a key role in tumor development by regulating M2 macrophage polarization (Roca et al. 2009a, b). M2 macrophage polarization correlates with high expression of CCL2 in tumor, and leads to the deterioration of the tumor (Qian and Pollard 2010).

Bindarit (2-methyl-2-[(1-[phenylmethyl]-1H-indazol-3-yl)methoxy] propanoic acid) is a small molecule that has been shown to be an anti-inflammatory molecule in various animal models (Guglielmotti et al. 1993, 2002; Ialenti et al. 2011; Ramnath et al. 2008; Zoja et al. 1998). It is able to prevent the chronicity of inflammation and thus decrease the cytotoxic effects of inflammation (Baggiolini and Dahinden 1994; Conductier et al. 2010; Lloyd et al. 1997; Luster and Rothenberg 1997). Bindarit had been proven safe and efficient by phase II trials in type 2 diabetic nephropathy and lupus nephritis patients.

Bindarit inhibits the synthesis of C–C chemokines including CCL2, CCL7, and CCL8 (Mirolo et al. 2008). Treatment of Bindarit has been shown to lead to a dramatic reduction of urinary CCL2 and albumin excretion (Ble et al. 2011; Ruggerenti et al. 2010). Since CCL2 plays a critical role in cancer-related inflammation, it is possible that Bindarit treatment might inhibit the cancer-related inflammation and relieve the pain. As Bindarit has been shown to be a safe drug, it would be clinically valuable to assess its effect in inhibiting cancer and relieving cancer-related pain.

The inhibition of CCL2 expression by Bindarit treatment is controlled by multiple pathways that are related to the promoter region of CCL2. Nuclear factor (NF)- κ B pathway is one of the pathways that have been shown to be involved in CCL2 expression (Boekhoudt et al. 2003; Ping et al. 2000). It has also been reported that Bindarit inhibits phosphorylation of both I κ B α and p65, which are two downstream factors in the NF- κ B pathway (Mora et al. 2012). p65 functions at the promoter of CCL2 and it is possible that Bindarit inhibits expression of CCL2 by inhibits phosphorylation of p65. Phosphorylation of I κ B α is also inhibited by Bindarit. Therefore it is possible that Bindarit might function at a common upstream site of I κ B α and p65. It would be interesting to investigate the upstream target(s) of Bindarit in this pathway.

Materials and Methods

Mouse Bone-Cancer Model

Experiments were performed with 30 adult male athymic nude mice (Shandong University Animal Centre), approximately 7–8 weeks old, weighing 25–30 g, received an intraperitoneal inoculation of breast sarcomatous Walker 256 cells (Shanghai Pharmaceutical Industry Research Institute, Shanghai, China). After 1 week, cells in the ascites were collected and resuspended in normal saline to a final concentration of 2×10^7 cells/mL. Bone cancer was then established by inoculating Walker 256 cells (2×10^5 cells, 10 μ L) into the intramedullary space of the mouse femur. Control mice ($n = 20$) were injected with heat-killed cancer cells. The protocol was approved by the Committee on the Ethics of Animal Experiments of The Second Hospital of Shandong University. All surgery was performed under 50 mg/kg intraperitoneal sodium pentobarbital (Sigma-Aldrich, St. Louis, MO, USA) anesthesia, and all efforts were made to minimize suffering.

Behavioral Assays

The analyses of the behavioral experiments were made by the experimenters blinded to the experimental groups. Measurements of spontaneous nocifensive behavior, mechanical allodynia, and thermal hyperalgesia were performed for 28 days after tumor inoculation during the experimental period. Bindarit (Nanjing Chemlin Chemical Industry Co., Nanjing, China) was prepared as 100 mM stock solution using DMSO as vehicle, which was then further diluted to assay specific concentrations. Effects of Bindarit were measured on day 7 through 28 after tumor inoculation. Paw withdrawal latency (PWL) was measured for both paws. In brief, rats were allowed to acclimate for 30 min in an inverted clear plastic chamber on a glass surface. Radiant heat was applied to the plantar surface of each hind paw using an automatic plantar analgesia tester (Institute of Biomedical Engineering, Chinese Academy of Medical Science, Tianjin, China). The heat intensity was adjusted, so that the PWL was 14 ± 2 s in the normal rats. A cutoff of 20 s was established to prevent tissue damage. Each paw was tested three times, and the latencies for each paw were averaged. Animals were tested before tumor inoculation to assess basal values. The same procedure was performed on days 7, 14, 21, and 28 after tumor inoculation. A nociceptive score was assigned by adapting the classic method of measuring nociceptive responses to the injection of formalin (Sigma-Aldrich, USA). Thus, a score of 0 was assigned when the paw was in normal contact

with the floor, 0.5 when the paw was flinched, 1 when the paw was lifted, and 2 when the paw was licked. The numbers of each behavior during the observation time (600 s) were counted and were multiplied by their respective scores. The final score was determined by the summation of the multiplied respective scores. Animals were tested before tumor inoculation to assess basal values. The same procedure was performed on days 7, 14, 21, and 28 after tumor inoculation. Mechanical hyperalgesia was measured using a single rigid filament attached to a handheld transducer (automatic plantar analgesia tester; Institute of Biomedical Engineering, Chinese Academy of Medical Science, Tianjin, China). Animals were acclimated to their surroundings daily for 10 min for three consecutive days in a plexiglass box on a metal grid surface prior to testing. On the testing days, rats were allowed to acclimate for 5–10 min. A rigid filament was pressed perpendicularly against the medial plantar surface of the hind paw with an increasing force. Brisk paw withdrawal or paw flinching accompanied by head turning, biting, and/or licking upon application of an increasing force was considered as a positive response. The paw withdrawal threshold was defined as the minimal force (g) required to evoke the cited positive responses. Each hind paw of rats was tested three times and the data were averaged. The interval between consecutive tests of the same paw was 5 min. The same procedure was performed on days 7, 14, 21, and 28 after tumor inoculation.

Radiography

High-resolution X-ray images of the mediolateral plane of the ipsilateral (cancer or vehicle-injected) femur were obtained following short term anesthesia of mice with ketamine/xylazine (0.005 ml/g, 50 mg/10 kg, Western Medical, Arcadia, CA, USA; Sigma-Aldrich). All radiographic image quantifications were obtained in a blinded fashion.

Number of Individual Cancer Cell Colonies and Overall Tumor Burden Quantification

Exploratory studies established that by 60 days following the initial injection of breast tumor cells into the mouse femur, less than 5% of the entire area of bone contains normal hematopoietic bone marrow. The radiographs were enlarged, so that each individual cancer cell colony was easily visualized, assigned a number, traced, the location and the area of the viable cancer cell colony (the translucent area) captured, and transferred to Image Pro Plus (Media Cybernetics, Rockville, MD, USA). The number of cancer colonies in each region was calculated as was the total tumor area in each tumor-bearing femur. These measurements were exported to Excel, where the average area and average number of

cell colonies of each treatment group were then determined (bone cancer + vehicle vs. bone cancer + Bindarit).

Flow Cytometry

Mouse single-cell suspensions were made from peripheral blood in a model of bone cancer. These cells were stained with indicated specific conjugated antibodies and subjected to flow cytometry, and cell sorting as described using LSR II (BD Bioscience, Franklin Lakes, NJ, USA) and FACS Aria (BD Bioscience, USA) flow cytometers, respectively. For intracellular cytokine staining, single-cell suspensions were further stimulated for 4 h with phorbol myristate acetate (PMA) plus ionomycin in the presence of monensin and then subjected to intracellular staining and flow cytometry.

RT-PCR

Total RNA was extracted using Trizol RNA extraction kit (Invitrogen, USA) following manufacturer's protocols. The quantity and quality of extracted RNA samples were determined by BioAnalyzer 2100 (Agilent, USA). The cDNA was synthesized with the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher, USA). The RT-PCR was performed with GoTaq Green Master Kit (Promega, USA) following manufacturer's protocols.

Luciferase Assay

The HEK293 cells (2×10^5) were seeded on 24-well plates and transfected with interleukin (IL)-6 promoter reporter plasmid by the standard calcium phosphate precipitation. In the same experiment, empty control plasmid was added to ensure that each transfection receives the same amount of total DNA. To normalize for transfection efficiency, 1 μ g of pRL-TK promoter Renilla luciferase reporter plasmid was added to each transfection. Luciferase assays were performed with a dual-specific luciferase assay kit (Promega, Madison, WI, USA). Firefly luciferase activities were normalized based on Renilla luciferase activities.

Statistical Analysis

Data were presented by mean $\pm$ SEM. Data were analyzed for statistical significance by Prism (GraphPad Inc., La Jolla, CA, USA). One-way (for data in Figs. 1, 4) or two-way ANOVA (for data in Figs. 2, 3, 5) analyses followed by a Tukey post hoc test were performed as appropriate. Significances were determined as $p < 0.05$.

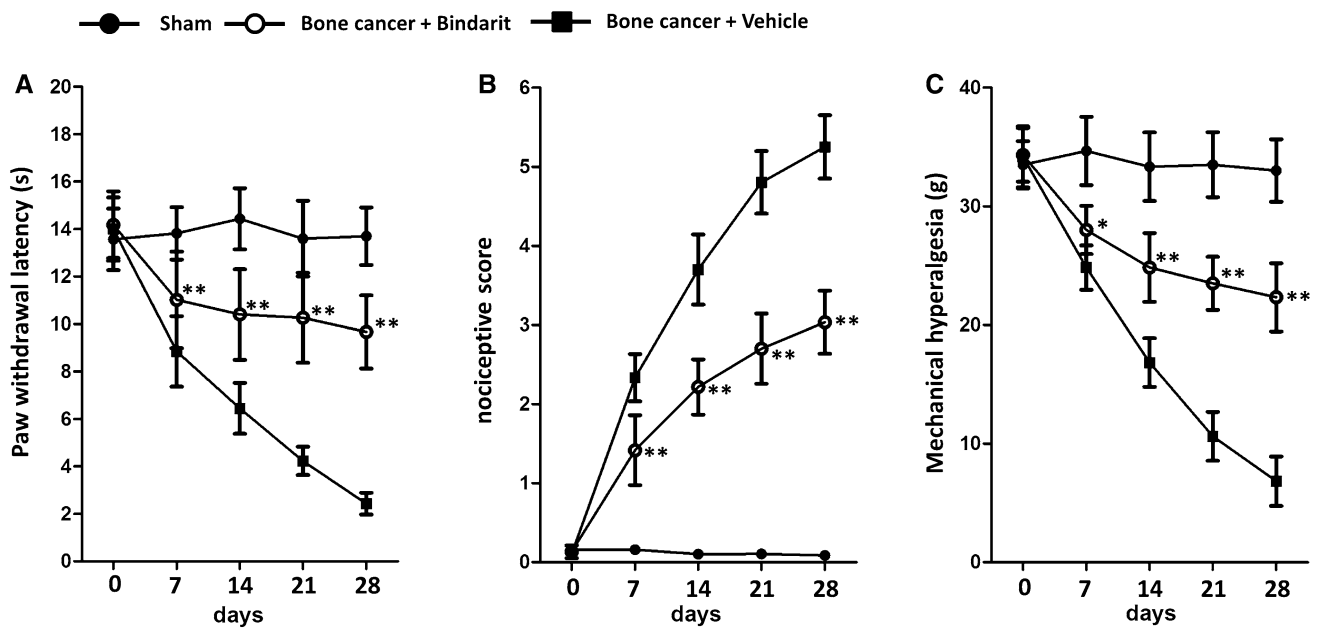


Fig. 1 Continuous administration of Bindarit significantly alleviated bone-cancer pain. Continuous administration of vehicle or Bindarit (100 mg/kg) was performed on day 7 after tumor inoculation, a single dose per day for 21 days. Sham group (sham) was treated with vehicle. Test was assessed on the days 7, 14, 21, and 28 after tumor inoculation. Baseline (0) was tested before tumor inoculation. Pain

behaviors were determined by thermal hyperalgesia (a), spontaneous nociceptive behavior (b), and mechanical allodynia (c). Data are presented as mean $\pm$ SEM values and representative of at least six independent experiments. Statistical analyses represent variations in experimental replicates. * $p < 0.05$; ** $p < 0.01$

Fig. 2 Continuous administration of Bindarit significantly reduces the number of individual cancer cell colonies and overall tumor burden. **a** Radiographs of bone-cancer femur showed that Bindarit group looks much better than vehicle group at the day 60 after tumor inoculation. Treatment of bone tumor-bearing animals with Bindarit significantly reduced the total tumor burden area (mm²) (b) and number of cancer cell colonies (c) in the femur as compared to vehicle-treated animals. Data are presented as mean $\pm$ SEM values and representative of at least six independent experiments. Statistical analyses represent variations in experimental replicates. ** $p < 0.01$

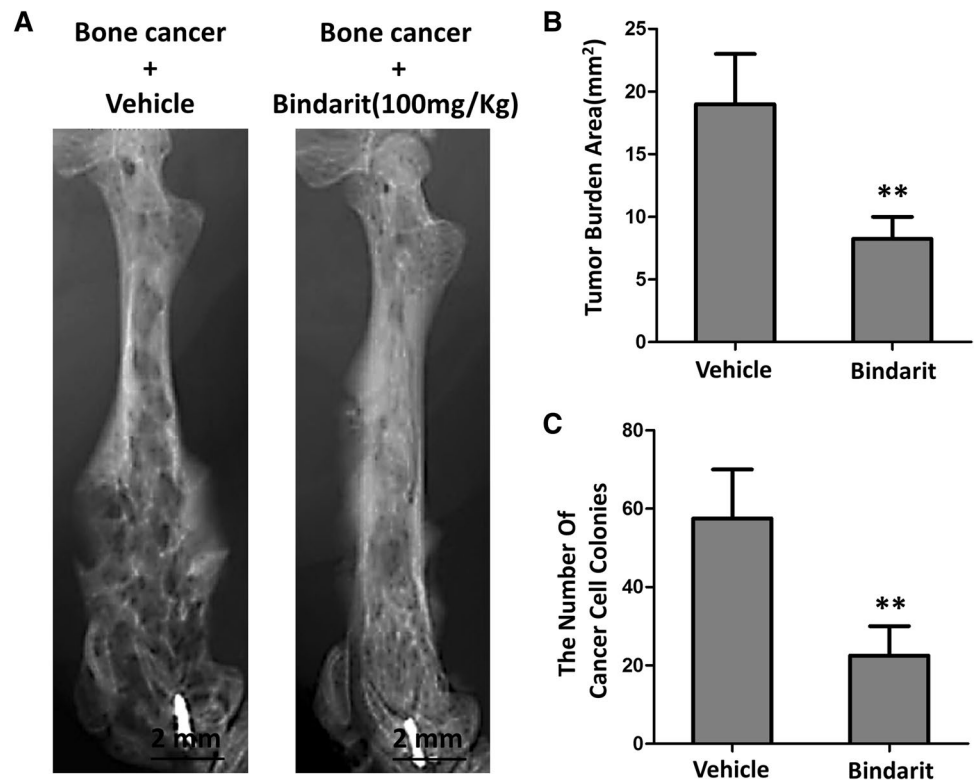


Fig. 3 Bindarit attenuates monocyte mobilization in a model of bone cancer. Flow cytometry gating strategies were used to define IM ($CD115^+/CD11b^+/CD16^-/CX3CR1^{low}$) and RM ($CD115^+/CD11b^+/CD16^+/CX3CR1^{hi}$) in peripheral blood

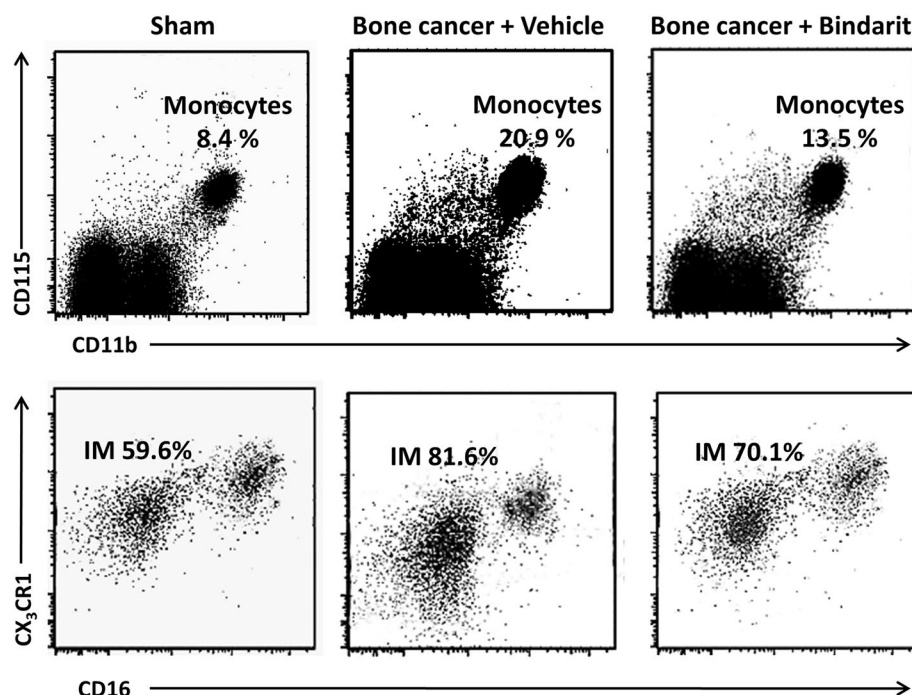
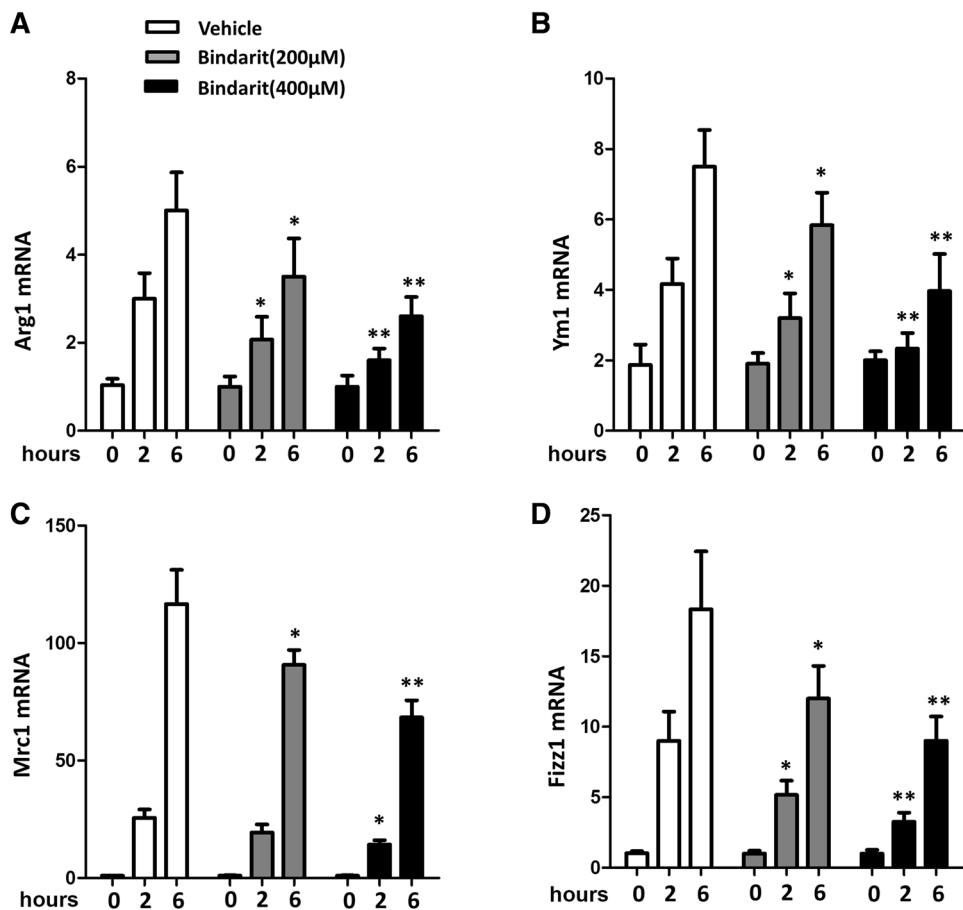


Fig. 4 Bindarit reduced the levels of cytokines produced by macrophage. **a–d** qRT-PCR analysis of Arg1 (**a**), Ym1 (**b**), Mrc1 (**c**), and Fizz1 (**d**) using macrophages in peripheral blood from bone-cancer mice under 0, 200, and 400 μ M Bindarit at 0, 2, and 6 h after IL-4 stimulation. Data are presented as fold relative to the Actin mRNA level. Data are presented as mean $\pm$ SEM values and representative of at least three independent experiments. Statistical analyses represent variations in experimental replicates. * $p < 0.05$; ** $p < 0.01$



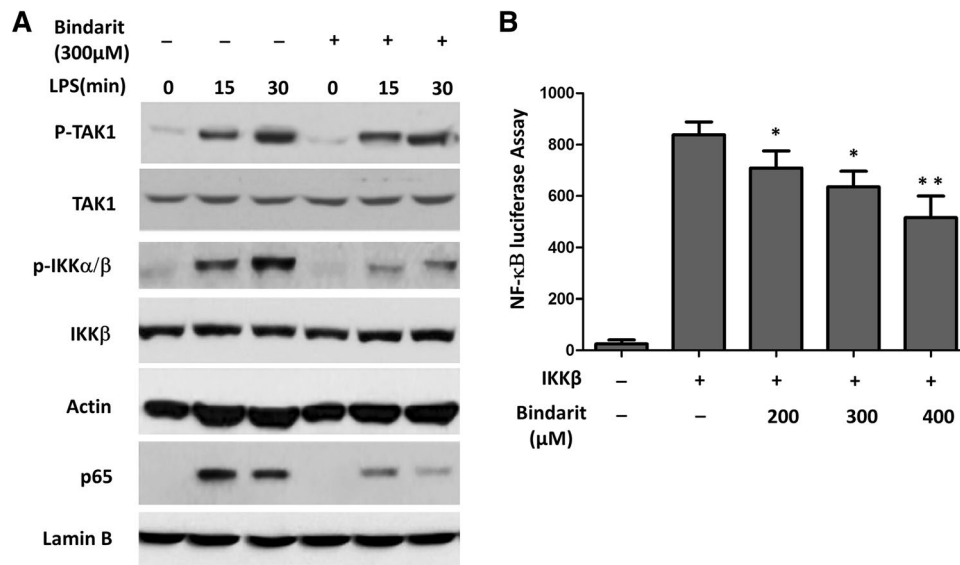


Fig. 5 Activation of NF-κB is impaired in macrophage treated with Bindarit for 24 h. **a** IB analyses of TAK1, IKKα/β phosphorylated (P-) and total proteins in cytoplasmic or p65 nuclear extracts of macrophage stimulated with lipopolysaccharide (LPS) for the indicated time periods with or without Bindarit (300 μM) for 24 h; **b** HEK293 cells (2×10^5) were transfected with the IL-6 promoter

luciferase plasmid and co-transfected with expression plasmid as indicated. Cells were incubated with Bindarit (300 μM) for 2 h before harvest. Luciferase assays were performed 36 h after transfection. Data are representative of three independent experiments. * $p < 0.05$; ** $p < 0.01$

Results

Bindarit Relieves Bone-Cancer-Related Pain

As CCL2 is related to pain, we tested whether Bindarit relieves pain in the bone-cancer mice. Cancer cells were inoculated to mice, and 3 days after inoculation 100 mg/kg of Bindarit was fed to the mice. Bindarit dose exhibited protective effect against bone-cancer-induced pain and inflammation based on our pilot study (data not shown). Seven days after the inoculation of the cancer cells, three different pain sensitivity tests (thermal hyperalgesia, spontaneous nocifensive behavior, and mechanical hyperalgesia) were carried out. As shown in Fig. 1A, mice inoculated with bone-cancer cells showed significantly lower PWL than the control mice (Sham) in thermal hyperalgesia test from 7 days after the inoculation. Strikingly, feeding Bindarit to the mice inoculated with bone-cancer cells significantly increased the PWL, compared to feeding with vehicle only. Treatment of Bindarit also significantly improved the performance of the mice in spontaneous nocifensive behavior test (Fig. 1b) and in mechanical hyperalgesia test (Fig. 1c). These data suggest that treatment of Bindarit significantly relieves the pain caused by bone cancer.

Bindarit Treatment Reduces Cancer Cell Colonies and Overall Tumor Burden

Next, we sought to investigate whether Bindarit has any tumor suppression effect. To do this, 60 days after tumor inoculation and Bindarit administration, the bone of the mice was checked by X-ray radiography. As shown in Fig. 2a, there were fewer cancer cell colonies and smaller tumor burden area at the bone of the mice continuously treated by Bindarit. Quantified tumor burden areas and numbers of cancer cell colony are shown in Fig. 2b, c, respectively. These data suggest that Bindarit treatment suppresses tumor development.

Bindarit Treatment Reduces Monocyte Mobilization in the Peripheral Blood

As we mentioned before, high expression of CCL2 increases monocyte mobilization, which in turn reduces survival rate of cancer patients. As Bindarit is a CCL2 inhibitor, we next investigated whether Bindarit treatment functions in regulating peripheral monocyte mobilization. Monocytes were sorted out from bone-cancer mice treated with or without Bindarit by flow cytometry. As shown in Fig. 3, bone-cancer mice have more monocytes in the peripheral blood compared

with sham mice (20.9 vs. 8.4%). Strikingly, in the peripheral blood of bone-cancer mice treated with Bindarit, the monocytes level decreased to 13.5%, indicating Bindarit reduced monocyte mobilization in peripheral blood.

Bindarit Treatment Down-Regulates *in Vitro* M2 Macrophage Polarization

We then tested whether Bindarit plays any role in M2 macrophage polarization. Macrophages (2×10^5) were sorted out from the peripheral blood of bone-cancer mice and treated with different concentrations of Bindarit after IL-4 stimulation. Total mRNAs then were extracted and qPCR was carried out to determine the expression levels of M2 macrophage markers Arg1, Ym1, Mrc1, and Fizz1. As shown in Fig. 4a, treatment of 200 μ M Bindarit 2 h after IL-4 stimulation significantly decreased the level of Arg1 mRNA. Treatment of 400 μ M Bindarit decreased the Arg1 mRNA level to a greater extent, indicating the Bindarit functions in a dose-dependent manner. Similarly, mRNA levels of other M2 macrophage polarization markers Ym1, Mrc1, and Fizz1 were also down-regulated after Bindarit treatment (Fig. 4b–d). Taken together, these results showed that Bindarit significantly inhibited M2 macrophage polarization *in vitro*.

Bindarit Down-Regulates CCL2 Expression by Inhibiting Phosphorylation of I κ B α and p65

Next, we sought to identify the target protein(s) of Bindarit. It has been reported that Bindarit inhibits phosphorylation of both I κ B α and p65 (Mora et al. 2012). It is also known that p65 functions at the promoter of CCL2 raise a possibility that Bindarit inhibits expression of CCL2 by inhibiting phosphorylation of p65. We hypothesize that Bindarit might target upstream factor(s) of I κ B α and p65. To test this possibility, we examined the phosphorylation of TAK1 and IKK β , two upstream factors in this signaling pathway. Consistent with previous report, western blotting showed that the phosphorylation of nuclear p65 is significantly reduced after Bindarit treatment. The phosphorylation of TAK1 is not affected by Bindarit treatment, while phosphorylation of IKK β is dramatically reduced under Bindarit treatment (Fig. 5a), indicating that IKK β is the target of Bindarit. NF- κ B luciferase assay further confirmed this conclusion (Fig. 5b).

Discussion

Bindarit is a safe compound that inhibits the expression of inflammatory chemokine CCL2. Given the roles of CCL2 in cancer-related inflammation and pain, we hypothesize that

Bindarit might help cancer patients against inflammation and cancer-related pain. To test this hypothesis, we employed a bone-cancer mice model. Our results show that Bindarit significantly relieved the pain caused by bone-cancer inoculation. Importantly, we show that Bindarit significantly suppressed bone-cancer development. After Bindarit treatment, the number of cancer cell colonies was drastically reduced. Moreover, the overall cancer burden area was also smaller in Bindarit-treated mice than in untreated mice. Consistent with the *in vivo* results, our *in vitro* assays showed that Bindarit reduced monocyte mobilization in the peripheral blood as well as M2 macrophage polarization. Although it has been reported that Bindarit inhibits the protein expression of inflammatory chemokine CCL2, the underlying mechanism is not well understood. To figure out the targets of Bindarit, we examined the expression level and phosphorylation level of multiple factors in the NF- κ B pathway, which is implicated in CCL2 expression. Our results showed that, in addition to I κ B α and p65, IKK β phosphorylation was also down-regulated after Bindarit treatment, while TAK1 phosphorylation stayed unaffected. Taken together, our data revealed a possible pathway for Bindarit to function in controlling cancer development and relieving cancer-related pain. Bindarit inhibits CCL2 expression by decreasing phosphorylation of IKK β , I κ B α , and p65. Reduced CCL2 level then inhibits monocyte mobilization in the peripheral blood and suppresses M2 macrophage polarization, which then lead to less inflammatory pain and delayed cancer development.

The pain relieving effect of Bindarit is convincing. Three different assays have been employed and all showed significant differences between the groups with or without Bindarit treatment, suggesting a dramatic pain relieving effect of Bindarit. Our results showing that Bindarit treatment helps to relieve cancer-related pain as well as to suppress cancer development suggest that Bindarit might work as an additional drug for cancer patients. A single drug that inhibits cancer development and relieves pain at the same time is of great clinical values. Besides the pain related to cancer itself, many cancer patients also suffer from pains caused by chemotherapy or radiotherapy. It would be interesting to test whether Bindarit can help to relieve those kinds of pains.

Our results show that Bindarit suppresses monocyte mobilization in peripheral blood and M2 macrophage polarization provides further evidences that Bindarit has a cancer suppression function. It is well known that increased monocyte mobilization in the peripheral blood is a risk factor for the survival of cancer patients. Thus, our results suggest that Bindarit might help to increase the survival rate of cancer patients.

It has also been shown that M2 macrophage polarization is a hallmark of cancer development and reverting M2 TAM to M1 has been proposed to be an important anti-cancer

immunotherapy strategy (Mills et al. 2016). Our result that Bindarit reduces M2 macrophage polarization is consistent with the previous studies and further supports the possibility that Bindarit might be an anti-cancer drug.

It has been well established that Bindarit functions in inflammation by inhibiting the expression of the chemokine CCL2. The facts that Bindarit reduces the phosphorylation level of the NF- κ B transcription factor p65 and that p65 regulates the expression of CCL2 lead to a proposal that Bindarit inhibits CCL2 expression by regulating the phosphorylation of p65. However, although NF- κ B pathway has been shown to be involved in CCL2 expression regulation, p65 might not be the only factor that is affected by Bindarit. Our results show that in addition to I κ B α and p65, phosphorylation of IKK β , but not TAK1, is also inhibited by Bindarit. These data not only identified a new target of Bindarit, but also confirmed the role of NF- κ B pathway in CCL2 expression and inflammation. These findings are consistent with the roles of Bindarit in relieving cancer-related pain and in inhibiting tumor development in bone-cancer mice model. This consistency suggests the potential of Bindarit as a anti-tumor and pain relief drug. It would be of great importance for clinic to investigate whether Bindarit functions in pain relieving and tumor suppressing in mice models for other kinds of cancers. It would also be interesting to study the mechanism for Bindarit to regulate the phosphorylation of the NF- κ B transcription factors. In addition, NF- κ B might not be the only pathway that is affected by Bindarit. Further study is required to investigate other targets of Bindarit. A high-throughput proteomic study might be helpful to address this question.

In conclusion, our results show that Bindarit inhibits monocyte mobilization in the peripheral blood and reduces macrophage M2 polarization. Moreover, Bindarit treatment relieves cancer-related pain and suppresses cancer development in a bone-cancer mouse model. Bindarit inhibits phosphorylation of IKK β as well as I κ B α and p65, which provides new insights to the mechanism for Bindarit inhibiting inflammatory chemokine CCL2 and the cancer suppression effect of Bindarit. Taken together, our findings suggest that Bindarit is a potential treatment for cancer patients.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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