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The interaction between RUNX2 and core binding factor beta (CBFβ) as a potential therapeutic target in canine osteosarcoma

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Abstract

Osteosarcoma remains the most common primary bone tumor in dogs with half of affected dogs unable to survive one year beyond diagnosis. New therapeutic options are needed to improve outcomes for this disease. Recent investigations into potential therapeutic targets have focused on cell surface molecules with little clear therapeutic benefit. Transcription factors and protein interactions represent underdeveloped areas of therapeutic drug development. We have utilized allosteric inhibitors of the core binding factor transcriptional complex, comprised of core binding factor beta (CBFβ) and RUNX2, in four canine osteosarcoma cell lines Active inhibitor compounds demonstrate anti-tumor activities with concentrations demonstrated to be achievable *in vivo* while an inactive, structural analog has no activity. We show that CBFβ inhibitors are capable of inducing apoptosis, inhibiting clonogenic cell growth, altering cell cycle progression, and impeding migration and invasion in a cell-line dependent manner. These effects coincide with a reduced interaction between RUNX2 and CBFβ and alterations in expression of RUNX2 target genes. We also show that addition of CBFβ inhibitors to the commonly used cytotoxic chemotherapeutic drugs doxorubicin and carboplatin leads to additive and/or synergistic anti-proliferative effects in canine osteosarcoma cell lines. Taken together we have identified the interaction between components of the core binding factor transcriptional complex, RUNX2 and CBFβ, as a potential novel therapeutic target in canine osteosarcoma and provide justification for further investigations into the anti-tumor activities we describe here.

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Data Availability Statement

All data can be made available upon reasonable request.

Conflict of Interest

The authors declare no conflicts of interest.

Keywords

canine sarcoma; osteosarcoma; novel therapeutic targets; transcription factor; RUNX2; core-binding factor beta (CBFβ)

Introduction

The treatment for osteosarcoma (OSA) has remained unchanged since the addition of adjuvant cytotoxic chemotherapy regimens (consisting of methotrexate, doxorubicin and cisplatin/carboplatin) to amputation or limb-sparing approaches in the late 1970s.[1,2] Although these advances on OSA treatment represented an improvement of the survival rates in dogs and humans in the non-metastatic disease setting, 10-15% after 2 years and 65-70% after 5 years, respectively, little advancements have been achieved in patients with metastatic and/or relapsed disease despite great efforts to improve the prognosis in these patients through treatment intensification.[3–5] Hence, there is an unmet need to find new therapeutic targets in order to increase the survival rates not only in patients with metastatic OSA but also with non-metastatic disease.

Among molecular targets investigated for therapeutic intervention in human OSA are cell surface receptor tyrosine kinases that are overexpressed, such as insulin-like growth factor 1 receptor (IGF1R), epidermal growth factor receptor 2 (HER-2/ERBB2), platelet-derived growth factor receptor (PDGFR) and hepatocyte growth factor receptor (HGFR/MET),[5] yet there is still not strong evidence supporting their therapeutic effectiveness.[6–10] In canine OSA, the multi-kinasetargeting drug toceranib phosphate has been evaluated in the adjuvant setting with no observable therapeutic benefit. A retrospective evaluation of dogs with macroscopic pulmonary metastases found no improvement to outcome in dogs treated with toceranib.[11] Another study prospectively evaluated the addition of toceranib to metronomic cyclophosphamide/piroxicam therapy following amputation and adjuvant carboplatin; no improvement in disease free interval (DFI) or overall survival (OS) was noted with the addition of toceranib.[12] These studies in human and canine patients exemplify the difficulty in developing targeted therapies against cell surface receptors in OSA and underscore the importance of identifying alternate therapeutic targets for this disease.

Transcription factors (TFs) are multi-protein complexes that serve as convergence points for intracellular signaling pathways and ultimately lead to modulation of gene expression. Historically, TFs as targets for cancer therapy has been a little-developed field but there is a growing interest in creating therapeutic strategies to alter the function of TFs as some of the most relevant oncogenes are in fact TFs, such as mutant p53 and MYC.[13] RUNX2 is a transcription factor that is critical in normal skeleton development by regulating chondrocyte maturation and osteoblast differentiation.[14] RUNX2 forms a heterodimer with Core-binding factor β (CBFβ), conferring stability to RUNX2 and enhancing Runx2-dependent transcriptional modulation; this interaction is essential for the inhibition of osteoblast differentiation by metastatic breast cancer cells and is associated with malignant phenotype of other tumor types.[15,16] RUNX2 and CBFβ protein expression is deregulated in OSA and levels are increased in cell lines and tissues and RUNX2 overexpression is involved in the

chemoresistance of human OSA cells.[17,18] Canine OSA tumors also have increased copy number of the RUNX2 locus and highly express the RUNX2 protein.[19,20] Furthermore, silencing of RUNX2 in human OSA cell lines sensitizes them to doxorubicin-induced apoptosis via c-Myc activity.[21] Thus, the heterodimer RUNX2/CBFβ may be a promising candidate as a therapeutic target for this disease in both species.

Recently, Illendula *et al* have developed small molecules capable of allosterically inhibiting the binding of CBFβ to RUNX transcription factors.[22] These compounds have demonstrated anti-tumor activity in leukemia and ovarian cancer cell lines, compromising cell viability, cell cycle and migration/invasiveness capabilities.[22,23] With these novel compounds, we have examined the effects of inhibiting CBFβ/RUNX2 interaction in canine OSA cell lines and the mechanisms underlying these effects. We found that small molecule inhibitors of CBFβ/RUNX2 binding decrease cell viability and clonogenic growth, arrest cell cycle, impair metastatic capabilities and trigger apoptotic cell death in canine OSA cell lines. These effects are likely due to reduced CBFβ/RUNX2 interaction and subsequent decreased expression of RUNX2 target genes. Altogether, our findings support the CBFβ/RUNX2 interplay as a viable, druggable target in OSA and provide valuable information for potential future clinical development of these compounds for this disease.

Methods

Cell lines and culture conditions

The previously validated and characterized canine OS cell lines Abrams, Grade, HMPOS (p53 mutant) and D-17 (p53 wild-type) were generously supplied by Dr. Dawn Duval (Flint Animal Cancer Center, Colorado State University, Fort Collins, CO).[24–27] Cells were routinely cultured in Corning® RPMI-1640 culture medium with L-Glutamine (Mediatech Inc., Manassas, VA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA) and penicillin (50 U/mL)/streptomycin (50 μg/mL) (Mediatech Inc). Cell cultures were maintained at 37°C in a humidified environment with 5% CO_2 and all assays were performed in this culture media.

Core binding factor (CBF) beta inhibitors

The small molecule inhibitors of CBFβ/RUNX binding AI-10-104 and AI-14-91, and control compound AI-4-88 employed in this work were synthetized by Dr. J.H. Bushweller's laboratory as previously described.[22] All compounds were dissolved in dimethyl sulfoxide (DMSO).

Cell viability

Cell proliferation and viability were evaluated by means of the fluorometric, bioreductive resazurin assay (Sigma-Aldrich, St. Louis, MO). Following a preliminary experiment to determine optimal cell numbers, cells were seeded in 96-well plates (Corning® Costar™, Corning, NY) at $4x10^3$ (24 hour assay), $2.5x10^3$ (48 hour assay) and $1.5x10^3$ (72 hour assay) cells per well. We performed two experimental approaches: 1) cells were treated with vehicle control, CBFβ inhibitors, or negative control compound for 48 hours in a range of concentrations (0, 20, 30, 50, 60, 75 or 100 μM); and 2) and cells were treated with 20 μM

CBFβ inhibitors (approximate IC_{50} from 48 hour proliferation assays), control compound, or vehicle for 24, 48 or 72 hours. All the treatments were performed in triplicate and repeated three times. Following the treatment, culture medium was replaced and cells were incubated with 10% resazurin in fresh culture media for 1-2 hours. Fluorescence was then measured at excitation/emission 560/590 nm on a microplate reader (Synergy HI, BioTek, Winooski, VT).

Clonogenic assay

Abrams, HMPOS and D-17 cells were treated with 20 μM CBFβ/RUNX inhibitors, control compound, or vehicle for 24 hours. Cells were subsequently detached by trypsinization and 300 single cells per well in 2 mL RPMI culture medium were seeded in triplicate in a 6-well plate (Corning® Costar™). Cells were incubated to form colonies for 7 days. Finally, colonies were gently washed with warm IX PBS and then fixed with 95% ethanol:acetic acid solution (3:1), stained with crystal violet (0.5% w/v) and manually counted using a microscope. Representative images of the assays were taken with a digital image analyzer (FluorChem E, ProteinSimple, San Jose, CA).

Caspase-3/-7 activity

Measurement of the enzymatic activities of caspase-3/-7 was carried out with a fluorometric SensoLyte® Homogeneous AMC Caspase-3/-7 Assay Kit (AnaSpec, Fremont, CA), according to manufacturer's instructions. Cells were treated with 20 μM CBFβ inhibitors, control compound, or vehicle for 48 hours in a 96-well microplate (Corning® Costar™). Fluorescence intensity was measured using 354 nm excitation and 442 nm emission detection on a fluorescence microplate reader (Synergy HI, BioTek).

Bivariate Annexin V/PI analysis

Abrams and D-17 cells were collected after 48 hours treatment with 20 μM CBFβ inhibitors or control compound or vehicle, and subsequently washed twice with IX Binding Buffer and stained employing the eBioscience™ Annexin-V Apoptosis Detection Kit FITC (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Stained cells were processed on a CytoFLEX S flow cytometer (Beckman Coulter, Indianapolis, IN) and data were analyzed using FlowJo v4.0 software (FlowJo LLC, Ashland, OR). The HMPOS and Grade cell lines were not included in this analysis because they have a stably transfected nuclear red protein which was found to interfere with the compensation for AnnexinV/PI flow cytometric analysis.

Cell cycle analysis

Cell cycle was analyzed in D-17 and Abrams cells treated with 20 μM CBFβ inhibitors or control compound or vehicle for 24 hours. Following the treatment, cells were washed in PBS and fixed in cold 70% ethanol drop by drop while vortexing and then incubated at −20°C for 2 hours. Subsequently, cells were washed twice in stain buffer (PBS, 2% FBS) and stained using a PI/RNase Staining Buffer (BD Pharmingen™, BD Biosciences, San Diego, CA). Stained samples were evaluated on a CytoFLEX S flow cytometer (Beckman Coulter) and data were then analyzed using FlowJo v4.0 software (FlowJo LLC).

Cell migration and invasion assay

HMPOS (4.5×10^4) or D-17 (1.0×10^5) cells in FBS-free medium were added to the inserts of Corning® Matrigel® Invasion chambers (Coming, Bedford, MA) and culture medium containing 10% FBS was added to the lower chamber. Following the attachment of the cells to matrigel (around 1 hour after plating), cells were treated with 20 μM CBFβ inhibitors, control compound, or vehicle and incubated at 37°C. After 20 hours, non-invading cells were removed from the upper surface of the insert, those on the lower surface were fixed with 95% ethanol:acetic acid and then stained with crystal violet. Images (seven random 20x fields) were taken using a Leica DM2000 phase-contrast inverted microscope (Leica Mycrosystems). Cells were treated in duplicate and assayed separately. Cell migration was evaluated in parallel employing Corning® BioCoat™ Control Inserts with 8,0pm pores without matrigel.

Quantitative RT-PCR

All four canine OS cell lines were treated with CBFβ inhibitors (20 μM), control compound (20 μM), or vehicle. After 24 hours, total RNAs were isolated using TRIzol™ Reagent (Invitrogen), according to manufacturer's instructions. Reverse transcription was performed employing a Quantitec® Reverse Transcription Kit (Qiagen Inc., Germantown, MD) on a SimpliAmp Thermal Cycler (Applied Biosystems®, ThermoFisher Scientific, Waltham, MA). Quantitative RT-PCR (qRT-PCR) assays were performed in duplicate employing a QuantiFast® SYBR® Green PCR Kit (Qiagen) on an AriaMx Realtime PCR System (Agilent Technologies, Santa Clara, CA). Sequences of canine gene primers are shown in Table 1. The geometric mean of housekeeping genes Rps5, Gapdh and *Hnrnph1* was used to normalize the expression of genes of interest. The threshold cycle (C_T) was determined and relative gene expression was calculated using the comparative Ct method $2^{-\Delta(\Delta CT)}$.[28]

Immunoblotting for protein expression analysis

Whole-cell protein lysates from all four untreated cell lines were extracted, quantified and immunoblotted as previously described.[29] Following protein transfer and blocking, PVDF membranes were incubated with primary antibodies: anti-RUNX2, anti-CBFβ, anti-H3 (Cell Signaling, Boston, MA) or anti-β-actin (ThermoFisher Scientific); and then with a horseradish peroxidase-conjugated secondary anti-rabbit or anti-mouse antibody (Invitrogen). Immunolabelling was detected employing Luminata™ Forte Western HRP substrate (Millipore, Billerica, MA) and was visualized with a digital chemiluminescent image analyzer (FluorChem E, ProteinSimple, San Jose, CA).

Co-immunoprecipitation of CBFβ/RUNX2

Protein lysates (500 μg) extracted from HMPOS (high basal RUNX2 expression) and D-17 (lower basal RUNX2 expression) treated with 20 μM CBFβ inhibitors for 6 hours, were incubated with anti-RUNX2 (2μg, Cell Signaling) overnight at 4°C under agitation, followed with a 4 hours incubation (4°C in a rotator mixer) with Protein A-Sepharose® CL-4B beads (GE Healthcare, San Ramon, CA). Next, immunonoprecipitates were washed three times with protein extraction buffer. Loading Buffer 2X was added to the samples and boiled at 95°C for 5 min in order to denature the proteins and separate them from the beads and

antibody. Lastly, samples were analyzed by immunoblotting as explained above and PVDF membranes were incubated with anti-CBFβ (Cell Signaling). IgG protein (detected ≈55 kDa) was employed as loading control protein.

Combination of CBFβ inhibitor and chemotherapeutic drugs

The dose-effect of AI-14-91 (AI; active CBFβ inhibitor) and its combination with Doxorubicin (Dox) or Carboplatin (Carbo) on the proliferation of canine OSA cell lines was analyzed by Chou-Talalay method for drug combination which is based on the median-effect equation.[30,31] Canine OSA cell lines plated in 96 well microplates were treated with AI (2.5-40 μM), Dox (12-500 ng/mL) or Carbo (12.5-500 μg/rnL) and combinations of AI with Dox or Carbo in a constant concentration ratio (Dox/Carbo:AI, 5:1). Following 48 hour incubation at 37°C, cell viability/proliferation was evaluated using Resazurin Assay as described previously. Combination index (CI), dose-reduction index (DRI), dose-effect levels of cell growth inhibition (ED50-ED95), and median-effect dose (Dm) were calculated using CompuSyn software (ComboSyn Inc, Paramus, NI). CI values <1, 1 and >1 suggest a synergistic, additive or antagonistic effect, respectively. DRI indicates the dose reduction of individual components allowed in a synergistic combination at a given effect level.[31]

Data and statistical analysis

All experiments were repeated independently at least twice and technical replicates in each independent experiment (at least in duplicate) were average and yielded a value for a biological replicate. Data (mean ± SEM) were statistically analyzed with GraphPad Prism® v7.04 (GraphPad Software, San Diego, CA). Comparisons of the effects of CBFβ inhibitors, negative control and vehicle control were compared by one- or two-ways ANOVA test followed by a Bonferroni multiple comparison test. For all comparisons, a P- value ≤0.05 was considered significant.

Results

RUNX2/CBFβ interaction is disrupted in canine OSA cells by CBFβ inhibitors, compromising cellular viability and clonogenic cell growth

Cellular viability/proliferation was analyzed in canine OSA cells (Abrams, HMPOS, D-17 and Grade) treated for 48 hours with CBFβ inhibitors (AI-10-104, AI-14-91) or the negative control compound (AI-4-88) employing a Resazurin Assay. Our results demonstrated that both AI-10-104 and AI-14-91 significantly decreased canine OSA cell viability/proliferation in a concentration-dependent fashion compared to AI-4-88 ($P<0.001$) with the HMPOS and Abrams cell lines. Cell proliferation was also significantly inhibited ($P<0.001$) with D-17 and Grade cell lines but this inhibition was already drastic with the lowest concentration used (20 μM) without enhanced effect with higher concentrations (Figure 1A). Given these results, we decided to employ 20 μM for all the compounds in the remaining experiments of this study. Cell proliferation was also analyzed in canine OSA cells treated with 20 μM CBFβ inhibitors or negative control compound at 24, 48 and 72 hours after treatment in order to evaluate the time-dependent effects. Our resazurin assays revealed a time-dependent anti-proliferative activity in cells treated with AI-10-104 and AI-14-91, with slight recovery in Grade cells exposed to AI-14-91 after 72 hours (Figure 1B). The negative control

compound did not exert a negative effect in cellular viability in cells treated up to 72 hours. Moreover, we analyzed the long-term effect on cell proliferation by means of clonogenic assays (Figure 1C). Our findings showed that both surviving fraction and colony formation in canine OSA cells treated with CBFβ inhibitors were significantly suppressed when compared to AI-4-88 or vehicle control ($P<0.01$). To support that the anti-proliferative effects in canine OSA cell lines by CBFβ inhibitors were associated with a disruption of CBFβ/RUNX2 interaction, we performed an immunoprecipitation of RUNX2 and then examined CBFβ levels by immunoblotting. After confirming that all canine OSA cell lines express both proteins (Figure 2A), we selected HMPOS and D-17 to perform the co-immunoprecipitation due to their different *p53* status (mutant and wild type, respectively). Our findings showed a decreased interaction of CBFβ and RUNX2 in HMPOS and D-17 after 6 hours of treatment, although this reduction was greater in D-17 cells (Figure 2B).

Small molecule inhibitors of CBFβ/RUNX binding trigger apoptotic cell death in canine OSA cells

In order to elucidate whether the inhibition of canine OSA cell proliferation by inhibitors of CBFβ/RUNX involves apoptosis pathways, we analyzed caspase-3/-7 activity employing a fluorometric assay in canine OSA cells (HMPOS, D-17 and Abrams) after a 48 hour treatment (Figure 3A). Both AI-10-104 and AI-14-91 (20 μM) led to a significant increase in caspase-3/-7 activity in all canine OSA cells as compared to AI-4-88 or vehicle control ($P<0.01$). We further evaluated the apoptotic profiles in Abrams and D-17 cells treated for 48 hours (20 μM) using a bivariate PI/Annexin-V assay analyzed by flow cytometry. Small molecule inhibitors significantly enhanced late apoptosis ($PI^+/Annexin\text{-}V^+$) and necrosis ($PI^+/Annexin\text{-}V^-$) features in Abrams ($P<0.001$) and D-17 cells ($P<0.05$), compared to AI-4-88 or vehicle control. Furthermore, AI-10-104 and AI-14-91 significantly triggered early apoptosis (PI7Annexin-V^+) in D-17 cells ($P<0.001$). Unlike small molecule inhibitors of CBFβ/RUNX binding, AI-4-88 did not have any effect on either apoptosis profile or caspase-3/-7 activity in canine OSA cells (Figure 3B). Together, our results indicate that inhibitors of CBFβ/RUNX binding greatly trigger apoptotic cell death in canine OSA cells, partially explaining the decrease in cell viability and proliferation.

Cell cycle progression is affected by inhibitors of CBFβ/RUNX binding in canine OSA cells

To evaluate whether cell cycle progression is compromised in canine OSA cells by small molecule inhibitors of CBFβ/RUNX binding, cell cycle was analyzed by flow cytometry in Abrams and D-17 cells treated for 24 hours, after staining DNA with a PI/RNase buffer. Cell cycle progression was slowed in a cell line dependent manner. Cell cycle was significantly arrested in G1 phase in Abrams cells treated with AI-10-104 or AI-14-91 as compared to AI-4-88 or vehicle control ($P<0.001$), accompanied with a significant decrease of cells in G2 phase (Figure 4A; $P<0.05$). However, cell cycle progression in D-17 was differently influenced by inhibitors of CBFβ/RUNX depending on the compound. AI-10-104 led to a significant accumulation of D-17 cells in G2 phase ($P<0.05$) while cells in G1 phase are significantly reduced ($P<0.01$) (Figure 4B).

Small molecules inhibitors of CBFβ/RUNX binding suppress *in vitro* metastatic ability of canine OSA cells

The impact of inhibitors of CBFβ/RUNX binding, specifically AI-10-104, on migratory behavior and invasion capability of canine OSA cells were further examined, because both processes are critical for tumor progression and metastasis. Migration and invasiveness of AI-10-104-treated HMPOS and D-17 cells were significantly inhibited ($P<0.001$) after 20 hours of treatment (Figures 5A and 5B). While neither vehicle control nor AI-4-88 affected any of these processes in D-17 cells, the latter significantly suppressed the invasion capability of HMPOS cells. However, the calculated invasion index, which represents the percentage of migrating cells that are also capable of invasion through the Matrigel, showed that only AI-10-104 had a critical effect in invasiveness of HMPOS and D-17 cells (Figure 5C).

Small molecules inhibitors of CBFβ/RUNX binding regulate the gene expression of RUNX2 targets

We next evaluated the gene expression of RUNX2 targets in order to elucidate whether anti-tumor effects in canine OSA cells by small molecules inhibitors of CBFβ/RUNX binding are associated with the disruption in the interaction of these proteins and subsequent alterations in RUNX2-mediated gene expression. We analyzed the mRNA expression levels by qRT-PCR for three targets genes of RUNX2, including *Vegfa, Cdkn1a* (p21) and *Bglap* (osteocalcin), in Abrams, D-17, HMPOS and Grade cells treated with 20 μM AI-10-104, AI-14-91 or negative compound (AI-4-88), or vehicle control (Figure 6). We found that all four canine OSA cell lines showed a decreased expression of *Bglap* mRNA following the AI-10-104 or AI-14-91 treatment, with significant reduction seen in HMPOS and Grade ($P<0.05$). The mRNA levels of *Vegfa* were greatly decreased in Abrams and Grade although not significantly, while D-17 showed a significant enhanced expression ($P<0.01$) and *Vegfa* levels were not altered in HMPOS. With regard to *Cdkn1a* expression, while AI-14-91 induced a reduction in its expression in D-17 and Grade, AI-10-104 triggered a significant increase in Abrams and HMPOS ($P<0.01$).

CBFβ inhibitors synergize with chemotherapeutics in a drug- and cell-dependent manner

The Chou-Talalay method was employed to evaluate the effect of combining a CBFβ inhibitor (AI-14-91 -AI-) with traditionally used chemotherapy drugs for canine OSA treatment, such as Doxorubicin (Dox) and Carboplatin (Carbo). Table 1 summarizes the combination effects of AI, Dox and Carbo in canine OSA cell lines. The AI/Dox combinations were synergistic at all dose-effect levels in Abrams and Grade cells, except for ED50 in the latter. This synergistic effect was also showed in D-17 and HMPOS cells, although the combinations were only additive at the highest dose levels (>ED90). Regarding AI/Carbo, combinations were synergistic in a dose-dependent manner in Grade and Abrams cells, with the exception of ED50 in Abrams cells. Table 2 shows that cytotoxic activity of both chemotherapeutic drugs is improved when canine OSA cells are co-treated with the inhibitor of CBFβ/RUNX binding, AI-14-91, except in the HMPOS cell line. The DRI values reflect that doses of chemotherapeutic drugs may be decreased due to their synergistic actions. For example, to inhibit cell proliferation by 95% (ED95), the concentration of Dox

could be reduced 13.13-fold, 4.55-fold and 26.00-fold in Abrams, D-17 and Grade, respectively, when combined with AI. The concentration of Carbo can be reduced 6.04-fold, 1.72-fold and 5.55-fold to get the same inhibition of cell growth in Abrams, D-17 and Grade, respectively. However, the combination of AI with chemotherapeutic drugs did not exhibit a clear advantage in HMPOS cells.

Discussion

It is widely known that gene expression is profoundly altered in cancer, particularly those genes involved in regulating cellular survival, proliferation and self-renewal. In many cases, this altered gene expression in cancer is caused by a misregulation of transcription factors, due to a myriad of mechanisms. Therefore, transcription factors have emerged as promising targets for the development of new treatment approaches against cancer.[32,33] Numerous strategies to target transcription factors have been considered, including direct targeting of transcription factors, modulating degradation or expression levels, preventing DNA binding, and blocking protein/protein interactions.[34] However, transcription factors are challenging to target due to their typically flat and large interaction surfaces.[13,32] Identification of molecules that work through allosteric inhibition is one strategy to overcome the difficulty in targeting the interaction surfaces of transcription factor protein complexes. In this regard, Illendula *et al.* have described the development of small molecules that bind to CBFβ at a site that is not part of the interface for RUNX protein binding, alter binding dynamics and reduce the affinity of CBFβ for RUNX.[22] In their studies, the CBFβ inhibitors were shown to inhibit the growth of leukemia cells with AML-ETO and MLL-AF9 fusion proteins which have a requirement for RUNX1 activity. This growth inhibition was associated with reduced interactions between CBFβ and RUNX1, reduced RUNX1 occupancy on target genes and altered gene expression.[35] Furthermore, Shin *et al.* recently demonstrated that knockdown of RUNX2 or CBFβ induces apoptosis in some human OS cell lines, and identified potential downstream effectors responsible for this activity, including MYC.[36] Another recent study identified tumor suppressive activity of a microRNA through its targeting of RUNX2 in OS cells.[37] Thus, in this study we aimed to identify whether inhibiting the interaction between RUNX2 and CBFβ, using allosteric inhibitors of CBFβ, would be sufficient to induce anti-tumor effects in canine OSA cell lines or if this activity may require knockdown of RUNX2 levels. We identified both concentration- and time-dependent anti-proliferative effects for two active CBFβ inhibitors while the negative control compound had no effect on cell proliferation. Clonogenic cell growth was also reduced by exposure to CBFβ inhibitors. The anti-proliferative effects were found to be due, in part, to both increased apoptosis as well as inhibition of cell cycle progression. The effect of CBFβ inhibition on cell cycle progression was cell-line dependent; a significant increase in G1 with a reduction in G2/M was seen in the Abrams cell line while the opposite was true for the D-17 cell line which also showed a significant increase in the sub-G1 population. This may be explained, in part, by differences in the relative expression of *Cdkna* (p21), an essential G1 checkpoint control protein, following exposure to inhibitors.[38,39] We identified a significant increase in Abrams while the expression change in D-17 was less pronounced. Annexin V/PI staining showed differences between cell lines in the distribution of cells in early apoptosis, late apoptosis and necrosis, the overall effect in both cell lines was a significant increase in apoptosis.

We identified that all four of the cell lines utilized express RUNX2 and CBFβ and co-immunoprecipitation demonstrated that these proteins interact in canine OSA cells. We only recognized a slight reduction in the amount of CBFβ bound to RUNX2 in our study. This may have been due to sub-optimal sample timing, inhibitor concentration, or re-association of proteins during the early steps of the co-immunoprecipitation protocol. It is important to note that less than complete inhibition of the interaction was identified by Illendula *et al.* in their evaluation of CBFβ and RUNX1.[35] We did not identify a reduction in the levels of RUNX2 or CBFβ protein following treatment with inhibitors; this is interesting because the CBFβ interaction has been suggested as a mechanism to stabilize RUNX2 and prevent its degradation through ubiquitination.[40,41] It may be that the degree of inhibition was not complete enough to impact RUNX2 protein levels, yet was adequate to result in downstream effects such as reduced proliferation, increased apoptosis, reduced invasion and migration and altered gene expression. In this study, the alterations in expression of *Vegfa* following inhibitor treatment were cell-line dependent with reduced expression only seen in the Abrams and Grade cell lines. Relative expression of *Cdkn1a* (p21) was also cell-line dependent with regard to the degree of change, though all cell lines demonstrated some degree of increased expression. Furthermore, all four cell lines showed a reduction in relative expression of *Bglap* (osteocalcin) following CBFβ inhibition. Thus, we have demonstrated altered expression of RUNX2 target genes following exposure of OSA cells to inhibitors of the CBFβ-RUNX2 interaction.

We also evaluated the effect of combined CBFβ-RUNX2 inhibition and cytotoxic chemotherapy using drugs commonly employed in the treatment of OSA, given that RUNX2 has been implicated in the DNA damage response [42] In the Abrams and Grade cell lines, the addition of the CBFβ inhibitor to DOX or Carbo results in a synergistic anti-proliferative effect, particularly at the higher effect levels. Interestingly, in the D-17 and HMPOS cell lines these synergistic effects are not appreciated and may become antagonistic, particularly for the p53-mutant HMPOS cell line. However, given that these two cell lines had similar trends toward antagonism at higher effect levels, it is possible that p53 activity is not the determining factor in sensitivity to combinations of CBFβ inhibition and cytotoxic chemotherapy. Overall, these results suggest that the combination of cytotoxic chemotherapy and CBFβ inhibition may be beneficial in subsets of OSA, but may not provide therapeutic benefit in all cases. However, we have identified the interaction between RUNX2 and CBFβ as an important regulator of the malignant phenotype of canine OSA cells and provide evidence that inhibiting this interaction without the requirement for reducing RUNX2 or CBFβ levels results in anti-tumor effects, providing a framework for future investigations into the potential clinical utility of CBFβ-RUNX2 inhibitors as a therapeutic option for OSA.

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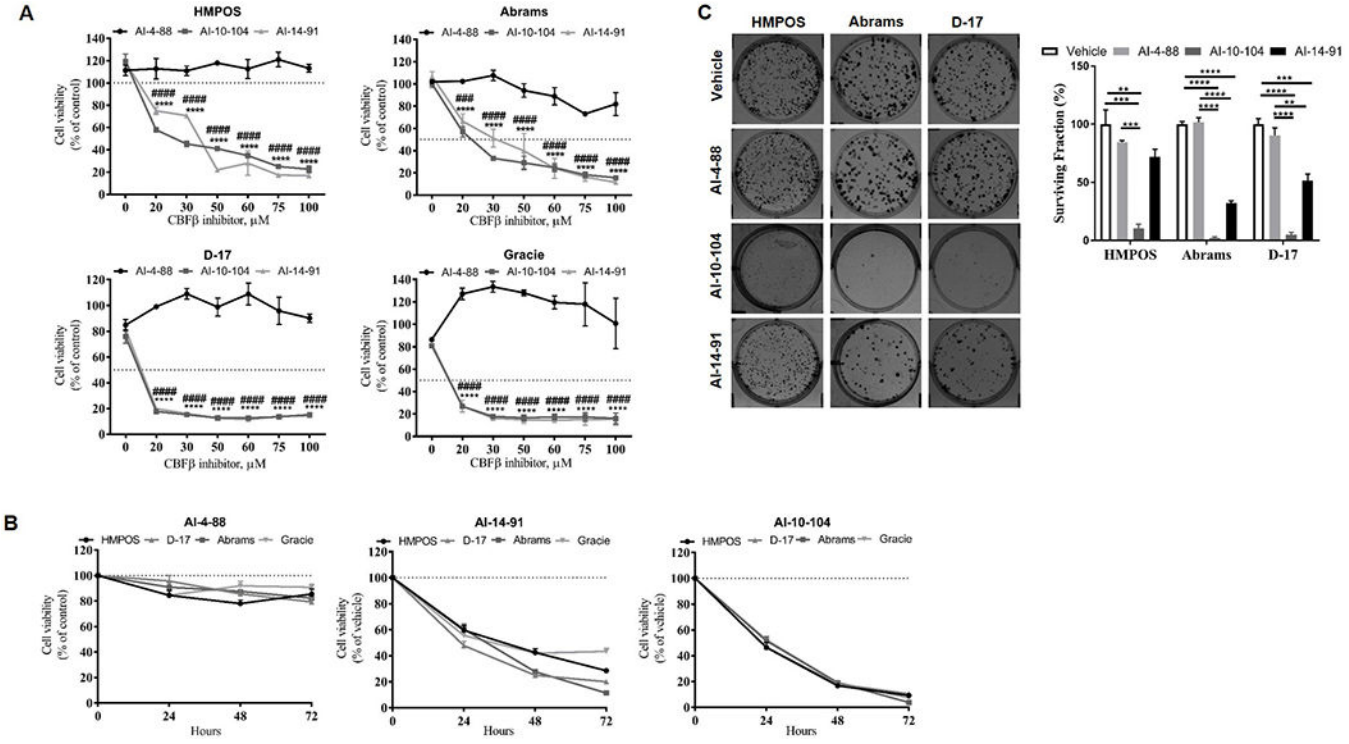

Figure 1. Cell proliferation and clonogenic growth is suppressed by small molecule inhibitors of CBFβ/RUNX2 binding in canine OSA cell lines.

Resazurin assay was used to assess cellular proliferation/viability in (A) cells treated with a range of concentrations (0-100 μM) of AI-10-104, AI-14-91 or AI-4-88 (negative control compound), or vehicle control for 48 hours, and (B) cells treated with 20 μM of AI-10-104, AI-14-91 or AI-4-88, or vehicle control after 24, 48, or 72 hours-treatment. (C) Representative images from clonogenic assays with respective surviving fractions. Data (mean±SEM) reported as percentage of control and analyzed by one-way or two-way ANOVA followed by a Bonferroni test (**p <0.01, ***p <0.001, ****p <0.0001).

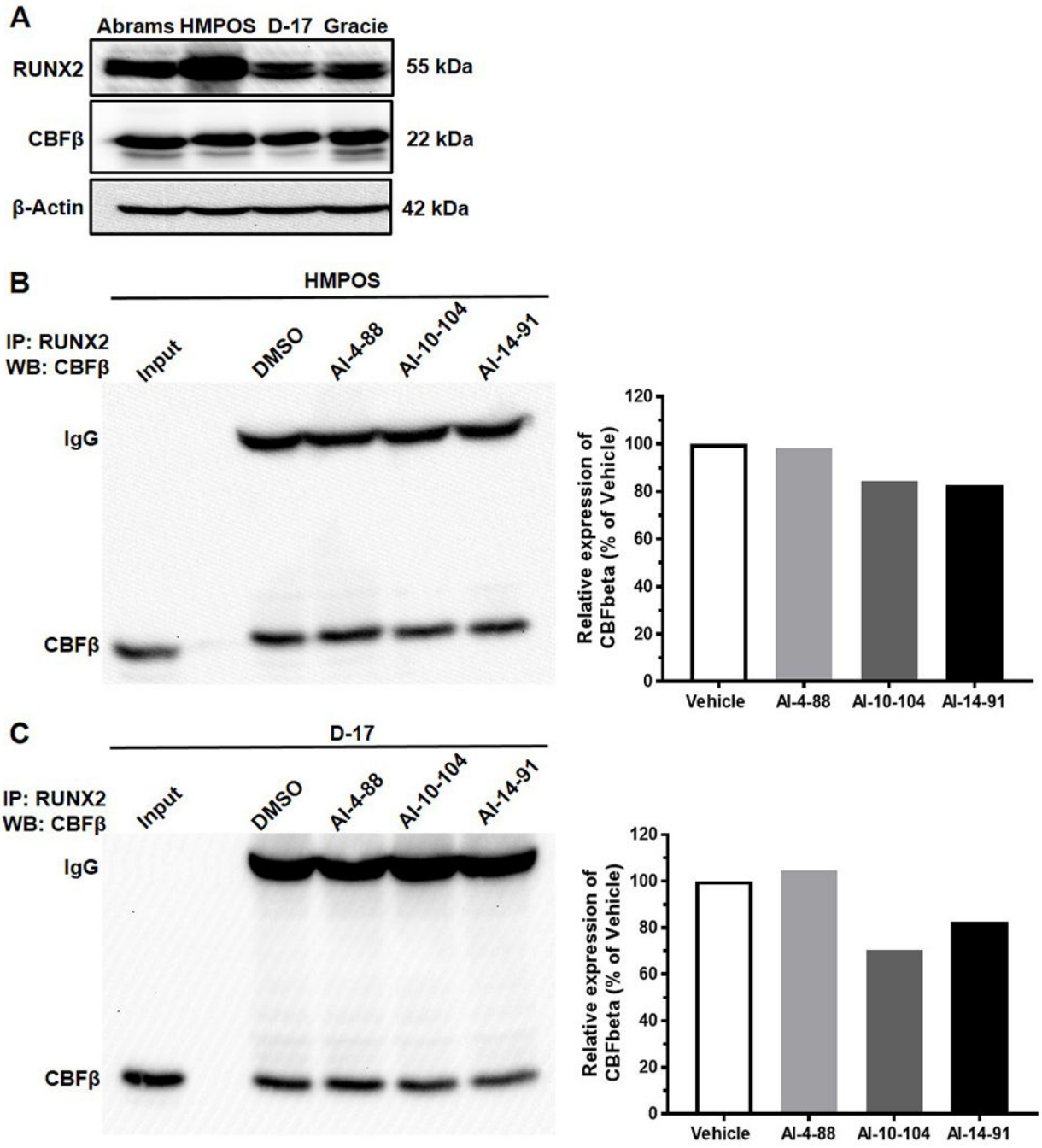

Figure 2. Inhibitors of CBFβ reduce CBFβ/RUNX2 binding in canine OSA cell lines.

(A) Representative western blot images of CBFβ (22 kDa) and RUNX2 (55 kDa) in untreated Abrams, HMPOS, D-17 and Grade cells. (B) Representative images of the immunoblotting analysis and histograms expressing quantification of CBFβ after immunoprecipitation of RUNX2 in protein lysates from HMPOS and D-17 cells treated with AI-10-104, AI-14-91 or AI-4-88 at 20 μM, or vehicle control for 6 hours.

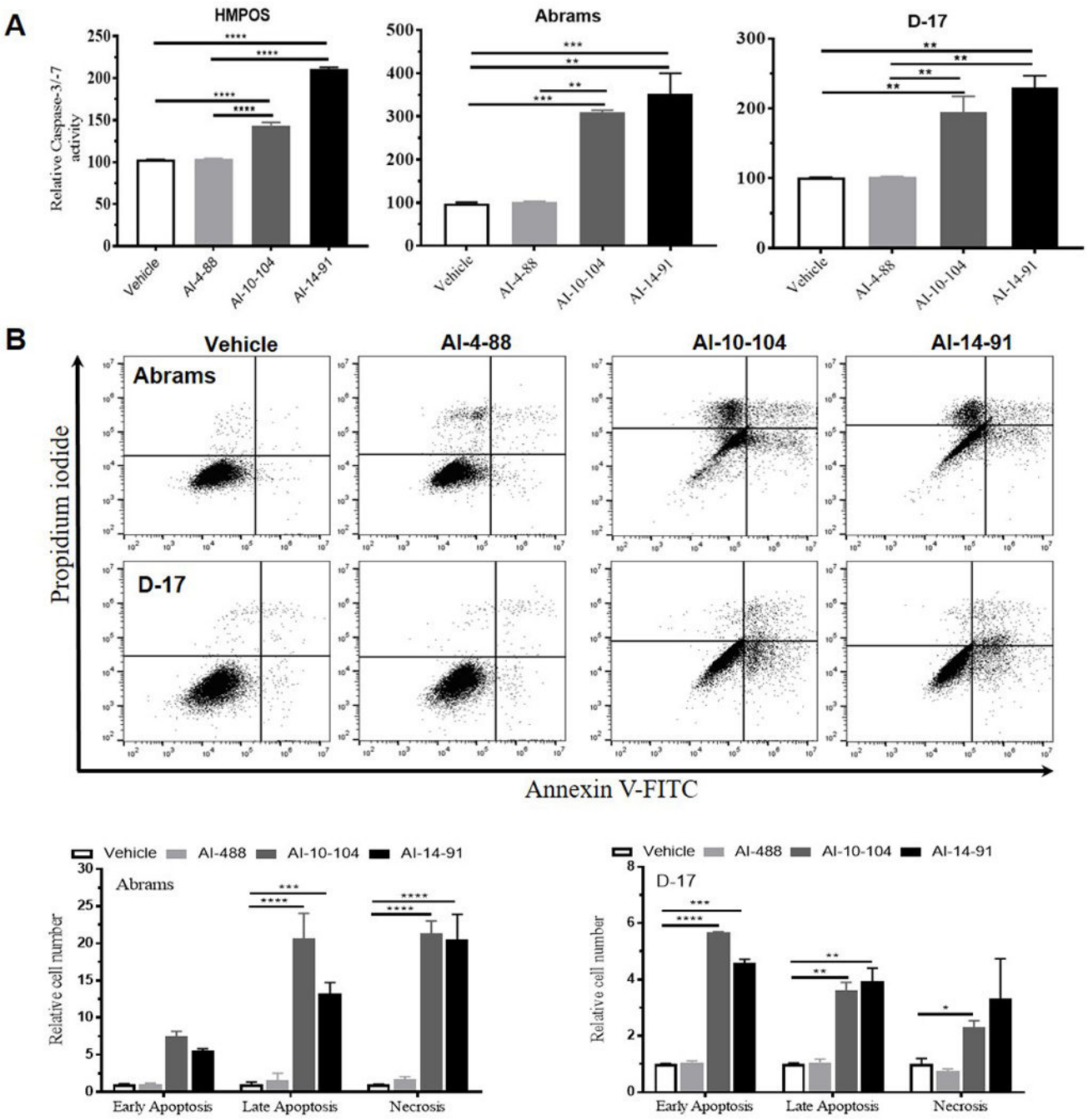

Figure 3. Necrotic and apoptotic cell death was triggered in canine OSA cell lines treated with small molecule inhibitors of CBFβ/RUNX2 binding.

Canine OSA cells treated with AI-10-104, AI-14-91 or AI-4-88 at 20 μM, or vehicle control for 48 hours were employed to evaluate the cell-death by means of (A) a caspase-3/-7 activity assay and (B) a bivariate PI/Annexin-V analysis. Data (mean±SEM) were calculated as percentage of control (untreated cells) and analyzed by two-way ANOVA multiple test followed by a Bonferroni test (*p <0.05, **p <0.01, ***p <0.001, ****p <0.0001).

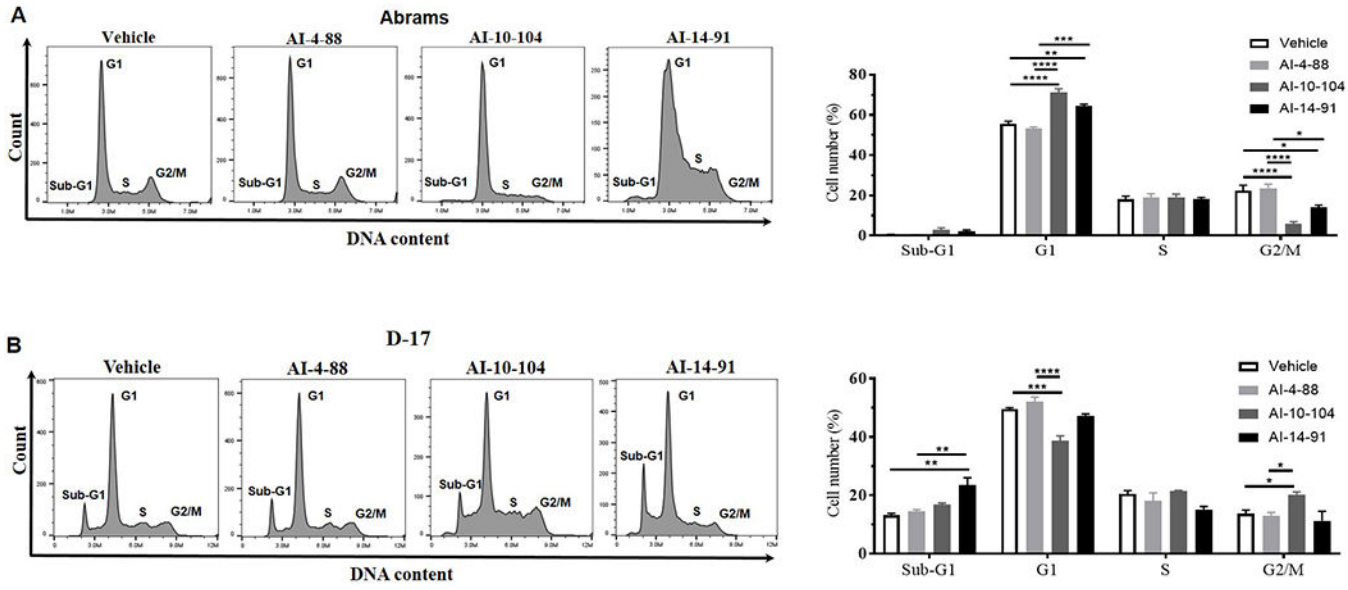

Figure 4. CBFβ inhibitors disrupted the cell cycle in canine OSA cells.

Flow cytometry histograms (left) and quantitation of cell cycle distribution (right) of (A) Abrams and (B) D-17 cells stained with PI after treatment with vehicle, or 20 μM AI-10-104, AI-14-91 or AI-4-88 (negative control compound) for 24 hours. Data (mean ±SEM) were calculated as percentage of control (untreated cells) and analyzed by two-way ANOVA multiple test followed by a Bonferroni test (*p <0.05, **p <0.01, ***p <0.001, ****p <0.0001).

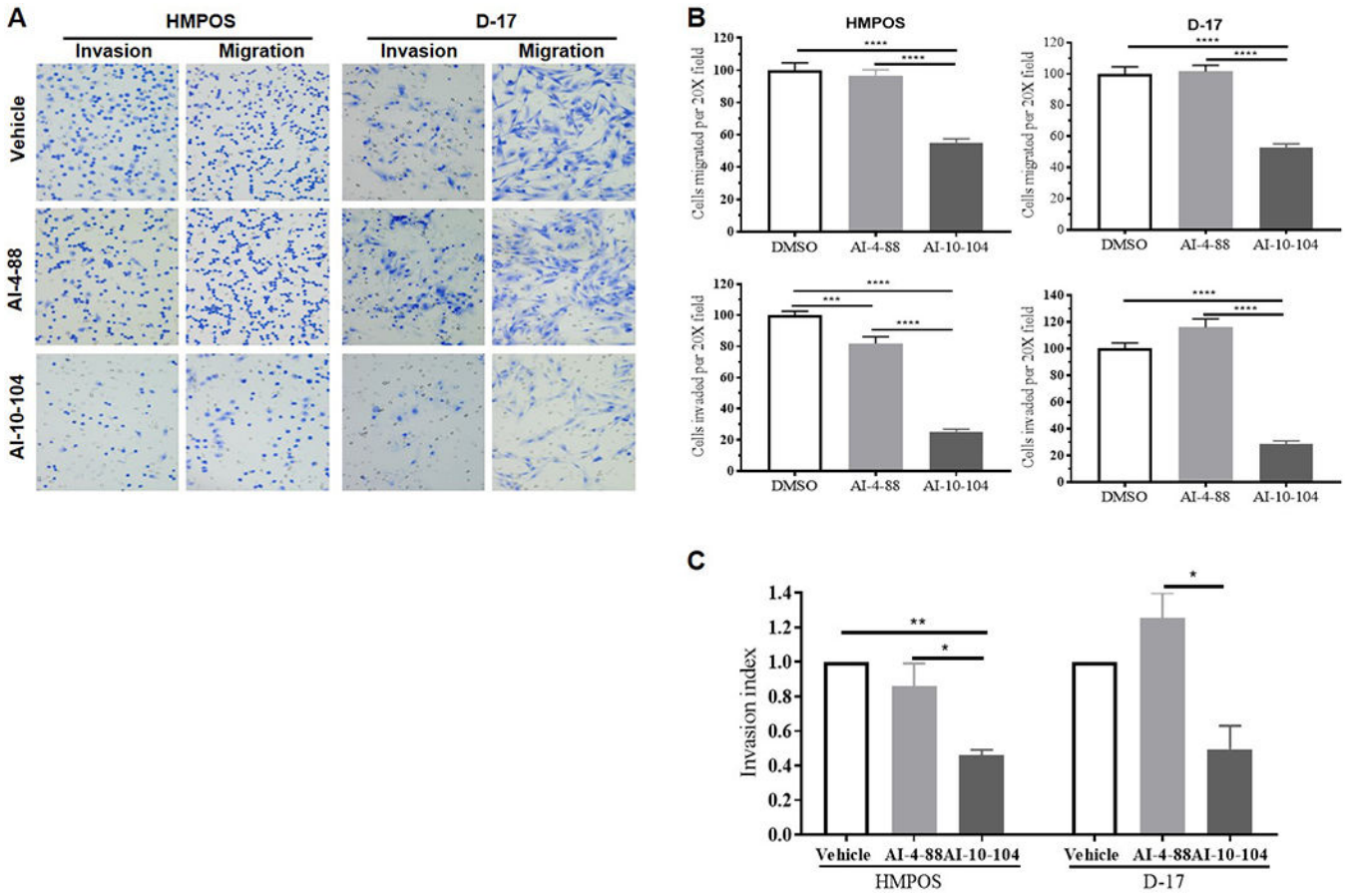

Figure 5. Migration and invasive capabilities were significantly inhibited in canine OSA cells by CBFβ inhibitors.

D-17 and HMPOS cells plated on upper matrigel-coated or control inserts were treated with 20 μM AI-10-104 or AI-4-88 (negative control compound), or vehicle and subjected to transwell invasion/migration assays for 20 hours. (A) Representative phase-contrast microscopy images (20X) of transwell invasion and migration assays. (B) Histograms expressing percentage of migrating and invading cells after 20 hours of incubation. (C) Invasion index was calculated by dividing percentage invasion of treated cells by the percentage invasion of vehicle cells. Data (mean±SEM) analyzed by one-way or two-way ANOVA followed by a Bonferroni test (*p <0.05, **p <0.01, ***p <0.001, ****p <0.0001).

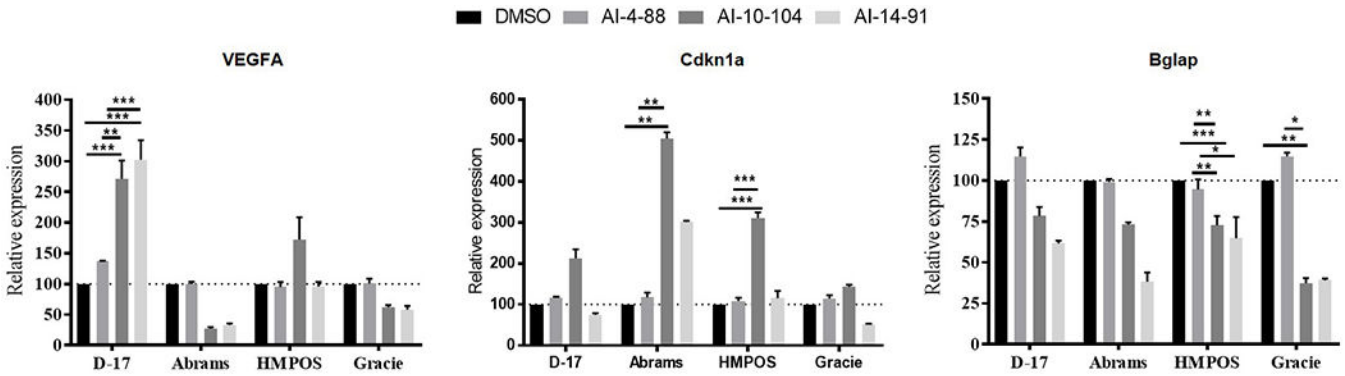

Figure 6. Small molecule inhibitors of CBFβ/RUNX2 binding modulated gene expression of RUNX2 targets in canine OSA cell lines.

Relative mRNA expression levels of VEGFA, Cdkn1a and Bglap were analyzed by qRT-PCR and normalized versus geometric mean of housekeeping genes GAPDH, Rps5, and Hnrnph1. Data (mean±SEM) were analyzed by two-way ANOVA multiple test followed by a Bonferroni test (*p <0.05, **p <0.01, ***p <0.001).

Table 1.

Canine primer pairs used in qRT-PCR.

Gene Symbol	Sequence	Sequence (5'→3')
Bglap	Forward	TTACCAGCGCTTCTATGGC
	Reverse	TATTCTGGAGCAGCTGTGATG
Cdkn1a	Forward	TGCTACATGGGCTAGTTGTG
	Reverse	CCAGGATGTTACAGGAGCTTAC
Vegfa	Forward	CTGACGAGATCGAGTACATCTTC
	Reverse	CACTCTAGGCCCTCATCATTAC
Rps5[43]	Forward	TCACTGGTGAGAACCCCCT
	Reverse	CCTGATTCACACGGCGTAG
Hnrnph1[44]	Forward	CTCACTATGATCCACCACG
	Reverse	TAGCCTCCATAACCTCCAC
Gapdh	Forward	AACATCATCCCTGCTTCCAC
	Reverse	AGACCACCTGGTCCTCAGTG

Table 2.

Combination study of CBFbeta inhibitor with conventional chemotherapeutic drugs in canine OSA cell lines.

CELL LINE	TREATMENT	PARAMETERS			CI VALUE				DRI VALUE			
		Dm	m	r	ED50	ED75	ED90	ED95	ED50	ED75	ED90	ED95
Abrams	DOX	33.84	0.91	0.82					2.10	4.16	8.25	13.13
	Carbo	10.02	0.68	1.00					0.82	1.73	3.64	6.04
	AI	12.75	1.76	0.93					5.23	4.07	3.16	2.67
	AI:DOX	19.31	2.11	0.87	0.81	0.49	0.31	0.23				
	AI:Carbo	14.63	1.25	0.96	1.41	0.82	0.59	0.54				
D-17	DOX	35.84	0.83	0.99					1.80	2.55	3.60	4.55
	Carbo	56.57	1.52	0.97					1.81	1.77	1.74	1.72
	AI	18.75	3.48	0.93					2.99	1.95	1.27	0.95
	AI:DOX	23.85	1.12	0.98	0.79	0.82	1.08	1.44				
	AI:Carbo	37.56	1.48	0.98	0.89	1.08	1.36	1.63				
Gracie	DOX	8.61	0.57	0.93					0.56	2.35	9.82	26.00
	Carbo	19.28	1.15	0.99					1.21	2.14	3.77	5.55
	AI	13.29	1.35	0.92					4.33	5.91	8.08	9.98
	AI:DOX	18.43	2.18	0.91	2.02	0.60	0.23	0.14				
	AI:Carbo	19.06	2.85	0.93	0.96	0.56	0.33	0.23				
HMPOS	DOX	1.88	0.77	0.80					30.98	2.57	0.21	0.04
	Carbo	6.85	2.07	0.94					0.75	0.84	0.94	1.02
	AI	15.67	2.59	0.98					4.26	4.30	4.34	4.36
	AI:DOX	0.08	0.28	0.64	0.03	0.45	6.15	38.40				
	AI:Carbo	12.87	2.64	0.96	1.58	1.43	1.29	1.21				

AI: AI-14-91, Dox: Doxorubicin, Carbo: Carboplatin, Dm: median-effect dose, m: slope, r: linear correlation coefficient of the Dm plot, (conformity), CI: Combination index (<1 synergism, 1 additive, >1 antagonism), DRI: dose-reduction index, ED: dose-effect levels of cell growth inhibition.