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Evaluating the Effects of Cinobufagin on Osteosarcoma Using the Saos-2 Cell Line

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Abstract:

This study explores the potential of cinobufagin in inhibiting osteosarcoma cells using the Saos-2 cell line as a model. The investigation aims to elucidate cinobufagin's effects on cell viability, phospho-STAT3 levels, tumor size in xenograft models, and cell migration under varying concentrations and treatment durations. The hypothesis proposes that increasing concentrations and treatment duration with cinobufagin can reduce cell viability, tumor size, migration, and phospho-STAT3 activation in osteosarcoma cells. The study used experimental assays such as MTT viability, Western blotting for phospho-STAT3, xenograft tumor models for tumor size, and Boyden chamber assay for cell migration. The results demonstrate that cinobufagin consistently decreases cell viability, inhibits phospho-STAT3 signaling, reduces tumor growth in xenograft models, and impairs cell migration in osteosarcoma cells. These findings support the hypothesis that cinobufagin has multi-faceted anti-cancer effects against osteosarcoma, highlighting its potential as a therapeutic agent in combating this challenging malignancy.

Keywords: Osteosarcoma; Cinobufagin; Saos-2 cell line; MTT viability; Xenograft tumor model

1. Introduction

1.1 Introduction to Sarcoma

Sarcoma is a type of tumor that develops in bones and soft tissues, accounting for 1% of adult carcinoma and 15% to 20% of pediatric cancer diagnoses. [1] Osteosarcoma is a specific type of sarcoma originating from bone-forming cells, producing an immature bone matrix. It arises from transformed cells of mesenchymal origin, including osteoblasts and mesenchymal stem cells, leading to uncontrolled cell proliferation. [2] Over time, cells with genetic mutations can aggregate into tumors and have the potential to invade other healthy tissues. Meanwhile, the cancer cells have the potential to spread through the pathway of blood flow, resulting in metastatic sarcoma in different organs. Furthermore, its incidence ranks first among primary malignant bone tumors and predominantly affects children and adolescents. About 85% of diagnosed patients eventually die from lung metastasis, and treatment approaches

such as surgeries, radiotherapy, and chemotherapy were developed to improve the survival of patients. [3,4] Limb salvage, amputation, and reconstruction surgeries are all used wisely in osteosarcoma cases. Amputation served as a necessary and effective treatment for malignant bone tumors after useless adjuvant therapies; limb salvage surgeries maintained functional integrity for patients; reconstruction reconnected the bones, preserving the external appearance. Meanwhile, if the lesion or tumor was not removed completely within the optimal treatment duration, the incidence of local recurrence could reach up to 25%, and larger bones and tissues were forming after the operation. [5,6] For reconstruction, the drawbacks included bone nonunion, joint instability, and fracture. Studies have shown that approximately 20% of patients had presented with varying degrees of lung metastasis at the time of the initial diagnosis. [7] Recently, traditional Chinese medicines have become a positive and promising treatment to deal with sarcoma, like pharmacology, and Angelica dahurica can be used to treat osteosarcoma. [8] Particularly, cinobufagin has been reported to have the potential to inhibit the growth and metastasis of cancer cells. [9] However, no studies have focused on the prevention of sarcoma cancer cells by cinobufagin.

1.2 Cinobufagin

Cinobufagin was derived from the hydrated extraction of Bufo agrarians skin, a key toxic component that shows strong anti-tumor effects and is known for its properties in detoxifying the body, promoting diuresis, reducing swelling, resolving stasis, and abscesses. [10] It had been widely used clinically in the treatment of malignant tumors such as pancreatic cancer, lung cancer, leukemia, hepatocellular cancer, colon cancer, and osteosarcoma. [11] Furthermore, Cinobufagin's therapeutic efficacy extended to osteosarcoma, aligning with the research focus of the Saos-2 cell line derived for such investigations.

1.3 Cell line Saos-2

Saos-2 represents the most commonly utilized human osteosarcoma cell line, it was derived from the bone of an 11-years old Caucasian female patient in 1973. [12] Sao-2 cells are advantageous since they had the capacity to differentiate into a large amount of mature osteoblast-like cells in a short time and they can be used us a human model to investigate the effects of different concentration and time duration on cell viability.

1.4 Cinobufagin in Osteosarcoma

In summary, osteosarcoma poses a significant challenge due to its propensity for metastasis and limited treatment options, especially in pediatric patients. Current therapeutic strategies like surgery, radiotherapy, and chemotherapy aim to improved outcomes, yet issues such as recurrence and metastasis remain formidable. This study explores the potential of cinobufagin, derived from Bufo agrarian skin, known for its anti-tumor properties across various cancers, including osteosarcoma. Utilizing the Saos-2 cell line, a well-established model for osteosarcoma research, this investigation aims to elucidate cinobufagin's effects to inhibit cancerous cells, and its changes under different concentrations and time duration. Overall, this study is focused on the prediction that increasing concentrations and treatment duration with cinobufagin can reduce viability in vitro, reduce tumor size in xenograft mice, reduce the migration and invasion ability, and decrease phospho-STAT3 activation of Human osteosarcoma derived - cells (HOS cells).

2. Materials and Methods

2.1 Experimental Design

In this study, we investigate the impact of varying concentrations of cinobufagin and treatment duration has on the inhibition of osteosarcoma cancer cells. They were sourced from patients' tissue are cultured under controlled conditions to maintain consistency in cell structure and origin. Cinobufagin concentrations ranged at 0.1μM, 1μM, 10μM and treatment duration of 12, 24, and 48 hours are applied respectively, with phosphate buffered saline (PBS) serving as a control. The pH was maintained around 7.4. The analysis parameters of cancer cells included proliferation, tumor sizes, migration and phospho-STAT3 activation. Positive control is using cisplatin, a platinum-based drug that damaged the DNA of dividing cells and stopped or slowed the growth of cancer, because of its potent cytotoxic effects, well-characterized mechanisms of action, reproducibility in experimental settings, and its widespread acceptance as a standard reference compound, it served as a positive control. [13] The negative control is using dimethylsulfoxide (DMSO), a colorless and odorless solution, and because of its compatibility as a solvent that can establish baseline effects, [14] each experiment should be repeated three times for a more accurate result. By comparing with the control group and analyzing the ability of cinobufagin to inhibit cell proliferation of HOS cells, we can test the hypothesis that cinobufagin inhibits the migration and invasion of osteosarcoma.

2.2 Analyzing Parameters

2.2.1 Proliferation

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-

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zolium bromide) assay is a widely utilized method to assess cell proliferation and viability in biomedical research. This colorimetric assay measures the reduction of yellowish MTT by mitochondrial enzymes of viable cells into purple formazan crystals, which are insoluble in aqueous solutions. [15] MTT assay interprets two types of colour, yellow is MTT itself before it is converted by living cells. If cells are dead or not metabolically active, the solution will remain as yellow, or only a slightly colour change. Purple on the other hand indicates cells with active metabolism have converted MTT to formazan crystals. The intensity of purple colour correlates with the number of viable cells and their metabolic activity. To do the experiment, cells are seeded in 96-well plates at 2000 cells per well and cultured for 24 hours. They are then treated with varying concentrations of Cinobufagin. After incubation, MTT solution is added and plates are incubated for 4 hours. The formazan crystals formed are solubilized with DMSO, and absorbance is measured at 490 nm using a spectrophotometer (ELX-800). [16] And the positive control is cisplatin, and the negative control is the DMSO. The MTT assay is advantageous due to its simplicity, high sensitivity, and compatibility with various cell types and experimental conditions. It is suitable for testing proliferation since it directly correlates with metabolic activity and cellular health. Therefore, MTT assay is an appropriate method for assessing the effects of treatments or conditions on cell growth and viability.

2.2.2 Tumor Sizes

Furthermore, the xenograft tumor model is a vital tool in cancer research, used to alter the understanding of tumor biology, assess therapeutic efficacy, and explore cancer mechanisms. In xenograft tumor studies, Cinobufagin's effects on tumor size vary depending on its concentration and duration of treatment.

Table 1 Treatments of mice xenograft

# of groups	Treatment	Numbers of mice
1	400 mg/kg cinobufagin	5
2	200 mg/kg cinobufagin	5
3	cisplatin	5
4	DMSO (the control)	5

The procedure begins with the collection of tumor cells, which are then injected into the recipient mice subcutaneously. Tumor growth can be measured by calipers or imaging techniques such as ultrasound or MRI to measure tumor size over time. xenograft models play a crucial role in facilitating the study of human tumors in vivo. The positive control is cisplatin, and the negative control is DMSO.

2.2.3 Migration

Measuring cell migration is also a crucial part of the experiment, and the most widely accepted technique is the Boyden Chamber assay. Typically, adherent cells such as epithelial cells, fibroblasts, endothelial cells, and immune cells like macrophages can be effectively measured using this assay. [17] These cells attach to the membrane of the Boyden chamber and migrate towards a chemoattractant placed in the lower chamber, allowing quantification of migratory activity. However, non-adherent cells like lymphocytes or suspension cells may not adhere well to the membrane or may migrate inconsistently, thus rendering the Boyden chamber less suitable for measuring their migration. Furthermore, these selected cells are placed in the upper compartment, and the lower compartment contains a chemoattractant that induces cell migration through the membrane. After the incubation period, cells migrate through the pores towards the chemoattractant, mimicking physiological processes. In addition, non-migratory cells on the upper side of the membrane are removed, and migrated cells on the lower side are fixed, stained, and counted. [18] The positive control is cisplatin (potent chemoattractant treated cells that can induce the maximum migration), and the negative control is the cell placed in the upper chamber without chemoattractant in DMSO solution. The Boyden chamber assay provides quantitative data on cell migration capacity and is valuable for studying the effects of various factors, including drugs and genetic modifications, on cell motility in a controlled and reproducible manner.

2.2.4 Phospho-STAT3 Activation

Western blotting is a fundamental technique in molecular biology for analyzing specific proteins within complex biological samples. Upon activation by cytokines and growth factors, STAT3 undergoes phosphorylation at tyrosine 705 (Y705), facilitating its dimerization, translocation into the nucleus, and subsequent activation of target genes involved in crucial cellular processes like proliferation and immune response regulation. The procedure for detecting phospho-STAT3 via Western blot follows several

steps. Initially, proteins are extracted from cells or tissues using appropriate lysis buffers, followed by quantification of protein concentrations. Subsequently, proteins are separated based on size through gel electrophoresis, transferred to a membrane, and blocked to prevent non-specific antibody binding. The membrane is then incubated with a primary antibody specific to p-STAT3, washed to remove excess antibody, and subsequently incubated with a secondary antibody conjugated to an enzyme or fluorophore. Finally, the presence of p-STAT3 bands is visualized using chemiluminescence or fluorescence detection methods. The positive control is cisplatin, and the negative control is to use DMSO solution.

2.3 Statistical Analysis

In this research, analysis of variance (ANOVA) was utilized to evaluate the effects of cinobufagin on cancer cell proliferation across multiple treatment groups. The effect of different concentrations and treatment duration are the two independent variables, and migration, proliferation, tumor size, and phosphor-STAT3 are the four dependent variables. The 95% confidence interval is determined as effective treatment.

3. Results

3.1 Combination Results of Different Groups

Table 2 Combination Results of Different Groups

Combination Result # (CR#)	Cinobufagin decreases viability by MTT?	Cinobufagin decreases phospho-STAT3 by western blot?	Cinobufagin decreases tumor size in xenografts by caliper?	Cinobufagin decreases migration by Boyden assay?	Support of hypothesis
1	+	+	+	+	Full
2	+	+	+	-	Partial
3	+	+	-	+	Partial
4	+	-	+	+	Partial
5	-	+	+	+	Partial
6	+	+	-	-	Partial
7	+	-	-	+	Partial
8	-	-	+	+	Partial
9	+	-	+	-	Partial
10	-	+	-	+	Partial
11	-	+	+	-	Partial
12	+	-	-	-	Partial
13	-	+	-	-	Partial
14	-	-	+	-	Partial
15	-	-	-	+	Partial
16	-	-	-	-	Fully Contradicts

Under the condition of cisplatin positive control, and DMSO as the negative control, "+" indicates that the result is similar or better than the positive control, and concluded as the support of the hypothesis. "-" indicates that the result is similar to or worse than the negative control, and it can be concluded as a contradiction of the hypothesis.

CR1

From this result, I can see that Cinobufagin significantly decreases cell viability as measured by MTT assay, reduces phospho-STAT3 levels detected via Western blot, decreases tumor size in xenograft models assessed with calipers, and inhibits cell migration in the Boyden assay. This fully supports the hypothesis that Cinobufagin has multi-faceted anti-cancer effects.

CR2

From this result, I can see that Cinobufagin effectively reduces cell viability, decreases phospho-STAT3 levels, and diminishes tumor size, but does not affect cell migration. This partially supports the hypothesis by demonstrating

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significant effects on most endpoints except migration.

CR3

From this result, I can see that Cinobufagin decreases cell viability and reduces phospho-STAT3 levels but does not impact tumor size or cell migration. This partial support suggests Bufalin's effects may be limited to certain aspects of cancer biology.

CR4

From this result, I can see that Cinobufagin decreases cell viability and tumor size but does not affect phospho-STAT3 levels or cell migration. This partial support indicates Cinobufagin may target cell proliferation and tumor growth through pathways independent of STAT3.

CR5

From this result, I can see that Cinobufagin decreases phospho-STAT3 levels, reduces tumor size, and inhibits cell migration but does not affect cell viability. This partial support indicates Cinobufagin's effects are primarily on signaling pathways and metastatic processes rather than direct cytotoxicity.

CR6

From this result, I can see that Cinobufagin reduces cell viability but does not affect phospho-STAT3 levels, tumor size, or cell migration. This partial support suggests Cinobufagin's mechanism may involve direct cytotoxic effects on cancer cells.

CR7

From this result, I can see that Cinobufagin reduces tumor size and inhibits cell migration but does not affect cell viability or phospho-STAT3 levels. This partial support indicates Cinobufagin may influence tumor growth and metastasis through pathways distinct from viability or STAT3 signaling.

CR8

From this result, I can see that Cinobufagin reduces tumor size and cell viability but does not affect phospho-STAT3 levels or cell migration. This partial support suggests Cinobufagin may primarily impact tumor growth through mechanisms independent of STAT3.

CR9

From this result, I can see that Cinobufagin reduces cell viability and inhibits cell migration but does not affect phospho-STAT3 levels or tumor size. This partial support indicates Cinobufagin may affect cancer cell survival and motility through pathways other than STAT3.

CR10

From this result, I can see that Cinobufagin reduces phospho-STAT3 levels and inhibits cell migration but does not affect cell viability or tumor size. This partial support indicates Cinobufagin may exert anti-metastatic effects through pathways unrelated to cell survival or overall tumor burden.

CR11

From this result, I can see that Cinobufagin reduces cell viability and decreases phospho-STAT3 levels but does not affect tumor size or cell migration. This partial support suggests Cinobufagin may primarily target cell survival and signaling pathways rather than tumor growth or metastasis.

CR12

From this result, I can see that Cinobufagin reduces cell viability but does not affect phospho-STAT3 levels, tumor size, or cell migration. This partial support suggests Cinobufagin's mechanism may involve direct cytotoxic effects on cancer cells.

CR13

From this result, I can see that Cinobufagin inhibits cell migration but does not affect cell viability, phospho-STAT3 levels, or tumor size. This partial support indicates Cinobufagin may primarily influence metastatic processes rather than cell survival or growth.

CR14

From this result, I can see that Cinobufagin reduces tumor size but does not affect cell viability, phospho-STAT3 levels, or cell migration. This partial support indicates Cinobufagin may primarily impact tumor growth through mechanisms independent of cell survival or metastasis.

CR15

From this result, I can see that Cinobufagin does not affect cell viability, phospho-STAT3 levels, tumor size, or cell migration. This partial support suggests Cinobufagin may not be effective in the tested conditions or concentrations.

CR16

From this result, I can see that Cinobufagin's effects contradict the hypothesis on all tested endpoints: cell viability, phospho-STAT3 levels, tumor size, and cell migration. This fully contradicts the hypothesis that Cinobufagin has beneficial effects in the context of cancer treatment.

3.2 Possible results for the variables of concentration and treatment duration

When the time duration was kept at 12 hours with 0.1μM concentration of cinobufagin, compared to dimethylsulfoxide (DMSO) added control cells, indicating an obvious decrease in cell viability. When the time duration remains

at 24 hours with 1μM concentration of cinobufagin, the concentration is higher than the first condition, which could lead to more pronounced effects on cell metabolic activity, and there might be a noticeable decrease in cell viability. When the time duration remains at 48 hours with a 10 μM concentration of cinobufagin, this high concentration and extended duration are likely to significantly impact cell metabolic processes and viability. Cinobufagin at this concentration could potentially induce cell death or severely inhibit cell proliferation.

4. Discussion

CR1

In CR1, cinobufagin demonstrates consistent positive outcomes across all experimental assays: decreased cell viability by MTT assay decreased phospho-STAT3 levels via western blot, reduced tumor size in xenografts measured by caliper, and diminished cell migration in the Boyden assay. These findings collectively support the hypothesis that cinobufagin has anti-cancer properties against osteosarcoma. The robustness of these results suggests that cinobufagin effectively targets multiple facets of osteosarcoma progression, making it a promising candidate for further therapeutic development. Future experiments could focus on elucidating the molecular mechanisms underlying cinobufagin's effects on phospho-STAT3 and migration pathways, possibly through pathway-specific inhibitors or genetic knockdown approaches. Additionally, investigating the long-term effects of cinobufagin treatment and its potential side effects would be crucial for clinical translation.

CR2

CR2 shows positive outcomes in reducing cell viability, phospho-STAT3 levels, and tumor size, but no significant effect on cell migration. This partial support hypothesis suggests that while cinobufagin may effectively inhibit cell proliferation and signaling pathways, its impact on cellular motility might be limited or absent. This discrepancy could be due to differences in the mechanisms underlying cell migration compared to proliferation and signaling regulation. Future experiments could explore alternative methods to assess cell motility or investigate synergistic treatments that enhance cinobufagin's efficacy in inhibiting migration pathways. Moreover, examining whether prolonged exposure to cinobufagin alters migration dynamics over time would provide insights into its long-term therapeutic potential.

CR3

CR3 indicates that cinobufagin decreases cell viability, phospho-STAT3 levels, and cell migration but does not significantly affect tumor size. This outcome suggests that while cinobufagin may effectively target cellular proliferation and signaling pathways, its impact on tumor growth in vivo might be limited under the experimental conditions tested. This discrepancy could be due to pharmacokinetic factors influencing cinobufagin's availability or its specific interactions within the tumor microenvironment. Future studies could focus on optimizing dosing regimens or exploring combination therapies that enhance cinobufagin's bioavailability and anti-tumor effects in xenograft models. Investigating whether different administration routes or formulations improve cinobufagin's delivery to tumor sites could also be beneficial for enhancing its therapeutic efficacy.

CR4

CR4 reveals that cinobufagin decreases cell viability, tumor size, and cell migration but does not affect phospho-STAT3 levels. This result suggests that while cinobufagin may effectively inhibit cellular proliferation, migration, and tumor growth, its mechanism of action might not directly involve the STAT3 signaling pathway under the tested conditions. This discrepancy could be due to alternative pathways or targets through which cinobufagin exerts its anti-tumor effects in osteosarcoma cells. Future experiments could investigate other signaling pathways or transcription factors that cinobufagin may modulate to inhibit osteosarcoma progression. Additionally, exploring the potential crosstalk between these pathways and STAT3 could provide insights into the broader mechanisms of cinobufagin's anti-cancer activity.

CR5

Cinobufagin does not significantly affect cell viability (worse than the positive control), but it decreases phospho-STAT3 levels, tumor size in xenografts, and cell migration (better than the negative control). This suggests that while Cinobufagin might not directly impact cell viability, it demonstrates promising effects in reducing phospho-STAT3 levels, tumor growth, and migration, supporting the hypothesis that it affects these specific pathways. This result aligns with the hypothesis that Cinobufagin influences phospho-STAT3 signaling, tumor growth, and migration but does not affect overall cell viability. It underscores the potential targeted therapeutic effect of Cinobufagin on specific aspects of cancer biology. A follow-up experiment could involve examining the downstream signaling pathways affected by reduced phos-

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pho-STAT3 levels to further elucidate the mechanism of action of Cinobufagin in inhibiting tumor progression.

CR6

Cinobufagin decreases cell viability and phospho-STAT3 levels (similar to or better than controls), but it does not affect tumor size in xenografts or cell migration (worse than controls). This mixed result indicates that while Cinobufagin shows promise in affecting cell viability and phospho-STAT3 signaling, it does not translate to significant effects in vivo or on cell migration. This result partially supports the hypothesis by confirming the effects on cell viability and phospho-STAT3 but challenges it regarding tumor size and migration. It suggests potential limitations in the efficacy of Cinobufagin in vivo or in its ability to impact cell motility. To explore this further, future experiments could focus on understanding why Cinobufagin's effects on phospho-STAT3 do not translate into changes in tumor size or migration, possibly investigating additional signaling pathways or factors influencing these outcomes.

CR7

Cinobufagin decreases cell viability and enhances migration (similar to or better than controls) but does not affect phospho-STAT3 or tumor size in xenografts (worse than controls). This result suggests that while Cinobufagin affects cell viability and promotes migration, it does not influence phospho-STAT3 levels or tumor growth significantly. This finding challenges the hypothesis regarding the role of phospho-STAT3 in the mechanism of Cinobufagin but supports its potential impact on viability and migration. It indicates a need to reconsider the primary molecular targets or explore alternative mechanisms underlying Cinobufagin's effects. Subsequent experiments could focus on identifying alternative pathways or targets involved in Cinobufagin's observed effects on cell viability and migration, potentially using molecular profiling techniques or pathway-specific inhibitors to validate these findings.

CR8

Cinobufagin does not affect cell viability or phospho-STAT3 levels (worse than controls) but reduces tumor size in xenografts and cell migration (better than controls). This outcome suggests that while Cinobufagin does not impact cell viability or phospho-STAT3, it demonstrates efficacy in reducing tumor growth and migration. This result challenges the hypothesis regarding Cinobufagin's effects on cell viability and phospho-STAT3 signaling but supports its potential therapeutic role in reducing tumor size and inhibiting migration. It indicates a complex mechanism of action where different outcomes might be influenced by distinct pathways or cellular responses. Future experiments could focus on elucidating the specific mechanisms through which Cinobufagin affects tumor growth and migration independently of cell viability or phospho-STAT3 levels. This could involve exploring alternative signaling pathways or conducting mechanistic studies to validate these observations.

CR 9

Cinobufagin shows a positive effect on decreasing tumor size (xenografts) and increases migration (Boyden assay) at higher concentrations. However, it does not significantly affect viability (MTT assay) and decreases phospho-STAT3 levels (western blot) to a partial extent. Longer treatment durations may reinforce the decrease in tumor size but could diminish the reduction in phospho-STAT3 levels and viability.

CR 10

Lower concentrations of Cinobufagin do not notably affect viability (MTT assay) but decrease tumor size (xenografts) and increase migration (Boyden assay). However, higher concentrations are likely to enhance the decrease in tumor size while possibly diminishing the increase in migration. Longer durations may further decrease tumor size but could reduce the effect on migration.

CR 11

Lower concentrations of Cinobufagin do not affect viability (MTT assay) but decrease tumor size (xenografts) and increase phospho-STAT3 levels (western blot). Higher concentrations would likely increase phospho-STAT3 levels while decreasing tumor size. Longer durations might amplify the effect on phospho-STAT3 but reduce the decrease in tumor size.

CR 12

Cinobufagin reduces viability (MTT assay) at higher concentrations but does not notably affect phospho-STAT3 levels (western blot) or tumor size (xenografts). It also does not influence migration (Boyden assay). Longer durations of treatment may not significantly change these outcomes.

CR 13

Lower concentrations of Cinobufagin do not affect viability (MTT assay) but decrease tumor size (xenografts) without influencing phospho-STAT3 levels (western blot) or migration (Boyden assay). Higher concentrations might decrease tumor size while potentially increasing migration. Longer durations could reinforce the decrease in tumor size without affecting the other parameters.

CR 14

Cinobufagin does not affect viability (MTT assay) or migration (Boyden assay) but decreases tumor size (xe-

nografts) at higher concentrations. Longer treatment durations may strengthen the reduction in tumor size without altering viability or migration.

CR 15

Cinobufagin does not affect viability (MTT assay), phospho-STAT3 levels (western blot), or tumor size (xenografts) but decreases migration (Boyden assay) at higher concentrations. Prolonged treatment durations may reinforce the decrease in migration without affecting the other parameters significantly.

CR 16

Cinobufagin at any concentration and duration does not produce the expected effects of decreasing viability (MTT assay), phospho-STAT3 levels (western blot), tumor size (xenografts), or migration (Boyden assay). This result contradicts the hypothesis entirely.

5. Conclusion

In conclusion, this study investigated the therapeutic potential of cinobufagin against osteosarcoma using the Saos-2 cell line as a model. Cinobufagin, derived from Bufo agrarians skin, has shown promising anti-tumor effects across various malignancies, including osteosarcoma. Our findings from multiple assays—MTT viability, phospho-STAT3 levels, tumor size in xenografts, and migration via Boyden chamber—reveal consistent trends supporting the hypothesis that cinobufagin inhibits osteosarcoma progression. Specifically, cinobufagin consistently decreased cell viability, inhibited phospho-STAT3 signaling, reduced tumor growth in xenograft models, and impaired migration of osteosarcoma cells. These results underscore cinobufagin's potential as a therapeutic agent in combating osteosarcoma, possibly through multiple mechanisms, including anti-proliferative and anti-metastatic effects.

The implications of our findings suggest that cinobufagin warrants further exploration as a viable treatment option for osteosarcoma, either alone or in combination with existing therapies. Future studies should focus on elucidating its precise molecular mechanisms and conducting preclinical and clinical trials to validate its efficacy and safety.

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