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Evaluation of the effects of cinobufagin on G361 melanoma cells and a comparison with paclitaxel

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Abstract

Introduction: The pathophysiology of melanoma is considerably complex. Wnt signaling via the β-catenin / transcription factor 7-like 2 / lymphoid enhancer-binding factor 1 (LEF1) complex, microphthalmia-associated transcription factor, tyrosinase-related protein-2, tyrosinase, and cyclin-dependent kinase 2 are reported to activate the transcriptional gene expression associated with pigmentation and the differentiation and proliferation of melanocytes and malignant melanoma cells. Cinobufagin, a common active ingredient in traditional Chinese medicine, has been approved in China as a chemotherapeutic agent for the treatment of liver and prostate cancer. The primary aim of this study is to evaluate the effects of cinobufagin, paclitaxel, and their combination (cinobufagin/paclitaxel) on melanoma cells, both individually and in combination, in the G361 melanoma cell line.
Methods: Within the study, half maximal inhibitory concentration doses were determined based on the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, caspase-3 levels were measured using an enzyme-linked immunosorbent assay method, and mRNA expression levels were analyzed using RT-PCR.
Results: The use of cinobufagin/paclitaxel was found to increase caspase-3 levels by more than the use of cinobufagin alone; moreover, cinobufagin treatment decreased the expression of the β-catenin, c-myc, and cyclin D1 genes, whereas cinobufagin/paclitaxel was found to increase the mRNA expression of all genes considered (Bax, caspase-3, Bcl2, β-catenin, c-myc, Wnt, and cyclin D1).
Conclusions: Cinobufagin can therefore be considered a promising natural pharmaceutical agent for the targeted treatment of cancers with high levels of LEF1.

Keywords: apoptosis, cinobufagin, lymphoid enhancer-binding factor 1 (LEF1), melanoma

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Introduction

Cutaneous melanomas are tumors that develop as a result of malignant transformations of pigment-producing melanocytes in the basal layer of the epidermis. In addition to the skin, melanomas can also develop in the uvea, gastrointestinal tract, genitourinary system, and meninges (1). Approximately 80% of all skin cancer–related deaths are attributed to melanoma; most cases are reported in developed countries, including European countries, Australia, New Zealand, and the United States (2). The World Health Organization reports an annual incidence of melanoma of approximately 160,000, with 48,000 associated deaths worldwide each year (3). Both individual risk factors and environmental factors contribute to the etiology of melanoma, and ultraviolet radiation, prolonged exposure to the sun / sunburn, and geographical location are the predominant environmental factors. Among personal factors, the leading causes of melanoma are skin phototype, the number of pigmented nevi, genetic factors, and immunosuppressive conditions (1, 4).

As with many other types of cancer, melanomas are an outcome of the transformation of protooncogenes into oncogenes as a result of mutations, and tumor suppressor genes are also involved. In melanomas, oncogenes such as *BRAF*, *NRAS*, and *KIT* become activated, and tumor suppressor genes such as *CDKN2A*, *p53*, and *PTEN* become inactivated (5). The pathophysiology of melanoma is considerably complex. Wnt signaling via the β-catenin / transcription factor 7-like 2 (TCF4) / lymphoid enhancer-binding factor 1 (LEF1) complex, microphthalmia-associated transcription factor (MITF), tyrosinase-related protein-2 (TRP-2), tyrosinase (Tyr), and cyclin-dependent kinase 2 (Cdk2) are reported to activate the transcriptional gene expression that is associated with the pigmentation, differentiation, and proliferation of melanocytes and malignant melanoma cells (6). LEF1 is a key transcription factor in the Wnt/β-catenin signaling pathway, which plays a critical role in melanoma progression, proliferation, and treatment resistance. Thus, LEF1 can be considered a promising molecular target for cancer therapy (6, 7).

Cinobufagin is a bufadienolide compound derived from Chan Su, a traditional Chinese medicine obtained from the dried secretions of the skin and parotid glands of toads. It is recognized as one of the major bioactive components responsible for the pharmacological effects of this preparation. Previous studies have demonstrated that cinobufagin exhibits a wide range of biological activities, including antitumor, pro-apoptotic, anti-proliferative, immunomodulatory, and cardiotonic effects. In particular, its anticancer activity has been attributed to the induction of apoptosis, inhibition of cell proliferation, and modulation of key signaling pathways involved in tumor progression (8). In China, cinobufagin has been approved as a chemotherapeutic agent for the treatment of liver and prostate cancers; it is also considered an important active ingredient in traditional Chinese medicine. Previous studies have reported the ability of cinobufagin to effectively

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suppress cell growth and progression in hepatocellular carcinoma, osteosarcoma, esophageal carcinoma, and colorectal cancer by inhibiting angiogenesis and inducing cell cycle arrest and apoptosis (9). It is notable, however, that very few studies have examined the effects of cinobufagin on melanoma cells. In 1992, paclitaxel was approved for the treatment of advanced ovarian cancer by the US Food and Drug Administration; subsequently, it has been widely used for the treatment of breast cancer, colorectal cancer, and squamous cell carcinoma of the bladder, and as a second-line antineoplastic agent for the treatment of melanoma (10).

The primary aim of this study is to evaluate the effects of cinobufagin and paclitaxel on melanoma cells, both individually and in combination, in G361 melanoma cells.

Methods

Chemicals and reagents

Cinobufagin (catalog no. P1632) and paclitaxel (catalog no. C3460) were purchased from TCI (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). The G361 melanoma cell line (catalog no. CRL-1424) was obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). A human caspase-3 enzyme-linked immunosorbent assay (ELISA) kit was purchased from BT-LAB (Bioassay Technology Laboratory, Shanghai, China). All chemicals used in cell culture were obtained from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany).

Cell culture

G361 melanoma cells were cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum, 5% glutamine, and 1% penicillin–streptomycin. The cells were maintained at 37 °C in a humidified incubator with 5% CO_2 and 100% humidity.

Cell viability assay and experimental groups

Cells (10,000 cells per well) were seeded in 96-well plates, with experiments performed in triplicate. The cells were treated with differing concentrations of cinobufagin (1 nM, 10 nM, 50 nM, 100 nM, 250 nM, 500 nM, and 1,000 nM) and paclitaxel (1 pM, 10 pM, 50 pM, 100 pM, 250 pM, 500 pM, and 1,000 pM) for 24 h, after which 10 µl of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay solution (5 mg/ml) was added to each well and incubated for 4 h at 37 °C under 5% CO_2. After incubation, 100 µl of dimethyl sulfoxide (DMSO) was added to each well, and the absorbance at 570 nm was measured using an automated reader Epoch (Biotek, Winooski, Vermont, USA). The half maximal inhibitory concentration (IC_{50}) values for cinobufagin and paclitaxel were calculated using GraphPad Prism version 8.0.1 (GraphPad Software, Inc., CA, USA) software. After the IC_{50} values were determined, the appropriate doses of cinobufagin and paclitaxel, both separately and together, were applied to the cells. Subsequently, four study groups were formed: the control group, the cinobufagin group, the paclitaxel group, and the cinobufagin/paclitaxel combination group. Using biochemical analyses, the effects of each treatment were evaluated to determine their potential as a treatment, with each assay performed in triplicate.

Protein analyses

The cells were washed twice with phosphate-buffered saline and then harvested using a lysis buffer containing a protease inhibitor cocktail (Roche Complete, Indianapolis, IN, USA). The lysates were centrifuged at 16,000 *g* at 4 °C for 15 min, and the concentration of proteins collected from the supernatants was determined using the TaKaRa BCA Protein Assay Kit (Takara Bio, Shiga, Japan).

Caspase-3 analysis

After the cells were incubated with drugs for 24 hours, the caspase-3 levels in the extracted protein were measured using an ELISA test in accordance with the manufacturer's instructions. The results were expressed as ng/mg protein.

Isolation of total RNA, reverse transcription, and quantitative polymerase chain reaction

Cells were incubated with appropriate reagents for 24 hours and then washed with phosphate-buffered saline. The relative expres-

Table 1 | **Oligonucleotide primer sequences and PCR programs.**

Genes	Primer sequences (5' → 3')	RT-PCR program	Cycles
Bax	F: 5'-CATGAAGACAGGGGCCCTTT-3'	95 °C, 30 s; 59 °C, 60 s; 72 °C, 30 s	40
	R: 5'-AAACACAGTCCAAGGCAGCT-3'		
Bcl2	F: 5'-ACAGGGTACGATAACCGGGA-3'	95 °C, 30 s; 59 °C, 60 s; 72 °C, 30 s	40
	R: 5'-CATCCCAGCCTCCGTTATCC-3'		
Caspase-3	F: 5'-GTGCTACAATGCCCCTGGAT-3'	95 °C, 30 s; 59 °C, 60 s; 72 °C, 30 s	40
	R: 5'-GCTGGATGCCGTCTAGAGTC-3'		
C-myc	F: 5'-ACTTCTACCAGCAGCAGCAG-3'	95 °C, 30 s; 59 °C, 60 s; 72 °C, 30 s	40
	R: 5'-GAGCAGAGAATCCGAGGACG-3'		
Cyclin D1	F: 5'-TGTGATGCTGGGCACTTCAT-3'	95 °C, 30 s; 59 °C, 60 s; 72 °C, 30 s	40
	R: 5'-GACAGACAAAGCGTCCCTCA-3'		
GAPDH	F: 5'-GATTTGGTCGTATTGGGCGC-3'	95 °C, 30 s; 59 °C, 60 s; 72 °C, 30 s	40
	R: 5'-AGTGATGGCATGGACTGTGG-3'		
Wnt1	F: 5'-CCCAAACAGACTCGCTAGCA-3'	95 °C, 30 s; 59 °C, 60 s; 72 °C, 30 s	40
	R: 5'-CTGGGAGAATGGGGGCATTT-3'		
β-catenin	F: 5'-TTGAAGGTTGTACCGGAGCC-3'	95 °C, 30 s; 59 °C, 60 s; 72 °C, 30 s	40
	R: 5'-GCCACCCATCTCATGTTCCA-3'		

Bax = BCL2-associated X protein, Bcl2 = B-cell lymphoma 2, Caspase-3 = cysteine–aspartic acid protease-3, C-myc = MYC proto-oncogene, bHLH transcription factor, *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase, Wnt1 = Wnt family member 1, β-catenin = beta-catenin.

sion of mRNA in the total RNA was isolated using a commercial kit (EURx GeneMatrix, Gdańsk, Poland, catalog no. E3598) in accordance with the manufacturer's instructions. The quantification and purity of the isolated RNAs were analyzed using the Epoch Take3 plate system (Agilent, USA) and a complementary DNA (cDNA) synthesis kit in accordance with the manufacturer's instructions. Subsequently, 1 µg of total RNA was used as the template for the reverse transcription polymerase chain reaction (RT-PCR); then 1 µl of cDNA was taken from each sample, and appropriate amounts of SYBR green PCR Master Mix and a primer (oligonucleotide) were added as described in the protocol. The expression of the target genes was normalized to that of the housekeeping gene *GAPDH*, and the relative quantification (RQ) was calculated using the ΔΔCt method as described by the following equation: $RQ = 2^{-\Delta\Delta Ct}$. The primer sequences used in the PCR and the PCR conditions are described in Table 1. For each assay, triplicate experiments of three biological replications were analyzed.

Statistical analysis

The results obtained from this study were analyzed using IBM SPSS Statistics (IBM Corp., Version 20.0. Armonk, NY, USA). The conformity of the variables to a normal distribution was tested using the Shapiro–Wilk test. Descriptive statistics were presented as the mean ± standard deviation for normally distributed data, and the median (min–max) for nonnormally distributed data. The statistical significance of differences between the study groups was examined using ANOVA for parametric variables and a Kruskal–Wallis test for nonparametric variables. A Student's *t*-test was conducted for parametric variables and a Mann–Whitney *U* test for nonparametric variables. A p value of < 0.05 was considered to indicate statistical significance.

Ethics committee approval

This research was carried out in accordance with the Declaration of Helsinki and received approval from the Noninvasive Clinical Research Ethics Committee of Afyonkarahisar Health Sciences University on March 4th, 2022 (approval no. 2022/3).

Results

Cell viability

The IC50 values of 24-hour treatments of cinobufagin and paclitaxel were determined as 212 nM and 573 pM, respectively (Fig. 1).

Caspase-3 levels

In the cinobufagin, paclitaxel, and cinobufagin/paclitaxel (combination) groups, caspase-3 levels were significantly higher in G361 cells than in the control group (p < 0.001), as shown in Table 2 and Fig. 2.

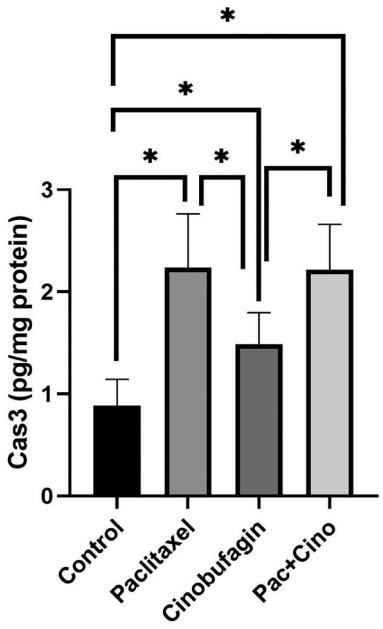

Figure 2 | In the cinobufagin, paclitaxel, and cinobufagin/paclitaxel (combination) groups, caspase-3 levels were significantly higher in G361 cells than in the control group (*p < 0.05).

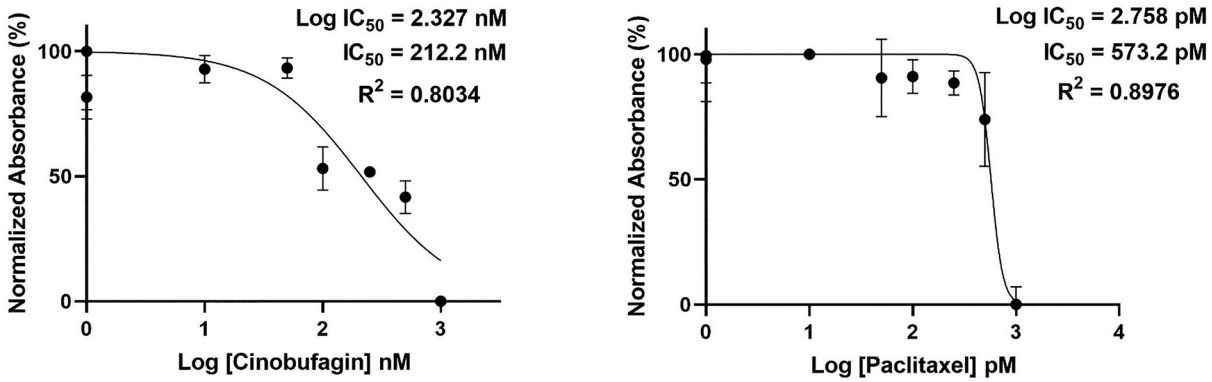

Figure 1 | The half maximal inhibitory concentration values of 24-hour treatments of cinobufagin and paclitaxel were determined as 212 nM and 573 pM, respectively.

Table 2 | Caspase-3 levels in different study groups.

Parameter	Group, mean ± *SD*			
	Control	Paclitaxel	Cinobufagin	Paclitaxel/cinobufagin
Caspase-3 (pg/mg protein)	0.8873 ± 0.2573	2.237 ± 0.5276[†]	1.49 ± 0.3076[‡,¶]	2.217 ± 0.4446[§,††]

[†]Statistically significant difference between control group and paclitaxel group (p < 0.001).
[‡]Statistically significant difference between control group and cinobufagin group (p = 0.026).
[§]Statistically significant difference between control group and paclitaxel/cinobufagin group (p < 0.001).
[¶]Statistically significant difference between paclitaxel group and cinobufagin group (p < 0.0043).
[††]Statistically significant difference between cinobufagin group and paclitaxel/cinobufagin group (p < 0.0056).
SD = standard deviation.

mRNA expression

The results show that paclitaxel treatment decreased only c-myc expression compared to the control group. In contrast, cinobufagin treatment reduced expression of the β-catenin, c-myc, and cyclin D1 genes. The combination treatment (cinobufagin/paclitaxel) increased the mRNA expression of all genes measured (Fig. 3, Table 3).

Table 3 | Relative mRNA expression levels of selected genes in G361 cells following treatment with paclitaxel, cinobufagin, or their combination. Values show fold changes relative to the untreated control group. Fold change values are dimensionless and represent relative mRNA expression normalized to *GAPDH* and control group levels (set as 1.0).

	Paclitaxel	Cinobufagin	Paclitaxel/cinobufagin
Bax	0.715	0.865	14.462
Bcl2	1.110	1.064	2.046
Caspase-3	1.136	0.615	15.825
C-myc	−0.346	−0.365	8.140
Cyclin D1	0.619	−0.604	9.775
Wnt	1.035	0.815	24.098
β-catenin	1.059	−0.571	5.333

Bax = BCL2-associated X protein, Bcl2 = B-cell lymphoma 2, Caspase-3 = cysteine–aspartic acid protease-3, C-myc = MYC proto-oncogene, bHLH transcription factor, Wnt1 = Wnt family member 1, β-catenin = beta-catenin.

Discussion

In this study, combined treatment with cinobufagin and paclitaxel resulted in a greater increase in caspase-3 levels compared to cinobufagin alone, indicating enhanced apoptotic activity. In addition, cinobufagin monotherapy significantly downregulated the expression of β-catenin, c-myc, and cyclin D1, which are key downstream targets of the Wnt/β-catenin signaling pathway. These findings are consistent with previous reports demonstrating that cinobufagin exerts its antitumor effects through inhibition of LEF1-mediated transcriptional activity and suppression of Wnt/β-catenin signaling in melanoma cells (9, 11).

The Wnt/β-catenin signaling pathway plays a central role in melanoma progression, proliferation, and therapeutic resistance. Aberrant activation of this pathway has been associated with increased tumor aggressiveness and poor clinical outcomes, largely through transcriptional regulation of oncogenic targets such as c-myc and cyclin D1 (6, 7). In line with these findings, the observed downregulation of these genes following cinobufagin treatment supports its potential as a targeted inhibitor of Wnt/β-catenin signaling.

Notably, the combination treatment led to an increase in mRNA

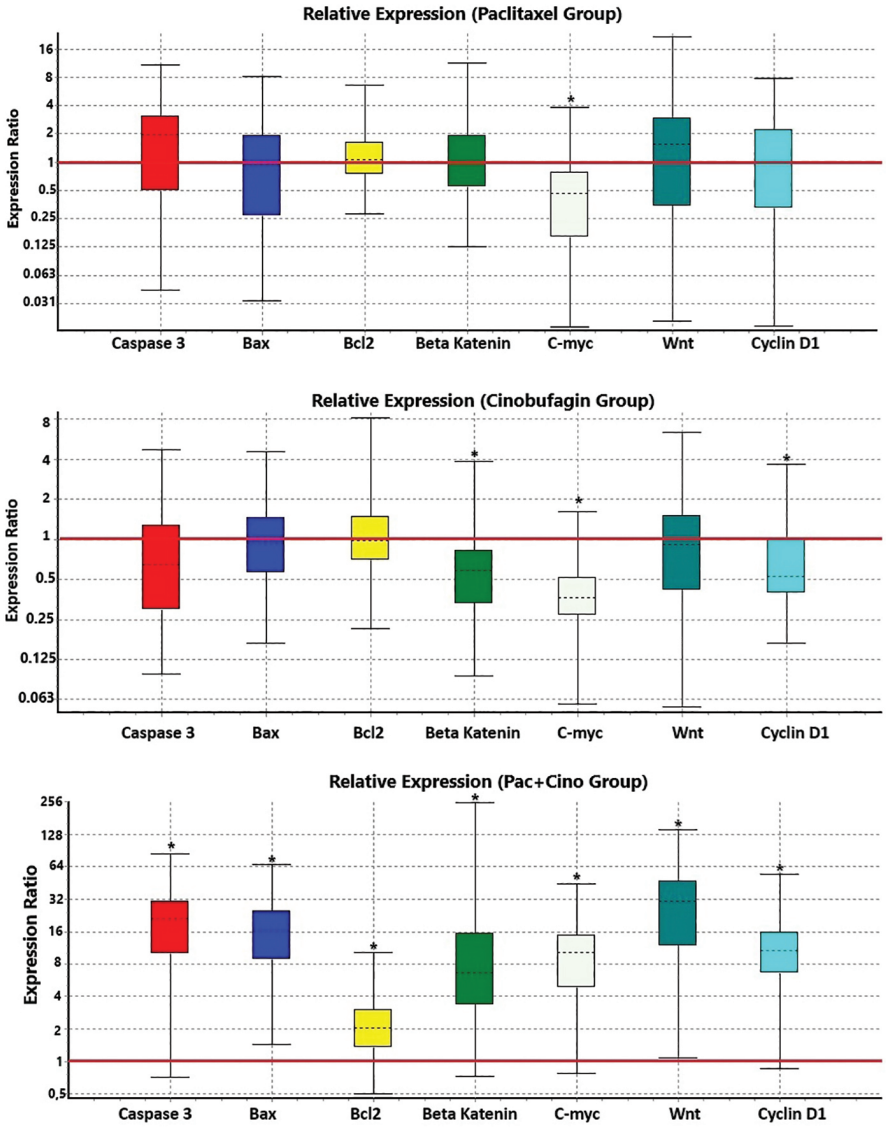

Figure 3 | Relative mRNA expression levels of genes. Values are expressed as mean ± standard deviation. All groups were compared to the control group, and the results were given as fold increase/decrease. REST 2009 software (Qiagen) was used for statistical analysis and graphing. The red line parallel to the *x*-axis shows the position of the control group, and $p < 0.05$ was considered statistically significant.

expression levels of all genes analyzed, which appears paradoxical given the observed pro-apoptotic effects. This apparent discrepancy may be explained by compensatory transcriptional activation or stress-induced feedback mechanisms triggered by cytotoxic treatment. Similar findings have been reported in previous studies, in which increases in mRNA levels did not correlate with protein expression due to post-transcriptional and post-translational regulatory processes (12). Therefore, the observed increase in mRNA expression may not necessarily reflect enhanced functional activity but rather a transient adaptive cellular response.

Current treatment strategies for melanoma have evolved significantly with the introduction of immune checkpoint inhibitors, including anti-programmed cell death protein 1 (PD-1) and anti-cytotoxic T-lymphocyte–associated protein 4 (CTLA-4) antibodies, as well as targeted therapies such as BRAF and MEK inhibitors. These approaches have markedly improved survival outcomes in advanced melanoma; however, the development of resistance remains a major clinical challenge (13). Consequently, there is an ongoing need for novel therapeutic agents and combination strategies that can overcome resistance and enhance treatment efficacy (14). Where possible, early treatment is recommended to prevent the metastasis and spread of melanoma because, after this has occurred, treatment becomes extremely difficult (11).

In recent studies, phytochemicals have become the focus of many research laboratories, with growing interest in natural products with anticancer properties owing to their low tissue toxicity (15). Several classes of phytochemicals modulate the various molecular mechanisms that contribute to melanoma development, including compounds such as fisetin, epigallocatechin-3-gallate, resveratrol, silymarin, curcumin, lupeol, honokiol, and tryptanthrin. Preclinical studies of phytochemicals, both alone and in combination with traditional cytotoxic and targeted therapies, have yielded promising results, although a literature review reveals very few studies investigating the effects of cinobufagin on melanoma cells (11, 15). A recent study reported the anticancer effect of cinobufagin to be associated with its inhibition of the LEF1-mediated activation of the Wnt/β-catenin signaling pathway in A375, A2058, and G361 melanoma cells. In addition, the expression of cancer-causing genes such as c-myc, cyclin D1, and axin-2, which are regulated by LEF1 and Wnt/β-catenin signaling, was reduced significantly by cinobufagin (9). Similarly, in this study, cinobufagin treatment was found to decrease the expression of the β-catenin, c-myc, and cyclin D1 genes. Previous studies have found that activation of the Wnt/β-catenin signaling pathway containing LEF1, TCF4, and β-catenin is increased considerably in melanoma. High nuclear β-catenin levels have also been observed in human melanoma samples, indicating that the activated canonical Wnt/β-catenin signaling pathway has an important role in the physiologies of melanoma and melanocytes (9). The importance of such a marker has been clearly emphasized in the literature because the increased gene expression is not reflected at the protein level and may be related to posttranslational changes and the duration of treatment/agent administration (12). Consequently, the observed increase in mRNA expression levels following combined cinobufagin/paclitaxel treatment is an important finding of this study and highlights the need to further investigate changes at the translational level. This apparent paradox may be explained by compensatory pathway activation or stress-induced transcriptional upregulation, which may not necessarily translate into corresponding changes in functional protein levels. In another recent study, cinobufagin was shown to inhibit cell survival and induce apoptosis in a dose-dependent manner in a case of uveal melanoma (16). Although the literature data on this subject are limited, cinobufagin would appear to be a useful pharmaceutical agent targeting cancers that express high levels of LEF1.

Paclitaxel is a tricyclic diterpenoid naturally occurring compound found in the bark and needles of the tree *Taxus brevifolia*. Owing to its unique anticancer mechanism, it is currently one of the most successful and widely used natural anticancer drugs throughout the world (10). Doo et al. reported that the combined use of the small molecule WNT974, which inhibits the Wnt/β-catenin signal in ovarian cancer cells, and paclitaxel led to improved immune parameters and reduced tumor growth compared to the use of either agent alone (17).

The efficacy of this combined treatment has been attributed to overlapping mechanisms of action: paclitaxel, which stabilizes microtubules, and β-catenin, which stabilizes mitotic spindles and facilitates centrosome separation. Inhibitors of the Wnt pathway may also be particularly useful in preventing chemoresistance. A recent study reported that low-dose paclitaxel combined with XAV939 (a Wnt inhibitor) induced higher levels of apoptosis in breast cancer cell cultures and model mice through the inhibition of Wnt signaling than when used alone, and suppressed angiogenesis, potentially leading to a higher level of therapeutic efficacy and fewer side effects (18). Zhang et al. evaluated the interaction between paclitaxel, bufalin, and cinobufagin in cultured hepatoma cells to identify the optimum combination and reported that the synergistic effects of the combination of bufalin and cinobufagin increased the potency of paclitaxel against tumor cells (19).

Despite these promising findings, several limitations should be acknowledged. First, protein expression levels corresponding to the mRNA targets analyzed were not evaluated, limiting the interpretation of transcriptional changes. Second, the relatively short treatment duration (24 hours) may not fully reflect long-term cellular responses. Further studies incorporating protein-level analyses and extended treatment periods are warranted to better elucidate the molecular mechanisms underlying the observed effects.

Conclusions

This study demonstrates that cinobufagin exerts significant effects on melanoma cells, particularly through modulation of the Wnt/β-catenin signaling pathway and induction of apoptosis. The enhanced apoptotic response observed with combination treatment suggests a potential synergistic interaction between cinobufagin and paclitaxel. These findings support the potential role of cinobufagin as a novel adjunctive therapeutic agent targeting LEF1-associated pathways in melanoma.

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