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Chemopreventive efficacy of stampidine in a murine breast cancer model

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Abstract

Background: The purpose of the present study was to examine the chemopreventive effect of Stampidine, an aryl phosphate derivative of stavudine, in side by side comparison with the standard anti-breast cancer drug paclitaxel in the well-established 7,12-dimethylbenz(a)anthracene (DMBA)-induced murine breast cancer model.

Methods: Groups of 20 female mice were challenged with the chemical carcinogen DMBA. DMBA-challenged mice were assigned to various chemoprevention treatments, including stampidine alone, paclitaxel alone, and stampidine plus paclitaxel according to the same treatment schedules for 25 weeks.

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Author contributions

KS, FMU and SQ were involved in the conception and design; CO, MT and KS performed the experiments; CO, IHO, MT, UK and BE were involved in analysis and interpretation of the data; SQ and CO performed statistical analyses; TKS and UK drafted the manuscript; KS and FMU wrote and revised the paper. All the authors read and approved the final version of manuscript. All authors agree to be accountable for all aspects of the work.

Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Results: Stampidine treatments resulted in substantially reduced numbers of mammary tumors, tumor weight as well as tumor size in DMBA-treated mice. Stampidine was as effective as paclitaxel in the model and their combination exhibited greater chemopreventive activity, as measured by reduced tumor incidence and improved tumor-free survival as well as overall survival of DMBA-treated mice. The length of time for the initial tumor to appear in DMBA-challenged mice treated with stampidine was longer than that of mice treated DMBA-challenged control mice. Tumors from mice treated with stampidine or stampidine plus paclitaxel displayed unique changes of a signature protein cassette comprised of BRCA1, p21, Bax, and Bcl-2.

Conclusion: Stampidine has potent chemopreventive activity and is as effective as the standard chemotherapy drug paclitaxel in the chemical carcinogenesis model of DMBA-induced murine breast cancer.

Keywords

stampidine; paclitaxel; apoptosis; breast cancer; mice

1. Introduction

Advanced and metastatic breast cancer patients are in urgent need for therapeutic innovations. Others and we previously reported a series of phosphoramidate substituted nucleoside analogs as potential anti-cancer drugs [1–7]. An aryl phosphate derivative of zidovudine showed promising activity in preclinical models of leukemia [8]. Likewise, another aryl phosphate derivative of zidovudine, 3’-Azidothymidine 5’-[*p*-Methoxyphenyl methoxyalaninyl phosphate], showed activity in preclinical models of breast cancer [9,10]. Notably, NUC-1031, a phosphoramidate modification of gemcitabine, was evaluated in a phase 1 clinical trial and provided early evidence for single agent activity [11].

Stampidine (5’-[4-bromophenyl methoxylaninylphosphate]-2’,3’- didehydro-3’-deoxythymidine) (CAS 217178-62-6), is an aryl phosphate derivative of stavudine [7,12]. Stampidine showed an acceptable nonclinical safety profile in both small and large animal safety pharmacology studies [12, 13]. No dose-limiting toxicities or treatment-emergent serious adverse events or drug-related fatalities were observed in a Phase 1 clinical study of Stampidine [13]. Stampidine has been shown to act as an epigenetic regulator of gene expression [14]. Stampidine has been also found to selectively methylate and silence several oncogenic transcription factor genes [14]. The purpose of the present study was to examine the chemopreventive effect of stampidine in an in vivo carcinogenesis model of breast cancer in mice that employs the chemical carcinogen 7,12-dimethylbenz(a)anthracene (DMBA).

2. Materials and methods

2.1. Animals

A total of 100 female BALB/c mice at the age of 50 days were used to demonstrate the effect of stampidine on breast cancer in DMBA-induced mice. Animals obtained from Firat University (Elazig, Turkey) were kept under standard conditions of humidity (55±5%), room temperature (22±2°C) and 12-hour light/dark cycles and had free access to water and food.

The study was performed at the Faculty of Veterinary Science of the Firat University in Elazig, Turkey with approval of the Animal Care and Use Committee (IACUC) at the Firat University (Approval No. 36–201102). All processes were done in agreement with the related law, the Animal Welfare Law, and the Public Health Service Policy.

2.2. Experimental design for mammary tumorigenesis in mice

Animals were divided into 5 groups (n=20 per group): (i) Control, mice received sesame oil only and saline; (ii) DMBA, mice received DMBA; (iii) DMBA+ Stampidine; Mice injected with stampidine (i.p.) three times per week (50 mg/kg BW) following DMBA administration; (iv) DMBA+Paclitaxel, mice injected with paclitaxel (i.p.) once per week (10 mg/kg BW) after DMBA administration [15]; (v) DMBA+Paclitaxel+Stampidine, mice injected with paclitaxel combined with stampidine after DMBA administration. DMBA was dissolved in sesame oil to give a 10 mg/ml stock concentration and mice were gavaged p.o. with 0.1 ml (total 1 mg) DMBA once a week for 6 weeks [16]. Sesame oil was used as a vehicle for administration of DMBA in all mice. Specifically, DMBA was dissolved in sesame oil to give a 10 mg/ml stock concentration and mice were gavaged p.o. with 0.1 ml (total 1 mg) DMBA once a week for 6 weeks. Controls included vehicle-treated mice that did not receive DMBA in order to isolate and fully appreciate the carcinogenic effects of DMBA in this mouse model. These mice were treated with sesame oil as a placebo and saline was used to clear the syringe of remaining sesame oil in order to standardize the amount of sesame oil administered. DMBA and paclitaxel (taxol) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Stampidine was prepared according to a service contract and in accordance with current Good Manufacturing Practices (cGMP) in the clinical manufacturing facility at the Division of Pharmaceutical Service at the College of Pharmacy at the University of Iowa (Iowa City, Iowa, U.S.A. also known as Iowa University Pharmaceuticals (IUP).

Mice were examined twice a week to check the development of mammary tumors by palpation from the first day of acclimatization until the end of the experiment and time of tumor appearance was recorded. The sacrifice and autopsy either of mice that died or of those that became moribund were performed during the experiment. All survivors were sacrificed by cervical dislocation at the end of 25 weeks. All tumors were counted and weighed. A 1 mm precision sliding caliper was used for measuring the size of tumors.

2.3. Protein levels

2.3.1. Material isolation—Mammary glands or tumor samples were harvested during necropsy and placed on dry ice and stored at −80°C. Protein samples from tumor specimens as well as normal mammary glands of healthy control mice were used. Multiple samples of equal amounts of mammary gland or tumor tissue were pooled from four animals. To extract the proteins, pooled tissue samples from each group were homogenized in 1 ml ice-cold hypotonic buffer A, containing 10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM MgCl2, 1 mM DTT, 0.1 mM EDTA, and 0.1 mM phenylmethylsulfonyl-fluoride (PMSF). 80 μl of 10% Nonidet P-40 (NP-40) solution was added to the homogenates and the mixtures were centrifuged for 2 min at 14,000 g. The precipitates, containing nuclei, were washed once with 500 μl of buffer A plus 40 μl of 10% NP-40, centrifuged, resuspended in 200 μl of

buffer C [50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 20% glycerol], and centrifuged for 30 min at 14.800 g and the supernatant was transferred into fresh tubes. The tissue homogenates were collected for Western blot analyses of BRCA1, p21, Bcl-2, Bax, caspase-3, and β-actin.

2.3.2. Western blot—Protein levels were measured by Western blotting method as described earlier [17]. Protein concentration was determined by the Lowry method. Equal amounts of protein (50 μg) from pooled tissue samples were electrophoresed (12% SDS-PAGE gels) and subsequently transferred to nitrocellulose membrane (Schleicher and Schuell Inc., Keene, NH, USA). The primary antibodies against BRCA1, p21, Bcl-2, Bax, caspase-3, and β-actin were obtained from Abcam (Abcam Inc., UK). Primary antibody was diluted (1:1000) in the same buffer containing 0.05 % Tween-20. The nitrocellulose membrane was incubated overnight at 4 °C with protein antibody. The blots were washed and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (goat anti-mouse IgG) at a dilution of 1:5000 (Abcam, Cambridge, UK). Blots were done at least four times to confirm data reproducibility. Protein levels were evaluated by densitometry using an image analysis system (Image J; National Institute of Health, Bethesda, USA), corrected with values determined on β-actin blots and showed as relative values compared with the control group.

2.4. Statistical analysis

Tumor incidence and number were evaluated by chi-square test while tumor weight and volume, serum, and protein levels data were examined by one-way ANOVA using SPSS statistical program (IBM, SPPS Version 21). Alterations among groups were detected by the Tukey's multiple comparisons. Furthermore, The Kaplan-Meier method, log-rank chi-square test was used to investigate for development of mammary gland tumors and tumor-free survival in each group. The protein levels were examined by one-way ANOVA followed by *post hoc* Tukey's test. Statistical significance was stated when $P<0.05$.

3. Results

3.1. Stampidine prevents development of mammary gland tumors and improves tumor-free survival in DMBA-treated mice

All 20 mice treated with DMBA only without any Paclitaxel or Stampidine developed mammary tumors at a median of 14 weeks (95% CI=11–16 weeks) (Figure 1). Stampidine treatments resulted in prevention or delayed appearance of mammary tumors in DMBA-treated mice. Only 15 of 20 mice in the Stampidine group developed tumors at a median of 18.5 weeks (95% CI=14-NA weeks) and this difference from the tumor incidence in control mice treated with DMBA only without any Paclitaxel or Stampidine was statistically significant (Log-rank $X^2=5.7$; $P=0.047$). Similar results were obtained with Paclitaxel (Median time to tumor appearance: 15.5 weeks; 95% CI=12-NA weeks). Only 13 of 20 mice treated with a combination of Stampidine plus Paclitaxel developed mammary tumors at a median of 19 weeks (95% CI=15-NA weeks) (Log-rank $X^2=8.5$; $P=0.008$). The tumor numbers per mouse were significantly lower ($P<0.001$) in DMBA-treated mice receiving Stampidine (mean±SE=1.3±0.2, N=15), Paclitaxel (mean±SE=2.3±0.2), or Stampidine +

Paclitaxel (mean±SE=1.8±0.2) than in DMBA-treated control mice not receiving Paclitaxel or Stampidine (mean±SE=4.6×0.2). The average sizes based on volume (Figure 2, $P < 0.05$ for DMBA vs Paclitaxel and $P < 0.01$ for DMBA vs Stampidine or Stampidine + Paclitaxel) or weight of tumors (Figure 3, $P < 0.001$ for all) as well as the total tumor load (Figure 4, $P < 0.001$ for all) in DMBA-treated mice receiving Stampidine, Paclitaxel, or a combination of Stampidine + Paclitaxel were significantly smaller than the average size of tumors in DMBA-treated control mice not receiving Stampidine or Paclitaxel.

Stampidine significantly improved the tumor-free survival (Log-rank X^2=8.6, P=0.003) as did Paclitaxel (Log-rank X^2=5.5, P=0.019) (Figure 5). No DMBA-treated control mouse not receiving Stampidine or Paclitaxel remained alive tumor-free beyond 15 weeks. By comparison, 40±11% of DMBA-treated mice also receiving Stampidine or Paclitaxel were alive without tumors by end of week 15 and 25±10% remained alive tumor-free until the end of the experiment at week 25 (Figure 5). The combination of Stampidine plus Paclitaxel appeared slightly more effective than Paclitaxel alone in improving the tumor-free survival outcome, but the observed difference was not statistically significant (Log-rank X^2=1.4, P=0.2) (Figure 5): 55±11 % of mice treated with Stampidine + Paclitaxel were alive by end of week 15 and 35±11% remained alive tumor-free until the end of the experiment at week 25.

3.2. Protein Expression profiles of Stampidine-Refractory Mammary Gland Tumors.

As shown in Figure 6, the $BRCA1^{high}P21^{low}$ mammary tumors in DMBA-treated mice not receiving Paclitaxel or Stampidine were characterized by a high level expression of the anti-apoptotic protein BCL2 as well as low level expression of the pro-apoptotic proteins BAX and Caspase-C. By comparison, the Stampidine refractory tumors emerging despite chemoprevention with Stampidine had low level expression of the anti-apoptotic proteins and higher level expression of the pro-apoptotic proteins. These findings are consistent with a model in which DMBA-induced malignant clones with low level expression of the anti-apoptotic protein BCL2 and increased expression of the pro-apoptotic proteins BAX and Caspase-C, albeit not as aggressive as their BCL2 overexpressing counterparts are capable of escaping the chemo-preventive effects of Stampidine. Hence, the resistance to Stampidine does not seem to relate to the anti-apoptotic machinery of the DMBA-induced malignant clones.

4. Discussion

Phosphoramidate substituted nucleoside analogs have emerged as a new class of promising anti-cancer drugs. A novel zidovudine derivative was recognized as a candidate anti-breast cancer agent that prevented bipolar mitotic spindle assembly. Notably, it prolonged cancer-free survival in the $MMTV^{neu}$ transgenic mouse model of HER2 positive breast cancer [9,10]. Likewise, NUC-1031, a phosphoramidate modification of gemcitabine, was evaluated a phase 1 clinical trial and exhibited promising single agent activity [11]. In the present study, we have evaluated the chemopreventive activity of a novel clinical-stage stavudine derivative, stampidine, in side by side comparison with the standard anti-breast cancer drug paclitaxel in the well-established 7,12-dimethylbenz(a)anthracene (DMBA)-

induced breast cancer model. Our results provide unprecedented evidence that stampidine impedes DMBA-induced carcinogenesis, significantly reduces the number and size of mammary gland tumors in DMBA-challenged mice and improves their tumor-free survival outcome. Intriguingly, the tumors developing despite stampidine chemoprevention displayed a distinctly more pro-apoptotic protein expression profile, which substantially differed from the protein expression profile of aggressive breast tumors developing in DMBA-challenged control mice. Hence, stampidine appears to prevent the development of aggressive breast cancers with an apoptosis-resistant protein expression profile. These insights may provide the foundation for new chemo-preventive strategies in which stampidine is applied to prevent the development of the most aggressive and difficult to treat forms of apoptosis-resistant breast cancer. Similar results were obtained for mammary gland tumors in paclitaxel-treated mice, which provides a cogent explanation as to why a combination of Stampidine and Paclitaxel did not abrogate breast cancer development in this model.

The exact molecular mechanism for the observed chemo-preventive activity of stampidine is unknown. Notably, stampidine has been found to selectively methylate several transcription factors found at hubs of correlated gene expression changes to cause widespread disruption of unique gene networks [14]. 821 probes showed increased methylation of 568 genes in the presence of stampidine [14]. Several genes silenced via methylation by stampidine were components of a network of associated oncogenic transcription factors that were silenced in the presence stampidine [14]. Of these, estrogen related receptor alpha (ERRα) is an orphan nuclear factor that is the main regulator of cellular energy metabolism. ERRα is overexpressed in a variability of tumors, including breast, ovarian, prostate, and cervical and is related with a more aggressive tumor and a worse outcome. ERRα is a critical regulator of cancer progress since it can meet the energy demands of proliferating cancer cells. High ERRα expression, specifically in breast cancer, is related to an increased rate of recurrence and a poor prognosis [18]. Retinoid orphan nuclear receptor alpha (RORA) is a member of family of orphan nuclear factors that play a role in the regulation of lipid and steroid metabolism, immune response and circadian rhythms involved in the pathogenesis of breast cancer [19]. Likewise, GATA-2 protein plays a vital role in the modulation transcription of genes involved in the development and proliferation of hematopoietic and endocrine cell lineages and is associated in breast cancer pathogenesis TFCP2 interacts with beta-catenin, improves the interaction between beta-catenin and TCF4, and triggers beta-catenin/TCF signaling [20–22]. TFCP2 has been described to interact with Polycomb protein RNF2 and has been implicated in tumorigenesis of pancreas cancer, colon cancer, and hepatocellular carcinoma (HCC). ZFP36L2 is zinc finger protein 36, C3H type-like 2 has been shown to increase cancer cell aggressiveness in pancreatic cancer [23]. In our future studies, we will seek to determine if the expression levels of these proteins, especially ERRa are altered by Stampidine during the course of DMBA-induced oncogenesis. It is currently also unknown whether or not the chemopreventive activity of Stampidine may in part be owing to a reduction of DMBA-induced oncogenic driver mutations contributing to mammary carcinogenesis, such as the previously reported somatic Ras gene mutations (e.g. $Hras^{CAA61CTA}$) [24, 25]. A separate comprehensive and unbiased analysis with next generation sequencing and whole exome comparisons of control mice treated with DMBA vs. test mice treated with DMBA as well as Stampidine would be required to explore such a

possibility as activating ras mutations are usually not involved in either the initiation or metastatic progression of human breast cancer. [26].

5. Conclusions

Stampidine exhibited potent chemopreventive activity and improve the tumor-free survival outcome of mice in a chemical carcinogenesis model of 7,12-dimethylbenz(a)anthracene (DMBA)-induced breast cancer. Our study uniquely demonstrates that stampidine both delays the development of and significantly reduces the number of mammary gland tumors developing in DMBA-challenged mice. The tumors developing despite stampidine chemoprevention were much smaller than those of DMBA-challenged control mice and displayed a distinctly more pro-apoptotic protein expression profile, which substantially differed from the protein expression profile of aggressive breast tumors developing in DMBA-challenged control mice. Hence, stampidine appears to prevent the development of aggressive mammary gland tumors with an apoptosis-resistant protein expression profile.

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Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers

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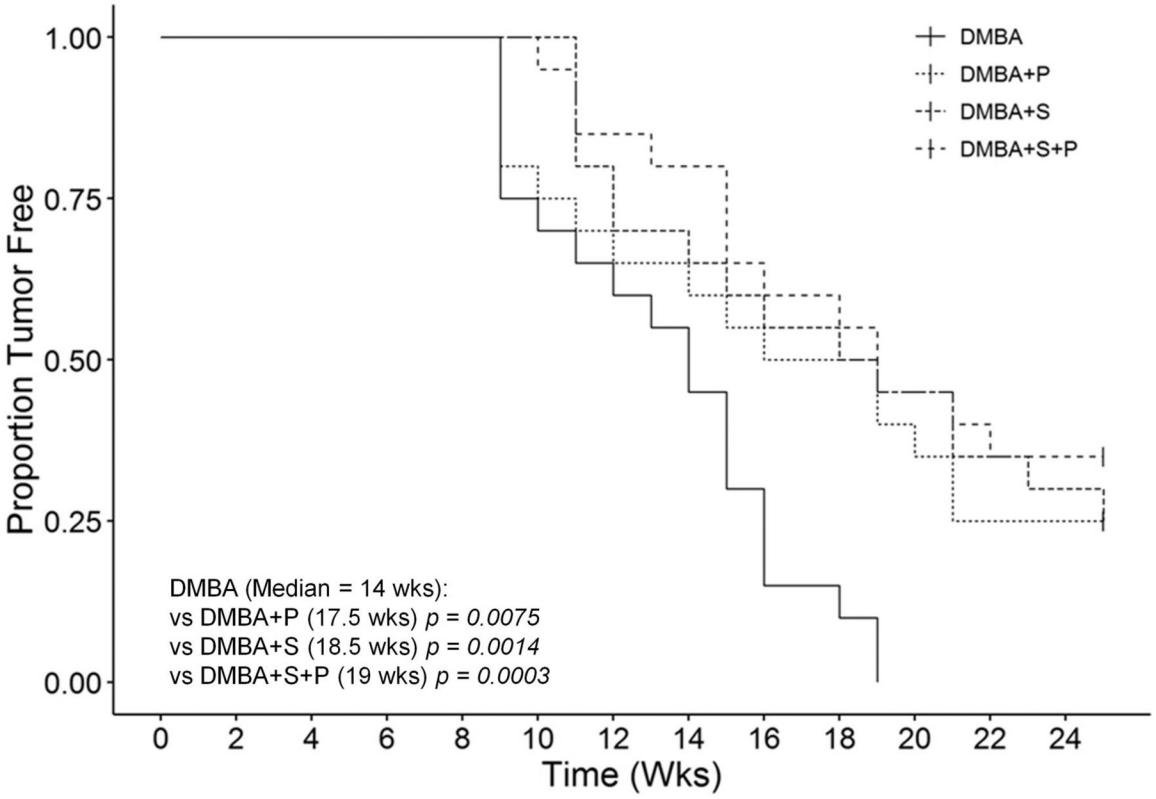

Figure 1. Effects of Chemoprevention with Stampidine, Paclitaxel, and Stampidine+Paclitaxel on Probability of Developing Mammary Gland Tumors in DMBA-Challenged Mice.

Depicted are the cumulative proportions of mice remaining tumor-free at the indicated time points. Treatment groups were stampidine (S), and paclitaxel (P), and combination of stampidine and paclitaxel (S+P) groups. DMBA, the mice were treated with DMBA; DMBA +Stampidine, the mice were treated with stampidine (50 mg/kg, three times a week, i.p.) following DMBA administration; DMBA+P, the mice were treated with paclitaxel (10 mg/kg, once a week, i.p.) following DMBA administration. Mice in the DMBA+P+S group were treated with stampidine (50 mg/kg, three times a week, i.p.) and paclitaxel (10 mg/kg, once a week, i.p.) following DMBA administration. All animals were sacrificed 25 weeks following DMBA exposure.

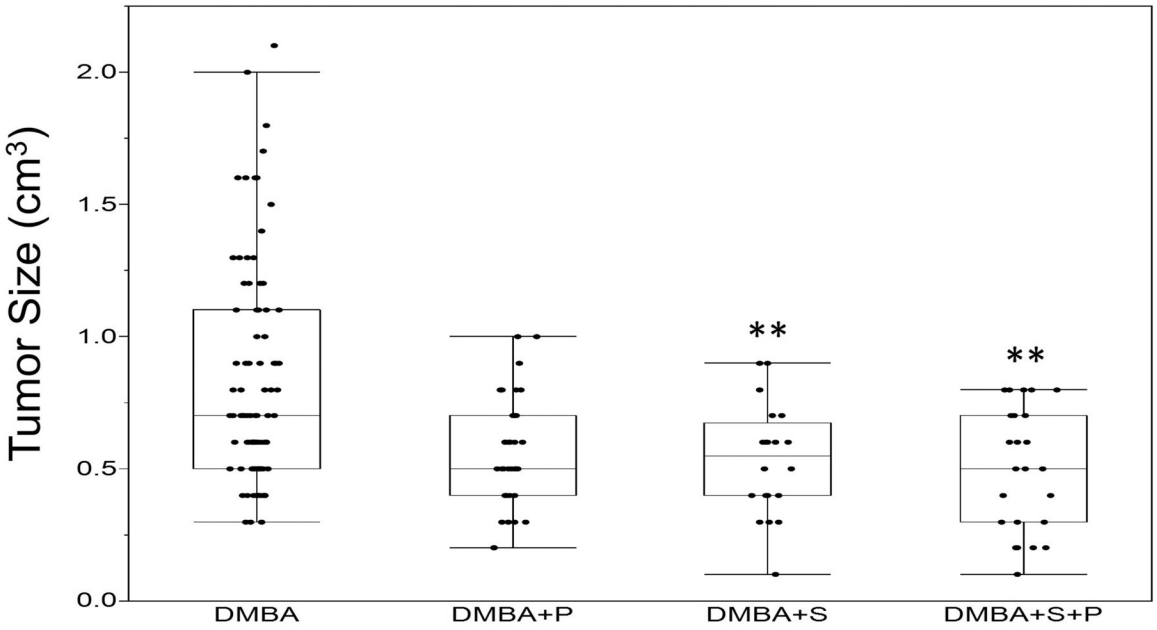

Figure 2. Prevention of development of large (as measured by volume) mammary gland tumors by chemoprevention with Stampidine, Paclitaxel, and Stampidine+Paclitaxel.

Depicted are the Box- and Whisker plots of the tumor sizes in various treatment groups. DMBA, the mice were treated with DMBA; DMBA+Stampidine, the mice were treated with stampidine (50 mg/kg, three times a week, i.p.) following DMBA administration; DMBA+P, the mice were treated with paclitaxel (10 mg/kg, once a week, i.p.) following DMBA administration. Mice in the DMBA+P+S group were treated with stampidine (50 mg/kg, three times a week, i.p.) and paclitaxel (10 mg/kg, once a week, i.p.) following DMBA administration. All animals were sacrificed 25 weeks following DMBA exposure.

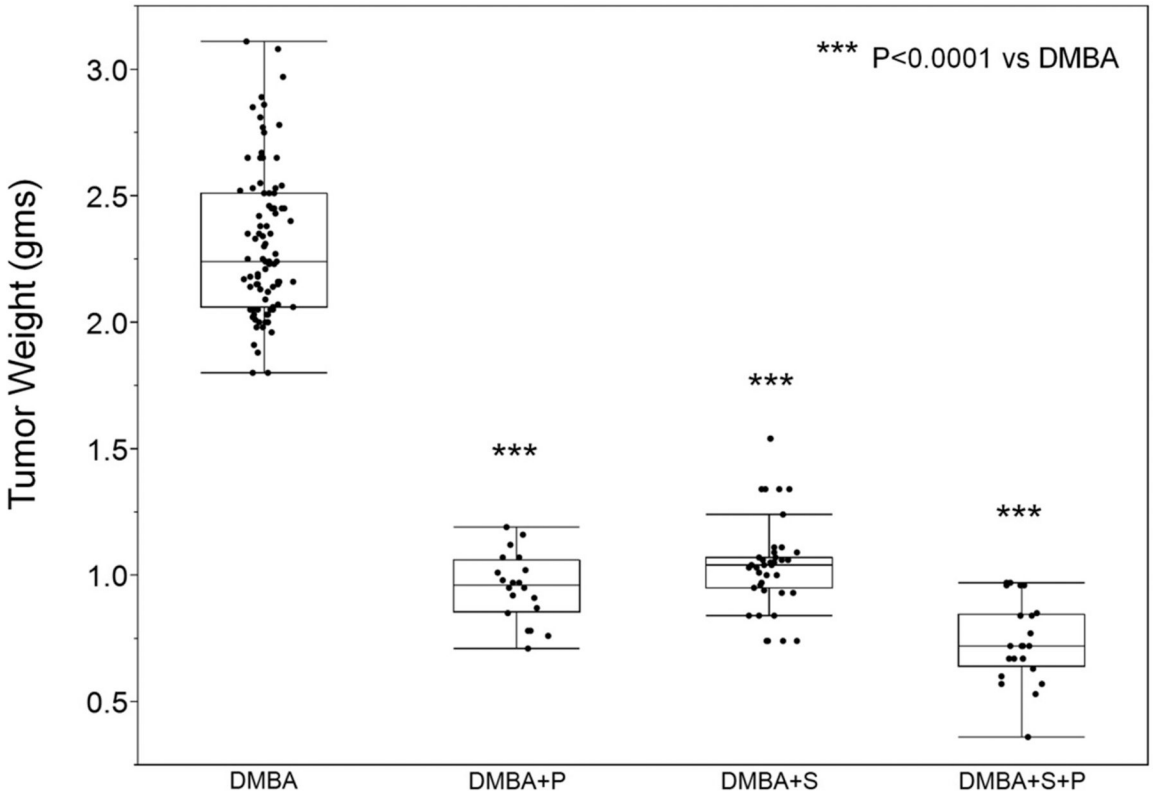

Figure 3. Prevention of development of large (as measured by weight) mammary gland tumors by chemoprevention with Stampidine (S), Paclitaxel (P), and Stampidine+Paclitaxel (S+P).

Depicted are the Box- and Whisker plots of the tumor sizes in various treatment groups. DMBA, the mice were treated with DMBA; DMBA+S, the mice were treated with stampidine (50 mg/kg, three times a week, i.p.) following DMBA administration; DMBA+P, the mice were treated with paclitaxel (10 mg/kg, once a week, i.p.) following DMBA administration. Mice in the DMBA+P+S group were treated with stampidine (50 mg/kg, three times a week, i.p.) and paclitaxel (10 mg/kg, once a week, i.p.) following DMBA administration. All animals were sacrificed 25 weeks following DMBA exposure.

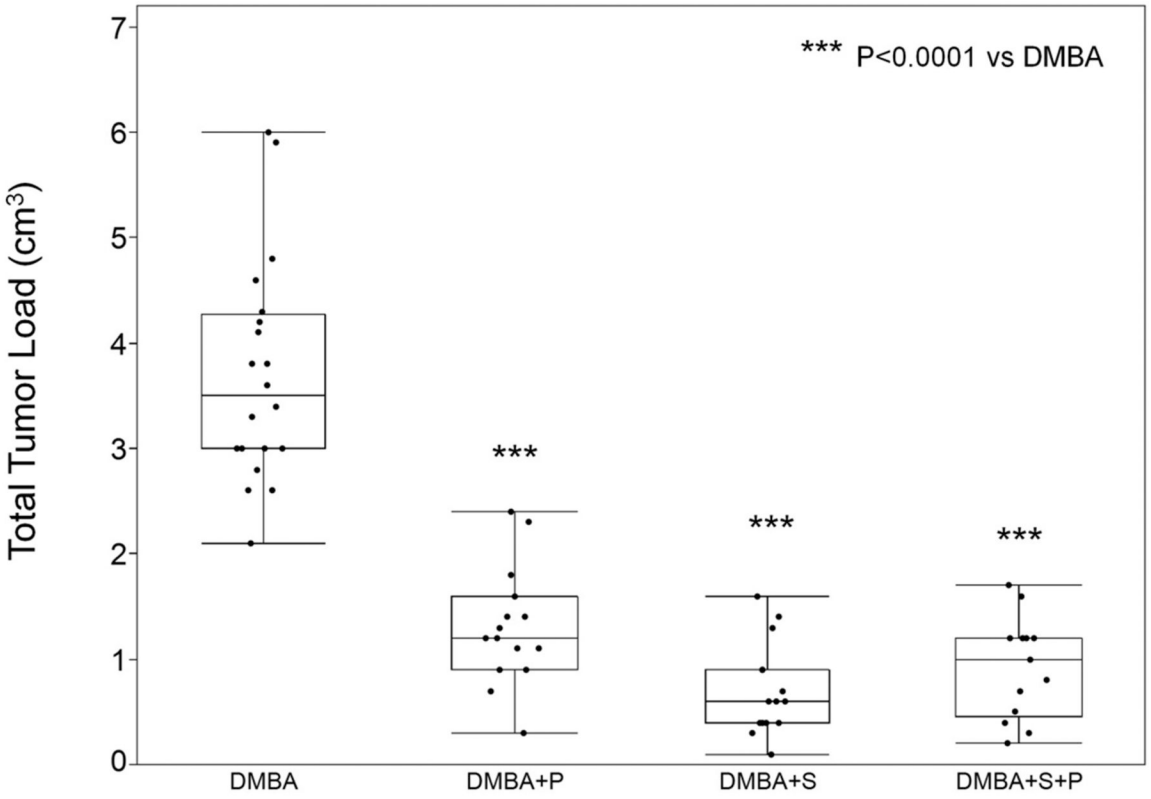

Figure 4. Comparison of the total tumor load of mice receiving chemoprevention with Stampidine, Paclitaxel, and Stampidine+Paclitaxel.

Depicted are the Box- and Whisker plots of the total tumor load per mouse (i.e. sum of the volume of all detected mammary gland tumors) in various treatment groups. DMBA, the mice were treated with DMBA; DMBA+Stampidine, the mice were treated with stampidine (50 mg/kg, three times a week, i.p.) following DMBA administration; DMBA+P, the mice were treated with paclitaxel (10 mg/kg, once a week, i.p.) following DMBA administration. Mice in the DMBA+P+S group were treated with stampidine (50 mg/kg, three times a week, i.p.) and paclitaxel (10 mg/kg, once a week, i.p.) following DMBA administration. All animals were sacrificed 25 weeks following DMBA exposure.

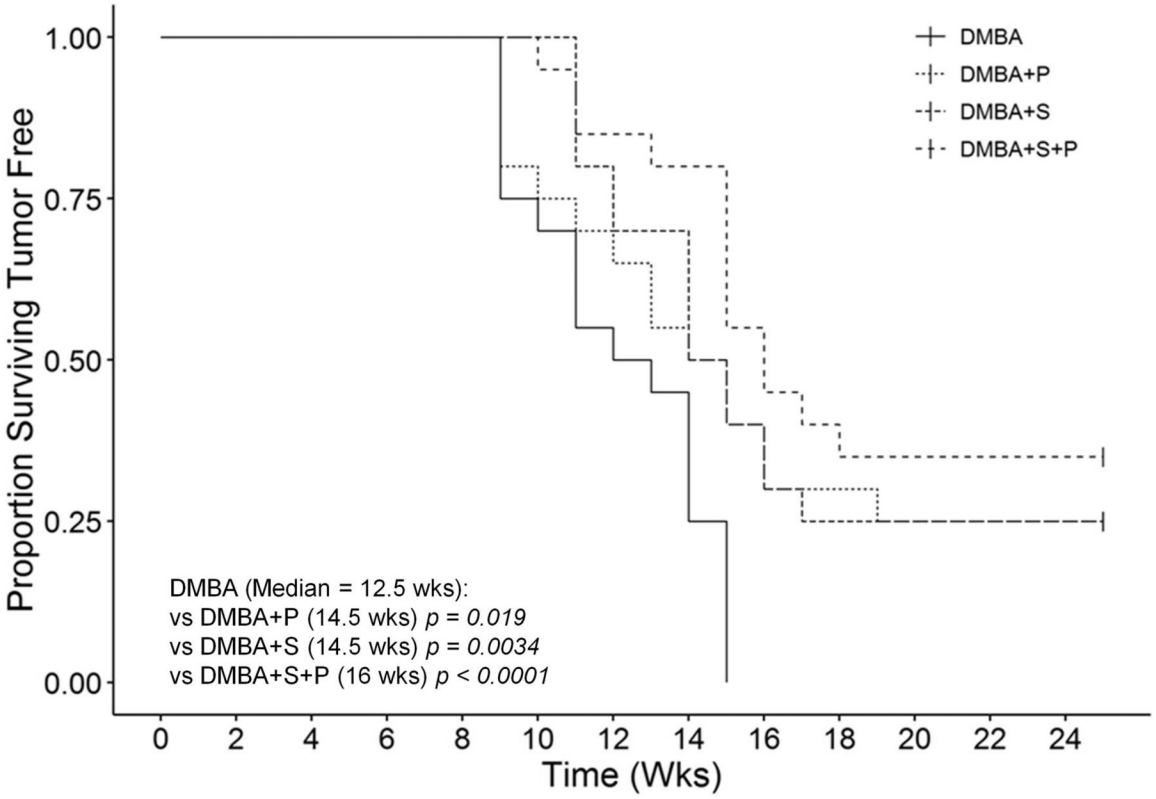

Figure 5. Effect of Stampidine-Based Chemoprevention on Tumor-Free Survival in DMBA Mammary Carcinogenesis Model.

All mice were challenged with 1 mg DMBA weekly for 6 weeks via gavage. Stampidine (50 mg/kg) was administered i.p 3x/week for 6 weeks following DMBA administration. Paclitaxel (10 mg/kg) was administered i.p weekly × 6 weeks either alone or in combination with Stampidine treatments following DMBA administration. Mice in the DMBA+P+S group were treated with stampidine (50 mg/kg, three times a week, i.p.) and paclitaxel (10 mg/kg, once a week, i.p.) following DMBA administration. Results are presented as the proportion of mice surviving tumor-free as a function of time. Depicted are the tumor-free survival curves for each chemoprevention group along with the median tumor-free survival times and log-rank P-value for the comparison of each treatment group with the DMBA control group.

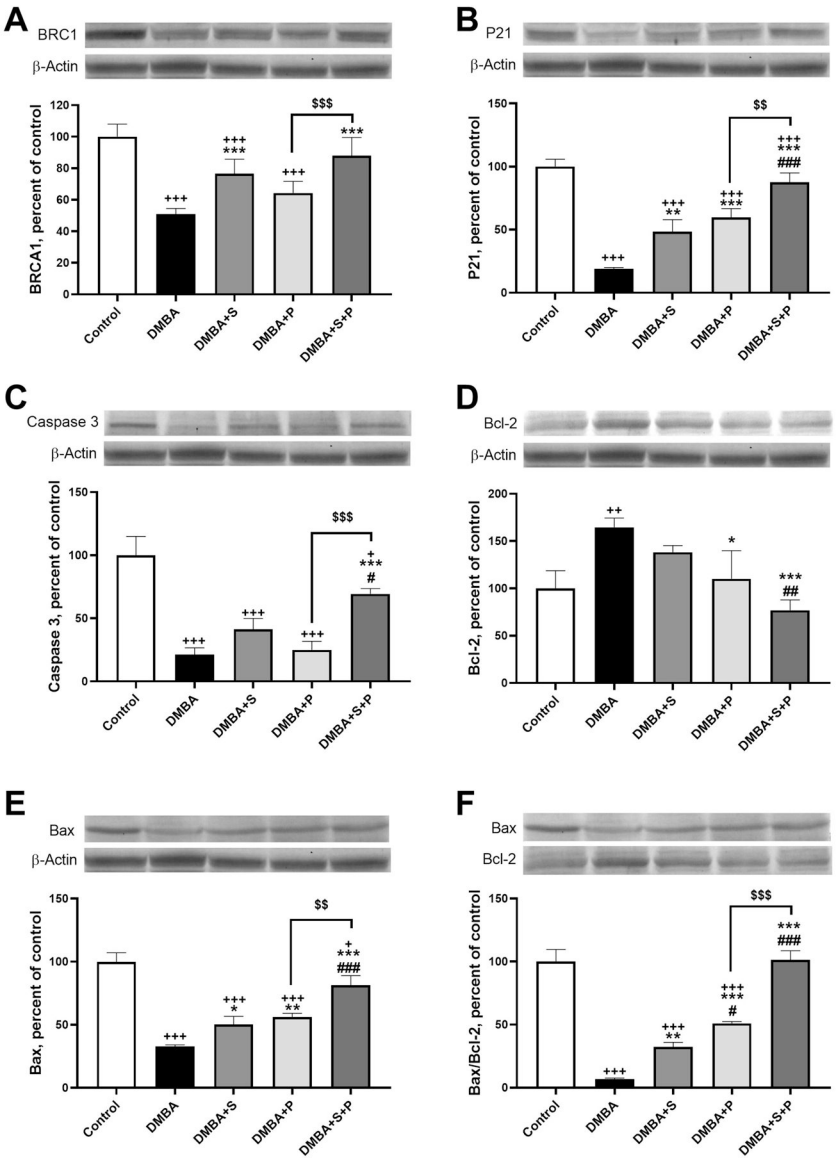

Figure 6. Protein expression profiles of mammary gland tumors developing in mice treated with DMBA alone or DMBA plus Stampidine (S), Paclitaxel (P), or Stampidine + Paclitaxel (S+P).

The depicted proteins are BRCA1 (Panel A), p21 (Panel B), Caspase 3 (Panel C), Bcl-2 (Panel D), Bax (Panel E), and Bax to Bcl-2 ratio (Panel F). Data are expressed as percent of the control value. The bar represents the standard deviation and mean. Blots were repeated at least 4 times (n=4), and a representative blot is shown. Actin was included to ensure equal protein loading. (ANOVA and Turkey's post-hoc test. Statistical significance between groups is shown by: $^{+}$ $P<0.05$; $^{++}$ $P<0.01$; $^{+++}$ $P<0.001$ compared as control group; * $P<0.05$; ** $P<0.01$; *** $P<0.001$ compared as DMBA group not receiving Stampidine or Paclitaxel; # $P<0.05$; ## $P<0.01$; ### $P<0.001$ compared as DMBA+S group; $ $ $P<0.01$; $ $ $ $P<0.001$ compared as DMBA+P group).