Interactive effects of 1, 25-dihydroxyvitamin D3 and soy protein extract (SPE) on oral cancer growth in vitro: evidence for potential functional relationships.

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ABSTRACT:

Background: Previous studies have found specific soy isoflavones (Genistein, Daidzein, Glycitein) demonstrate anti-tumor properties against several cancer types, including oral cancer. Few studies have evaluated whole soy extract, containing a combination of these isoflavones and other bioreactive compounds, which may function synergistically and more effectively against oral cancers. Preliminary work by this group has now demonstrated whole soy protein extract (SPE) inhibits oral cancer cell growth specifically and selectively, through independent cell-cycle and apoptotic pathways. However, more recent evidence now suggests that ingestion of vitamin D_3, either in dietary foods or supplements may potentiate the activity of soy components and their anti-tumor effects.

Objective: The primary goal of this study was to investigate the interactive and inter-connected effects of 1, 25-dihydroxyvitamin D_3 administration with the anti-proliferative effects of whole soy protein extract (SPE) on oral cancer and normal cell lines *in vitro*.

Methods: Three oral squamous cell carcinoma cell lines (SCC15, SCC25, and CAL27) were treated with 1, 25-dihydroxy Vitamin D_3 at physiological concentrations (10-125 nmol). Cell growth was then compared with cell treatment using soy protein extract (SPE) within the normal physiologic range (0 - 10 µM/L). Interactive effects were then evaluated using co-administration of SPE and 1, 25-dihydroxy Vitamin D_3. Quantitative RT-PCR was performed at various time points to determine any changes in mRNA expression for key cell cycle and apoptotic signaling

pathway regulators, including *p53*, *c-myc*, *ornithine decarboxylase (ODC)*, *caspase-2*, *caspase-8*, and *bax*.

Results: Administration of 1, 25-dihydroxy Vitamin D$_3$ induced distinct dose-dependent, growth-inhibitory effects in all three oral cancer cell lines examined. These inhibitory effects were comparable to the overall range of growth inhibition induced by SPE. However, the combined effects of co-administration were far greater, suggesting the presence of synergistic relationships between these components. In addition, these results indicate that either treatment alone appeared to modulate mRNA expression of oral cancer cell-cycle promoters *c-myc* and *OD*C, as well as the *caspase*-dependent apoptosis pathway, while only 1, 25-dihydroxy Vitamin D$_3$ administration appeared to influence the *bax* pathway.

Conclusion: These results suggest that co-administration with 1, 25-dihydroxy Vitamin D$_3$ and SPE may enhance their anti-tumor effects. This study may help to explain, in part, why balanced diets rich in fruits, vegetables, and soy protein, are associated with protection against development and progression of oral cancers, although further study is needed to develop specific public health recommendations for oral cancer treatment and prevention.

Key words: vitamin D, soy extract, whole soy protein, oral cancer, growth inhibition.

BACKGROUND:

Oral cancer: The main risk factors contributing to the development of oral cancers in the United States (US) are tobacco use (in the form of smoking), and alcohol consumption, which together may be responsible for up to 80% of this cancer risk [1-3]. Another important risk factor for oral and pharyngeal cancers (OPC) is infection with the human papillomavirus (HPV), which has been identified in a significant subset of these cancers [4, 5]. More recently, evidence has demonstrated that dietary intake may account for as much as 20-25% of the variability in OCP risk, with health-protective effects and reduced incidences associated with specific dietary components such as fruits and vegetables, soy proteins, coffee, fiber, folic acid, and the vitamins A, C, D, and E [6, 7].

Diet and nutrition: In addition to these health protective and cancer preventive effects, evidence now suggests that progression of oral cancer is also intricately linked with diet and nutrition, which can modulate the influence of all three major oral cancer risk factors, as well as cellular growth [8]. For example, dietary fruit or vegetable intake separately and independently reduces oral cancer risk or progression - even after adjusting for age, gender, or tobacco use and alcohol consumption [8, 9]. The efforts to identify and characterize specific dietary components with these potent chemo preventive and chemotherapeutic properties have led researchers to explore a large number of putative anti-cancer agents derived specifically from fruits, vegetables and legumes.

Oral cancer inhibition: Flavonoids and polyphenolics, carotenoids, dithioltiones,

glucosinolates, indoles, isothiocyanates, protease inhibitors, plant sterols, allium compounds, limonenes, selenium, vitamin C, vitamin E, and dietary fiber all have noted inhibitory effects on oral cancer [10-12]. The majority of studies focusing on dietary prevention and therapy for oral cancer have explored the role of flavonoids, part of a large family of polyphenolic compounds made by plants [13-19]. New evidence, however, has suggested that another subclass of flavonoids highly concentrated in soybeans and soy-containing foods (isoflavones) may also exhibit potent effects specific for oral cancers [20-24].

Soy isoflavones: Many of these studies have focused on specific isoflavones derived from soy, which include Daidzein, Genistein, and Glycitein, which are capable of inhibiting growth and proliferation of oral cancers [25-27]. Although significant, these effects were observed *in vitro* at supraphysiologic levels (> 10 µmol/L), which may be neither safe (*in vivo*) nor feasible through dietary consumption or nutritional supplementation. This group was among the first to demonstrate that soy proteins, in combination, may be useful and effective against oral cancers at concentrations that approximate physiologic serum levels (0-2 µmol/L) achievable through dietary or supplement intake, which may help to explain why diets rich in fruits, vegetables, and soy protein are associated with protection against development and progression of oral cancers [24]. There is some evidence suggesting that synergistic effects of multiple isoflavones in whole soy foods and soy extracts may be responsible for preventing the negative side effects observed with single agent administration to normal cells, as well as the expression of anti-cancer effects at much lower serum concentrations [28, 29].

Soy interactions with Vitamin D3: Researchers have focused on the direct, antitumor properties of soy isoflavones on oral cancers; however, few studies have evaluated the interactive effect of whole soy (or soy-derived isoflavones) via regulation of P450 enzymes, involved in the metabolism of Vitamin D. However, there is evidence to suggest this may be a significant regulatory pathway of anti-cancer action as the few studies completed to date have demonstrated that soy protein (both *in vivo* and *in vitro*) directly influences the expression of P450 enzymes in other cancers - thereby regulating cellular Vitamin D metabolism [30-32]. For example, CYP24 is often highly expressed in many cancers, which degrades newly synthesized or serum-derived levels of 1, 25-dihydroxyvitamin D3 [33-36]. These studies suggest that dietary intake of soy may result in a consistent down-regulation of CYP24, thereby enabling the functions and anti-cancer properties of Vitamin D [32, 37].

Vitamin D and oral cancer: Consistent with these laboratory findings, large-scale population studies clearly demonstrated that low serum vitamin D levels and vitamin D deficiencies are associated with much higher cancer risk [38, 39]. More specifically, epidemiologic and case-controlled studies have confirmed that low vitamin D levels and deficiency are strongly associated with OPC risk [40, 41]. The primary mechanism of Vitamin D action is mediated through binding of either 1, $25(OH)_2D$ (active form) or 25(OH)D (less active form) to the vitamin D receptor (VDR), which is a member of the nuclear receptor super family of steroid and thyroid hormones with gene regulatory and consequent anti-proliferative properties [42, 43].

Vitamin D mechanism: Binding of 1, 25(OH)$_2$D to the VDR (either in the cell nucleus or cytoplasm) promotes association of the VDR- 1,25(OH)$_2$D complex with the retinoid X receptor (RXR) [41, 42]. The 1, 25(OH)2D–VDR–RXR complex binds to vitamin D-response elements in DNA which operate to initiate gene transcription. Activation of the VDR by 1, 25(OH)$_2$D can restore or enhance pro-apoptotic effects in different cancer cells through transcriptional activation of bax and p-calpain, two effective pro-apoptotic proteins [44, 45]. Although much is known about these effects in other cancers, few studies to date have evaluated Vitamin D in oral cancers [46, 47].

Evidence for interactive effects: There is now evidence that many oral cancers may, in fact, exert their effects on cellular Vitamin D metabolism by changing the availability of (or affecting the ability to bind to) the VDR. New evidence demonstrates that some oral cancers may exhibit reduced VDR expression [48, 49]. In addition, Ras activation (common in many oral cancers) might also impair Vitamin D-mediated transcription. Cytochrome p450 (CYP24, the enzyme responsible for degrading vitamin D) exhibited the highest up-regulation (196-fold increase) from a screening of more than 4,500 genes in one oral cancer cell line [47]. This may provide some evidence consistent with the observation that CYP24 mutations may lower oral cancer risk compared with wild type, after adjusting for age, gender, alcohol consumption, and smoking status [50].

Based upon this information, the overall objective of this study is to evaluate the anti-cancer properties of soy protein extract (SPE), which is available as a dietary supplement and most closely resembles the whole soy food that most US adults are likely to consume. The effects of SPE against well-characterized oral cancer cell lines will be evaluated using *in vitro* analysis methods. These experiments will be performed in conjunction with administration of Vitamin D to determine the interactive effects of SPE on Vitamin D-induced oral cancer growth inhibition.

Based upon previous work that determined SPE and Vitamin D are each, independently sufficient to mediate the proliferative phenotypes of these cancers in this experimental model, the working hypothesis is that co-administration will synergistically amplify these effects. Using in vitro analysis methods, any quantitative differences in viability and growth of oral cancer cells will be measured. The specific aims will be to characterize the inhibition of oral cancer proliferation induced by SPE administration in combination with Vitamin D, as well as any effects on cellular viability. The activation and expression of the apoptotic regulators, caspase-2, caspase-8, and bax, will also be assessed.

MATERIALS AND METHODS:

Cell culture and cell lines: The human oral squamous cell carcinoma cell lines, CAL 27 (CRL-2095) and SCC25 (CRL-1628), were obtained from American Type Culture Collection (ATCC: Manassas, VA). CAL 27 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 4.0 mM L-Glutamine adjusted to contain 3.7 g/L sodium bicarbonate, 4.5 g/L glucose, and 110 mg/L sodium pyruvate, obtained from HyClone (Logan, UT). SCC25 cells were maintained in a 1:1 mixture of DMEM and Ham's F12 medium with 2.5 mM L-Glutamine,

modified to contain 15 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES), 0.5 mM sodium pyruvate, and 1.2 g/L sodium bicarbonate.

The normal oral gingival fibroblast cell line, HGF-1 (CRL-2014), was also obtained from American Type Culture Collection (ATCC: Manassas, VA). HGF-1 cells were maintained in DMEM with 4 mM L-Glutamine, adjusted to contain 3.7 g/L sodium bicarbonate and 4.5 g/L glucose, from HyClone (Logan, UT). All cell culture media was supplemented with 1% Penicillin (10,000 units/mL)-Streptomycin (10,000 μg/mL) solution and 10% fetal bovine serum (FBS) obtained from HyClone (Logan, UT). Cells were cultured in 75 cm^2 BD Falcon tissue-culture treated flasks (Bedford, MA) at 37°C and 5% CO_2 in humidified chambers.

Materials: 1, 25-dihydroxyvitamin D $_3$ (VitD$_3$) was obtained from GNC Preventive Nutrition® (Pittsburgh, PA). Proliferation and viability assays were performed in the appropriate complete media, with and without the addition of VitD$_3$ (10, 50 and 125 nmol). This concentration range (25 – 312.5 ng/mL) approximates the physiologic concentrations of the less active form of VitD$_3$, but supraphysiologic concentrations of the active form, which allowed for short-term *in vitro* effects to be observed [42, 51]. This also enabled comparison with the only other studies examining OPC cell lines [46, 47], which utilized similar concentration ranges (1, 10, 100 nmol or 2.5, 125, 250 ng/mL).

Soy protein extract (SPE) was obtained from GNC Preventive Nutrition® (Pittsburgh, PA). Equivalent amounts of SPE were used to approximate the low- (10 μg/mL), mid- (50 μg/mL), and high-range (100 μg/mL) concentrations of flavonoid extracts utilized in prior *in vitro* studies [16, 17, 19], which were 10, 50, and 100 μg/mL. In addition to total SPE, the concentration of soy isoflavones for each experimental condition was also calculated to determine if these concentrations were within the normal physiologic range (0 - 10 μM/L).

Quantitative analysis provided by GNC demonstrated each gram of SPE contained 0.86 – 2.6 mg of isoflavones, with 0.30-0.91mg/g Daidzein (all forms), 0.52-1.56 mg/g Genistein (all forms), and 0.04-0.13mg/g Glycitein (all forms), resulting in an approximate ratio of 7:12:1 (Daidzein, Genistein, Glycitein, respectively). The proportional molecular weights (MW) of the soy isoflavones in the SPE were calculated to be:

    Daidzein (MW = 254.24) x 7 = 1779.68
    Genistein (MW = 270.2) x 12 = 3242.40
    Glycitein (MW = 284.24) x 1 = 284.24
    Total (sum)   = 5306.32 / 20 = proportional, averaged MW 265.31

To derive physiologic concentration, the total amount of SPE used (mg/L) is multiplied by the percentage of isoflavones contained within this amount; the proportional amount of total isoflavone is then divided by the averaged MW to yield moles (M/L); this amount is converted to derive final isoflavone concentration (μmol/L) as follows:

    10 μg/mL SPE = 10 mg/L or 0.01 g SPE
    0.01 g SPE (0.0026 isoflavone concentration per g/SPE) = 0.000026 g total isoflavone

0.000026 g / 265.31 MW = 0.00000000979 mol/L (1,000,000) = 0.0979 µmol/L

10 µg/mL SPE = 0.0979 µmol/L total isoflavone	=	approximately 0.1µmol/L
50 µg/mL SPE = 0.4895 µmol/L L total isoflavone	=	approximately 0.5µmol/L
100 µg/mL SPE = 0.979 µmol/L total isoflavone	=	approximately 1.0 µmol/L

Previous studies have demonstrated normal physiological levels of total plasma soy isoflavones vary between 1 and 2 µmol/L [52, 53], although higher concentrations have been observed among Asian populations [54, 55]. Based upon this information, the range of soy isoflavone concentrations utilized in this study (0.1 – 1.0 µmol/L) was within the normal physiologic range. The individual concentrations for the active soy isoflavones are as follows:

	10 µg/mL	**50 µg/mL**	**100 µg/mL**
SPE	2 µM/L	10 µM/L	20 µM/L
Daidzein	0.0357 µM/L	0.185 µM/L	0.357 µM/L
Genistein	0.057 µM/L	0.289 µM/L	0.577 µM/L
Glycitein	0.005 µM/L	0.023 µM/L	0.046 µM/L

Proliferation: Proliferation assays were performed in the appropriate complete media, with and without the addition of SPE, VitD$_3$ or both, prior to the start of each experimental assay. In brief, cells were plated in Corning Costar high-throughput, 96-well assay plates (Corning, NY) at a concentration of 1.2 x 10^4 cells per well, which roughly approximates 30-40% confluence per well at the onset of each assay. Proliferation was subsequently measured after three days. Cultured cells were fixed after 72 hours or day 3 using 50 µL of 10% buffered formalin, and were stained with crystal violet 1% aqueous solution (Fisher Scientific: Fair Lawn, NJ). The relative absorbance was measured at 630 nm using a Bio-Tek ELx808 microplate reader (Winooski, VT). Data was analyzed and graphed using Microsoft Excel (Redmond, WA) and SPSS (Chicago, IL). Three separate, independent replications of each experiment were performed.

Statistics: Comparisons of the effects of treatments were made using two-tailed *t*-tests with α = .05. All samples were analyzed using two-tailed *t*-tests as departure from normality can make more of a difference in a one-tailed than in a two-tailed t test [56]. As long as the sample size is moderate (> 20) for each group, quite severe departures from normality make little practical difference in the conclusions reached from these analyses. Multiple analyses involving two-sample *t*-tests have higher probability of Type I errors, leading to false rejection of the null hypothesis (H$_0$). To confirm the effects of these experiments, one-way analysis of variance (ANOVA) was used to assess statistical significance, $p < 0.05$. In addition, the data were analyzed after each experimental set to determine whether if the growth inhibition was normally distributed. This analysis revealed the cumulative probably of these data falling within a normal distribution was 0.99379 or 99.38% (α = 0.0125). Because the outcome measures were tested against a three (individual tests of SPE or VitD$_3$concentration) or four (combination of SPE and

VitD$_3$ concentrations) predictors, a Bonferroni-adjusted significance level of α = 0.0125 was used to account for the increased possibility of type I error (α = 0.05 / 4 = 0.0125).

Survival and viability: Prior to plating cells for proliferation assays, aliquots of trypsinized cells were stained using Trypan Blue (Sigma: St. Louis, MO), and live cells were enumerated by counting the number of Trypan-blue negative cells using a VWR Scientific Counting Chamber (Plainfield, NJ) and a Zeiss Axiovert 40 inverted microscope (Gottingen, Germany). At each time point (d1-d3), several wells were processed using the Trypan stain, and live cells were enumerated using this procedure.

RT-PCR: RNA was isolated from 1.5 x 10^7 CAL27, SCC25 and SCC15 cells at baseline (day 0), after 24 hours (day 1), and 72 hours (day 3) following administration of VitD$_3$ combined with SPE at the indicated concentrations; lowest concentration [L] and the Growth Inhibitory Maximum (GI$_{MAX}$) or highest [H] concentration using ABgene Total RNA Isolation Reagent (Epsom, Surrey, UK) and the procedure recommended by the manufacturer. RT-PCR was performed on total RNA with the ABgene Reverse-iT One-Step RT-PCR Kit (ReadyMix Version) and a Mastercycler gradient thermocycler (Eppendorf: Hamburg, Germany). The following mRNA primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [17], *p53* [17], *c-myc* [8], *ornithine decarboxylase (ODC)* [57], *caspase-2* [58], *caspase-8* [59], and *bax* [58], synthesized by SeqWright (Houston, TX), were used:

p53 forward primer, ACCAGGGCAGCTACGGTTTC;
p53 reverse primer, CCTGGGCATCCTTGAGTTCC;
c-myc forward primer, TCCAGCTTGTACCTGCAGGATCTGA;
c-myc reverse primer, CCTCCAGCAGAAGGTGATCCAGACT;
ODC forward primer, AATCAACCCAGCGTTGGACAA;
ODC reverse primer, ACATCACATAGTAGATCGTCG;
caspase-2 forward primer, TGGCATATAGGTTGCAGTCTCGG;
caspase-2 reverse primer, TGTTCTGTAGGCTTGGGCAGTTG;
caspase-8 forward primer, GATATTGGGGAACAACTGGAC;
caspase-8 reverse primer, CATGTCATCATCCAGTTTGCA;
bax forward primer, GGTTTCATCCAGGATCGAGACGG;
bax reverse primer, ACAAAGATGGTCACGGTCTGCC;
GAPDH FORWARD: ATCTTCCAGGAGCGAGATCC;
GAPDH REVERSE: ACCACTGACACGTTGGCAGT;

RNA standard: GAPDH was obtained from control cells, human gingival fibroblasts (HGF-1) 0.3-0.5 x 10^6 cells/mL were used to establish the minimum threshold (CT) and saturation (CS) cycles required for calibration and concentration comparisons using relative endpoint (RE) RT-PCR. GAPDH signal detection above background or threshold (CT) required a minimum of ten cycles (C10), with saturation (CS) observed at C40. Based upon this data, RE-PCR was performed at C35, above the lower detection limit but below the saturation limit.

In brief, one (1) μg of template (total) RNA was used for each reaction. The reverse transcription step ran for 30 minutes at 47°C, followed by denaturation for 2 minutes at 94°C. Thirty-five amplification cycles were ran, consisting of 20 second denaturation at 94°C, 30 seconds of annealing at 58°C, and 6.5 minutes of extension at 72°C. Final extension was run for 5 minutes at 72°C. Reaction products were separated by gel electrophoresis using Reliant 4% NuSieve® 3:1 Plus Agarose gels (Lonza: Rockland, ME). Bands were visualized by UV illumination of ethidium-bromide (EtBr)-stained gels and captured using a Kodak Gel Logic 100 Imaging System and 1D Image Analysis Software (Eastman Kodak: Rochester, NY). Quantification of RT-PCR band densitometry was performed using Adobe (San Jose, CA) Photoshop imaging software, Image Analysis tools.

RESULTS:

A dose-dependent response relationship between growth inhibition and $VitD_3$ administration was observed in all three oral squamous cell carcinoma cell lines (CAL27, SCC25, SCC15) with increasing concentrations resulting in more robust inhibition of cellular proliferation (Figure 1). For example, the lowest concentration $VitD_3$ (10 nmol or 2.5 ng/mL) reduced growth significantly (compared with baseline controls) by 11%, 6.5%, and 12% in CAL27, SCC25, and SCC15 cells ($p<0.05$), respectively. Higher concentrations of $VitD_3$ (50 nmol or 125 ng/mL) induced greater growth inhibition, reducing proliferation by 18.6%, 38.8%, and 18.2%, respectively ($p<0.01$). The highest concentration (125 nmol or 312.5 ng/mL) induced the greatest effects, inhibiting proliferation by 29.1%, 43.1%, and 32.1%, respectively – which was determined to be the observed growth inhibitory maximum (GI_{MAX}) concentration. Higher concentrations induced ever-diminishing effects above this concentration 150 – 300 nmol or 375 -750 ng/mL (data not shown), which approximate physiologic levels of vitamin D intoxication [51].

Growth inhibition was also observed following SPE administration among all three cell lines, although these effects were not uniform. More specifically, the lowest concentration of SPE (10 μg/mL or 2 μM/L) was sufficient to inhibit growth significantly by 25.8%, 20.2%, and 15.2% in CAL27, SCC25, SCC15 cells, respectively ($p<0.05$). The growth inhibitory maximum (GI_{MAX}) concentration of SPE was observed at 50 μg/mL (10 μM/L), which inhibited growth by 39.1%, 29.2%, and 19.2% in CAL27, SCC25, SCC15 cells, respectively ($p<0.01$). However, the highest concentration evaluated (100 μg/mL or 20 μM/L) was not sufficient to inhibit CAL27 growth as intensely (-31.3%, $p<0.01$) and the effects on SCC25 and SCC15 were indistinguishable from those observed at 50 μg/mL ($p>0.05$). No greater effects were observed above this concentration range.

To assess any additive, interactive or synergistic effects, three-day growth assays were performed using of concomitant administration of $VitD_3$ and SPE combining the lowest [L] and the highest [H] GI_{MAX} concentrations (Figure 2). For example, the combined effects of the lowest concentrations of $VitD_3$ [L] and SPE [L] inhibited growth of all three cell lines more intensely than either component independently. This combination inhibited CAL27 growth by 41.8%, which is greater than the effects of $VitD_3$ [L] or SPE [L] individually (-18.6%, 25.8%, respectively). This combination resulted in similar growth inhibition among SCC15 cells (-

31.2%), which was stronger than either in isolation (-18.2%, 15.2%, respectively). However, this combination precipitated equivalent reductions (-37.1%) to VitD$_3$ [L] (-38.8%) in SCC25 cell growth, although this was more than what was observed under SPE [L] administration (-29.1%).
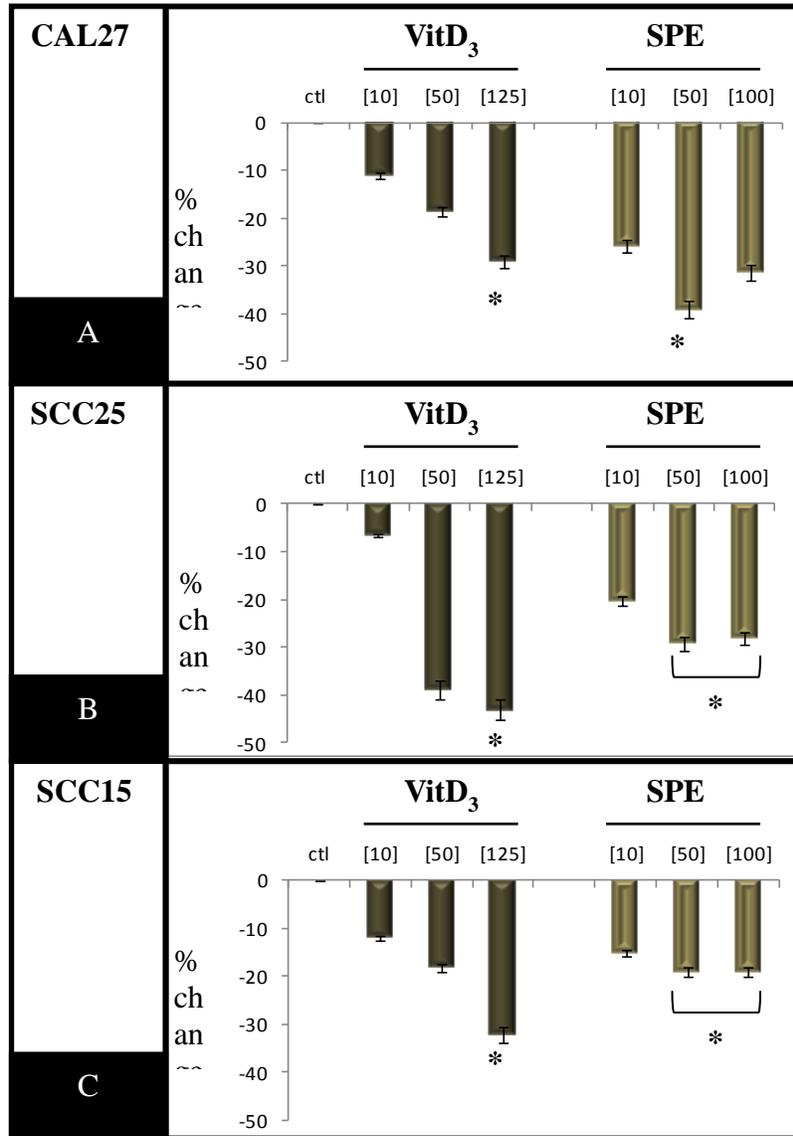

Figure 1. Oral cancer proliferation inhibition by VitD3, or SPE administration. (A) Three day growth of CAL27 was significantly inhibited by increasing concentrations of VitD$_3$ (10 nmol, -11%; 50 nmol, -18.6%, 125 nmol, -29.1%) and SPE (10 nmol, -25.8%; 50 nmol, -39.1%; 100 nmol, -31.3%) compared with baseline controls. (B) SCC25 growth was also inhibited by VitD$_3$ (10 nmol, -6.5%; 50 nmol, -38.8%, 125 nmol, -43.1%) and SPE (10 nmol, -20.2%; 50 nmol, -29.2%; 100 nmol, -28.1%). (C) SCC15 inhibition by VitD$_3$ (10 nmol, -12%; 50 nmol, -18.2%, 125 nmol, -32.1%) and SPE (10 nmol, -15.2%; 50 nmol, -19.2%; 100 nmol, -19.1%) was similar. Note: * denotes growth inhibitory maximum (GI$_{MAX}$).

Higher concentrations of SPE were then evaluated in combination with low-dose VitD$_3$ to ascertain any differential effects on cellular growth inhibition. Although the combination of VitD$_3$ [L] and SPE [H] reduced growth in all three cell lines, this inhibition was nearly equivalent to the effects observed with VitD$_3$ [L] and SPE [L]. In brief, CAL27 growth was

inhibited by 41.5%, which was greater than the effects of VitD$_3$ [L] (-18.6%) or SPE [H] (-39.1%) alone, but indistinguishable from the results of the first combination trial (-41.8%, $p>0.5$). A similar result was observed in SCC15 cells, which were inhibited by 33.2%, which was consistent with the previous combination (-31.2%, p>0.5), but greater than either VitD$_3$ [L] (-18.2%) or SPE [H] (-19.2%) in isolation. The growth of SCC25 cells was not significantly altered by the increased concentration of SPE (-32.1% versus -37.1%) and was similar to the results with either VitD$_3$ [L] (-38.8%) or SPE [H] (-29.2%) alone.

Interestingly, when the GI$_{MAX}$ concentration of VitD$_3$ [H] was combined with the GI$_{MAX}$ concentration of SPE [H], growth was nearly completely inhibited. Growth in CAL27 cells was inhibited by 83.1%, by 80.1% in SCC15 cells, and by 81.2% in SCC25 cells. These effects were significantly greater than any other combination or single treatment evaluated for all three cell lines ($p < 0.01$). Finally, each combination and the GI$_{MAX}$ concentrations for each variable were tested on the normal oral gingival cell line (HGF-1), which exhibited no growth inhibition, but rather stimulation of cell proliferation, which ranged between 18.2% and 41.2%.

To determine if the growth inhibitory effects of VitD$_3$ or SPE administration on these cell lines was due, at least in part, to alterations in cell survival or viability – each cell line and experimental treatment was analyzed at each time point of the proliferation assays and the viability assessed (Table 1). This analysis revealed that VitD$_3$ and SPE administration was sufficient to decrease viability among the oral cancer cell lines. More specifically, the baseline viability of CAL27 cells (94.4%) was lowered by VitD$_3$ [L] to 71.1% and more considerably by VitD$_3$ [H] to 50%. SCC25 (90.5%) and SCC15 (93.1%) viability was also diminished by VitD$_3$ [L] (72.3%, 68.2%) and more drastically by VitD$_3$ [H] (53.1, 61.3%). Although SPE administration was sufficient to reduce viability, these reductions were less dramatic and did not differ significantly between the low- and the high-concentrations. For example, SPE administration decreased CAL27 viability (94.4%) to 88.2% and 84.6% under SPE [L] and SPE [H] administration, respectively. SCC25 and SCC15 viability was similarly reduced by the addition of SPE [L] (85.2%, 84.7%) and SPE [H] (82.5%, 81.3%).

Table 1. Cell viability.

		VitD$_3$ [L]	VitD$_3$ [H]	SPE [L]	SPE [H]
CAL27	94.4%	71.1%	50.0%	88.2%	84.6%
SCC25	90.5%	72.3%	53.1%	85.2%	82.5%
SCC15	93.1%	68.2%	61.3%	84.7%	81.3%

		VitD$_3$ [L] SPE [L]	VitD$_3$ [L] SPE [H]	VitD$_3$ [H] SPE [L]	VitD$_3$ [H] SPE [H]
CAL27	94.4%	66.4%	63.3%	48.2%	23.4%
SCC25	90.5%	67.1%	62.6%	47.2%	19.6%
SCC15	93.1%	65.1%	61.8%	51.3%	14.9%

The GI_{MAX} concentration of $VitD_3$ [H] was then combined with the low-dose of SPE [L], which resulted in a significant change in growth inhibition in all three cell lines. CAL27 growth, for example, was inhibited by 45.1%, which was greater than the inhibition observed under either previous combination (-41.8% and -41.5%) or either alone (-29.1%, -39.1%). Similar results were observed for SCC15 (-47.3%) and SCC25 (-53.2%), which was also greater than either previous combination examined (SCC15: -31.2%, -33.2%; SCC25: -37.1%, -32.1%) or $VitD_3$ [H] (SCC15, -32.1%; SCC25, -43.1%) or SPE [L] (SCC15, -15.2%; SCC25, -29.1%).
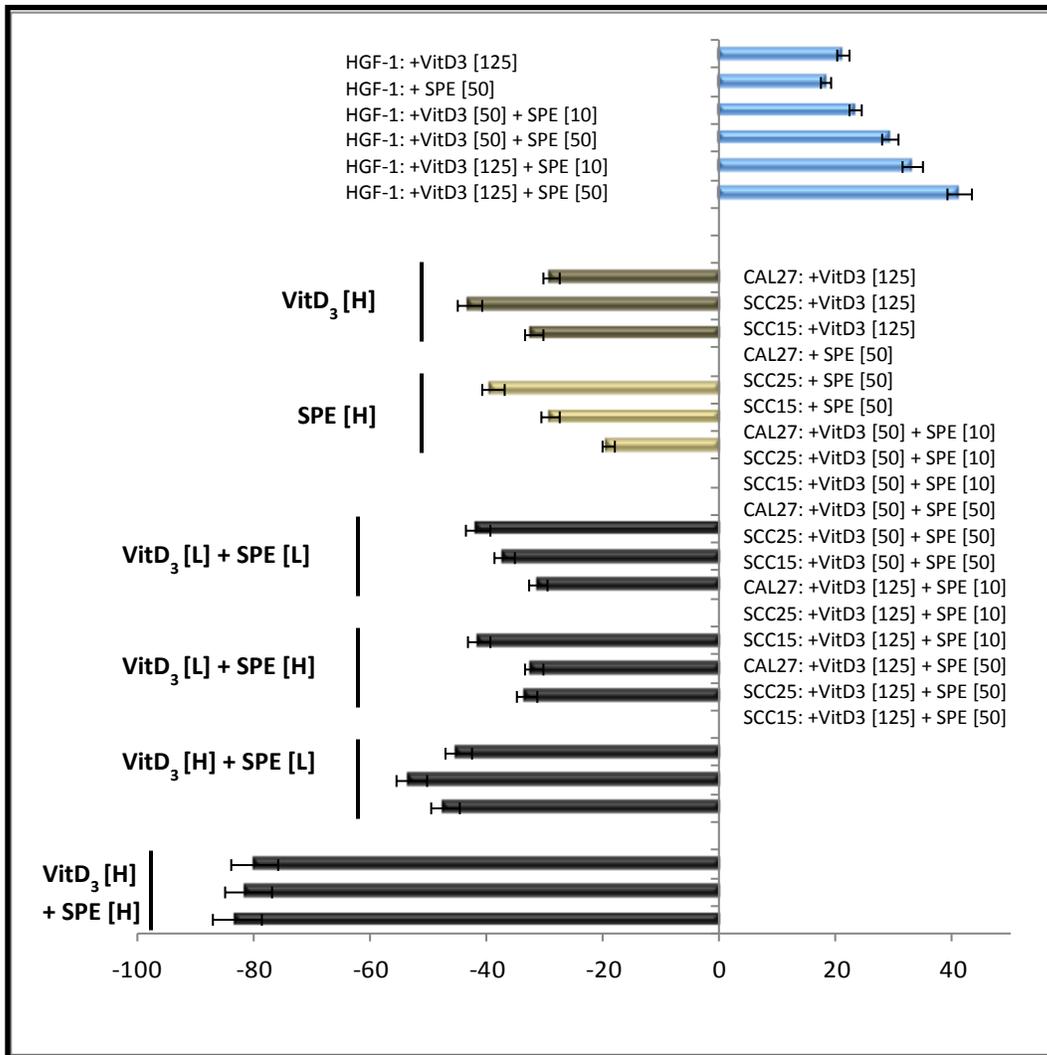

Figure 2. Effects of combined $VitD_3$, and SPE administration on proliferation. The combined effect of the lowest $VitD_3$ [L] and SPE [L] concentrations inhibited CAL27, SCC25, and SCC15 growth by 41.8%, 37.1%, and 31.2%. $VitD_3$ [L] and the GI_{MAX} concentration of SPE [H] inhibited CAL27, SCC25, and SCC15 growth by 41.5%, 32.1%, and 33.2%. The GI_{MAX} concentration of $VitD_3$ [H] combined with the low-dose of SPE [L] inhibited CAL27, SCC25, and SCC15 growth by 45.1%, 53.2%, and 47.3%. The GI_{MAX} concentrations of both $VitD_3$ [H] and SPE [H] inhibited growth more robustly in CAL27, SCC25, and SCC15 cells (-83.1%, -81.2%, and -80.1%).

The combined administration of VitD$_3$ and SPE had more variable effects. To illustrate, the viability of CAL27, SCC25 and SCC15 was reduced under VitD$_3$ [L] with either SPE [L] (66.4%, 67.1%, 65.1%) or SPE [H] (63.3%, 62.6%, 61.8%) administration to levels that were lower than either VitD$_3$ or SPE alone (but were not dissimilar between these treatment groups). However, the treatment with VitD$_3$ [H] combined with SPE [L] dramatically reduced viability among these three cell lines (48.2%, 47.2%, 51.3%). Furthermore, the combined treatment of these cells with VitD$_3$ [H] combined with and SPE [H] had more drastic effects on viability and cell survival (23.4%, 19.6%, and 14.9%).

To determine if the growth inhibitory and viability reducing effects of VitD3 and SPE administration were associated with differential gene expression, relative endpoint (RE) RT-PCR was performed on total RNA isolated from each cell line following combination therapy using oligonucleotide primers specific to the mRNA of critical apoptosis regulatory genes *caspase-2* (an apoptosis initiator), *caspase-8* (an apoptosis effector), and *bax*, as well as the cell-cycle regulatory genes *c-myc*, *ornithine decarboxylase* (ODC) and the tumor suppressor, *p53* (Figure 3). Three VitD$_3$ - SPE combinations were evaluated due to their differential effects on growth, as well as viability. These experimental conditions included VitD$_3$ [L] - SPE [L], VitD$_3$ [H] - SPE [L], and VitD$_3$ [H] - SPE [H]. This analysis revealed that the caspase initiator and effector, *caspase-2* and *caspase-8* exhibited barely detectable mRNA expression under VitD$_3$ [L] - SPE [L] administration, although expression increased markedly under both VitD$_3$ [H] - SPE [L] and VitD$_3$ [H] - SPE [H] administration. The expression of *bax* mRNA, which was easily observed in all cell lines in every condition, also increased under all three experimental treatment conditions.
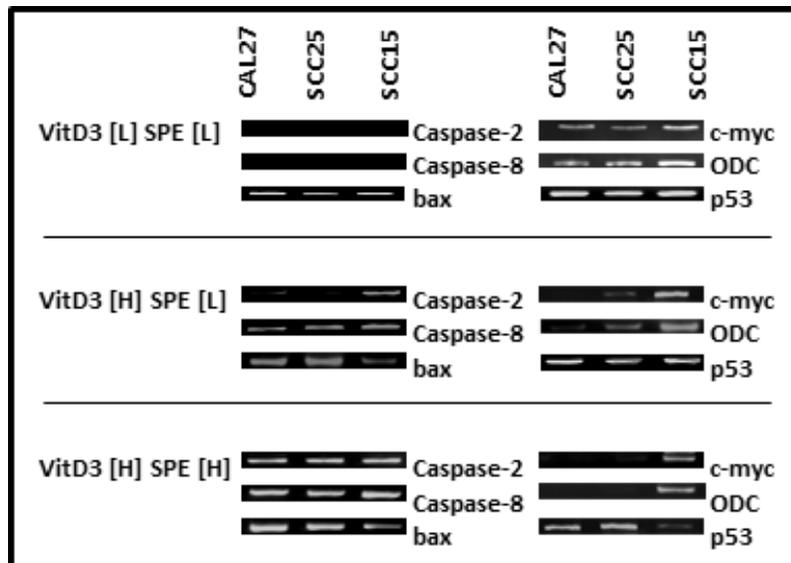

Figure 3. Relative endpoint RT-PCR analysis. Relative endpoint (RE) RT-PCR was performed on total RNA extracted from CAL27, SCC25 and SCC15 cells under combination treatments of VitD$_3$ [L] - SPE [L], VitD$_3$ [H] - SPE [L], and VitD$_3$ [H] - SPE [H]. These treatments induced mRNA expression of all apoptosis-related genes, *caspase-2*, *caspase-8*, and *bax*. The most marked changes in mRNA expression of cell-cycle related genes, *c-myc* and *ODC* were observed under VitD$_3$ [H] - SPE [H] administration.

An analysis of the cell-cycle regulatory genes, c-myc, ODC and the tumor suppressor p53 revealed active mRNA expression in all three cell lines under VitD$_3$ [L] - SPE [L] administration. However, the increased administration of VitD$_3$ [H] - SPE [L] and VitD$_3$ [H] - SPE [H] induced decreased expression of both *c-myc* and *ODC*. Interestingly, no observable changes in mRNA expression of the tumor suppressor p53, which has been found to be actively transcribed in all three cell lines, were observed except in SCC15 cells under the most concentrated treatment condition: VitD$_3$ [H] - SPE [H].

DISCUSSION:

The overall objective of this study was to evaluate the anti-cancer properties of soy protein extract (SPE), which is available as a dietary supplement and most closely resembles the whole soy food that most US adults are likely to consume as edamame, tofu, or other soy-based products. The effects of SPE against well-characterized oral cancer cell lines were evaluated using *in vitro* analysis methods in conjunction with administration of Vitamin D to determine the interactive effects of SPE on Vitamin D-induced oral cancer growth inhibition. Based upon previous work that determined SPE and Vitamin D are each, independently sufficient to mediate the proliferative phenotypes of these cancers in this experimental model, the working hypothesis was that co-administration would synergistically amplify these effects. These results of this study clearly demonstrated these anti-tumor effects, which are consistent with previous reports and observations. However, this may be the first study to evaluate the co-administration of SPE and Vitamin D, as well as the first to report synergistic effects on growth inhibition of oral cancer cell lines (under conditions of Vitamin D GI$_{MAX}$ concentrations).

Although this type of research has been extremely useful for identifying agents that are now clinically demonstrated as effective agents against specific cancers, a number of limitations must be considered when evaluating the outcomes of this study. First, one limitation of this and other preclinical studies involves the use of oral cancer cell lines, as there may be underlying dissimilar genetic mutations that might potentially influence the experimental outcomes. For example, the SCC25 cell line has been found to contain a deletion in the cdk1 promoter that contains a key transcriptional repressor region [60]. In addition, the CAL27 cell line contains a nonsense mutation in the SMAD4 gene, while SCC15 cells were found to harbor a missense mutation in SMAD2 - both signal TGF-β transduction proteins [61]. Finally, both CAL27 and SCC15 cells contain a single nucleotide polymorphism in the S100A2 gene, a calcium-binding tumor suppressor protein, although this does not appear to alter their propensity for growth, migration or invasion [62]. However, a growing body of evidence demonstrates that deregulation and reduced expression of key tumor suppressors, such as p16, in these cell lines may, in fact, be the result of hyper methylation - providing further justification to elucidate the interconnected roles of dietary components and transcriptional regulation in the growth and progression of oral cancers [63-65].

Another limitation to be considered is that SPE, unlike Vitamin D, contains multiple bioactive substances in addition to the soy isoflavones (Daidzein, Genistein, Glycitein), which may have direct effects on cellular behaviors despite their relatively low concentrations [66, 67]. These SPE components include, but are not limited to, phytates (inositol hexaphosphate), oligosaccharides, saponins, Kunitz inhibitor and Bowman-Birk Inhibitor (BBI) [68]. Some

previous studies have demonstrated that saponins were capable of exerting growth inhibiting effects on colon cancer cell lines *in vitro* [62, 69, 70]. In addition, other studies have demonstrated cytotoxicity and apoptotic effects on oral cancers *in vitro* using saponins [71-73]. However, these effects in animal and human models may be severely limited due to low absorption rates, as well as extremely low serum concentrations that are far lower than the experimental concentrations that were evaluated [66, 67].

Finally, some recent studies have demonstrated there may be possible adverse effects of elevated 25(OH)D concentrations on cancer risk in prostate, breast, pancreas, and esophageal cancers, suggesting that these effects may depend on dose, timing and duration of exposure, as well as tissue specific, lifestyle, and genetic factors [74, 75]. Although J- or U-shaped risk curves have been proposed to describe the noted associations in these studies, confounding factors present in the original studies are likely responsible for these findings [51, 74]. For example, outcomes of intervention trials of supplemental Vitamin D were inconclusive due to the lack of baseline vitamin D status reports of trial participants and consequent dose adequacy estimates [51, 74, 75]. This may suggest that studies focused on the dose administered, rather than their effect on alleviating deficiency, achieving adequacy, or adding to pre-trial adequate serum levels, might have significantly affected the dose-response curves and further complicated the interpretation of trial outcomes.

CONCLUSION:

Although many clinical studies have suggested Vitamin D status, intake, and supplementation may have significant effects on oral cancer risk, progression, and mortality, growing epidemiologic evidence now suggests that dietary patterns, which may include the intake of other cancer preventing foods (such as soy) may influence these effects [1, 6]. This study is among the first to examine the relationships between Vitamin D and soy administration in oral cancer cells, which provides specific information about the mechanisms and pathways that may be modulated along with growth inhibition and reduced viability. This study provides the first direct evidence of the magnitude of these relationships, which may be particularly useful to oncologists, oral health researchers, and nutrition epidemiologists as they analyze and develop rubrics for generalizing the health protective effects of diet and dietary supplements, as well as the most effective ancillary treatment options for patients with oral cancer.

List of abbreviations:

Soy protein extract (SPE); *ornithine decarboxylase (ODC)*, United States (US), oral and pharyngeal cancers (OPC), human papillomavirus (HPV), vitamin D receptor (VDR), retinoid X receptor (RXR), Dulbecco's Modified Eagle's Medium (DMEM), 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES), fetal bovine serum (FBS), 1,25-dihydroxyvitamin D_3 (VitD$_3$), molecular weights (MW), null hypothesis (H$_0$), analysis of variance (ANOVA), lowest concentration [L], growth Inhibitory Maximum (GI$_{MAX}$), highest concentration [H], glyceraldehyde 3-phosphate dehydrogenase (GAPDH), human gingival fibroblasts (HGF-1), minimum threshold cycle (CT), saturation cycle (CS), relative endpoint polymerase chain reaction (RE) RT-PCR, ethidium-bromide (EtBr), Bowman-Birk Inhibitor (BBI).

Competing interests:

The authors declare they have no financial or non-financial competing interests and report no conflicts of interest relevant to the contents of this article. No funding was obtained from any company or pharmaceutical organization.

Authors' contributions:

KK, MAK, and CJB conceived and designed this project and were responsible for performing the experimental assays, data collection, figure generation, and writing. Karl Kingsley, Mark Keiserman, Christine J Bergman.

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