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Research Article

Effects of Verapamil on Bone Cancer Cells *In Vitro*

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Abstract

Verapamil is a calcium channel blocker drug that inhibits the transmembrane flux of calcium ions and is used clinically in the management of cardiac arrhythmias. Several studies have proposed its role as an adjuvant in multi-drug chemotherapy due to its potential to decrease calmodulin activity in cancer cells and thus diminish drug resistance. However, newer studies have described the role of verapamil as an anti-cancer agent in the growth inhibition of several types of cancer cells. It is postulated that calcium signaling is a vital part of the survival of osteoblastic cells with alteration in the intracellular calcium levels leading to imbalances between several mechanisms ultimately resulting in inhibition of cell growth. The effects of verapamil (12.5-100 µg/ml) on the inhibition of cell growth of G292 osteosarcoma cells and normal HOB osteoblastic cells were studied *in vitro*. Moreover, the effects of this drug on growth factor induced effects in the G292 cells were also assessed. In order to evaluate potential verapamil releasing vehicles, we tested its release and effectiveness when incorporated in a nano Calcium Sulfate (nCS) delivery system. In higher doses, verapamil (50-100 µg/ml) significantly inhibited the activity of G292 osteosarcoma cells; however, it had no significant effect on normal HOB osteoblasts at these doses. Verapamil was also shown here to significantly inhibit Human Platelet Lysate (HPL) and Platelet Derived Growth Factor (PDGF) induced G292 cell activity. Additionally, it was found that nCS could release verapamil in a time-dependent manner and the released verapamil could inhibit G292 cell activity. In appropriately administered concentrations, verapamil may possess the potential to inhibit the activity of osteosarcomas and act as an anti-tumor agent down-regulating the effects of naturally occurring growth factors.

Keywords: Nanocalcium sulfate; Osteosarcoma; Verapamil

Introduction

Osteosarcoma (OS) is a rare but devastating bone tumour that mainly affects children and adolescents [1]. The 5-year survival rates for patients with localized disease are between 50 and 70%, but in

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patients with metastases, the prognosis remains poor (~20%). Today's standard therapy of OS includes neoadjuvant chemotherapy with doxorubicin, methotrexate and cisplatin followed by surgical resection of the primary tumour and adjuvant chemotherapy [2-5]. Patients who relapse show increased chemoresistance against currently used chemotherapy approaches [6-8]. With about three cases per million people and year, OS is a rare disease, which makes the evaluation of new potential drugs difficult [9]. This potentiates a need for exploring existing or newer drugs in the treatment of osteosarcoma.

Until recently, verapamil was employed as a drug in cancer therapy in order to overcome multi drug resistance [10]. Some studies have suggested a possible role for its use in the suppression of bone cell activity. As an example of the effects of verapamil on bone cellular activity the direct inhibitory action of calcium channel blockers on rat calvarial skeletal collagen synthesis was previously reported [11]. Likewise, Breslau et al., found that calcium channel blockers such as verapamil have an effect on calcium metabolism through the alteration of bone turnover rate and the inhibition of bone formation in hypertensive patients [12]. Although osteosarcoma cells such as the G292 cell line studied here have been shown to possess voltage activated calcium channels sensitive to verapamil [13,14], there does not appear to be studies reported on the effects of this drug on the viability and growth responses of these cells.

Since *in vivo* the microenvironment of an osteosarcoma is a complex niche with numerous growth factors interacting as both autocrine and paracrine factors [15], it must be recognized that a calcium inhibitor such as verapamil can be acting on many levels to affect the overall activity of osteosarcoma cells. Acknowledging that verapamil has been shown in other cells to affect a number of different mechanisms involving postreceptor signaling pathways such as those with protein kinase C, calmodulin and phosphodiesterase in addition to calcium channels depending upon its concentration [16], we studied several *in vitro* incubation conditions and drug concentrations.

We report here some of the possible interactions of growth factors and verapamil with G292 osteosarcoma cells with respect to their cell activity. Co-culture of G292 osteosarcoma cells with Human Platelet Lysate (HPL) and Platelet Derived Growth Factor (PDGF) enhanced their activity. However, when treated with verapamil at relatively high doses, this cellular activity was significantly decreased. Particularly, since verapamil at high *in vivo* concentrations can induce significant side effects, including high mortality [17] we present data on a possible local delivery system for verapamil that could potentially decrease its absorption into the blood stream and at the same time target an osteosarcoma site.

Materials and Methods

G292 osteosarcoma and HOB osteoblasts cell culture

G292 osteosarcoma cell line: The human osteosarcoma cell line, G292, clone A14181 (ATCC CRL-1423) was cultured in alpha MEM culture medium (Gibco, USA) supplemented with 10% Fetal Bovine Serum (FBS) and 1% antimycotic (Gibco, USA) in a humidified

incubator at 37°C and 5% CO₂ for 3 days until the cell culture reached confluence. The cells were washed with PBS (Gibco, USA) and trypsinized using 0.25% trypsin-EDTA solution (Gibco, USA). The cells were then centrifuged and re-suspended with fresh MEM medium before they were seeded for the test. For the experiments, passages 4 & 5 were used.

HOB osteoblastic cells: Human calvarial osteoblast cells, obtained from ScienCell, (Carlsbad, CA, USA, Catalog #4600) were incubated in alpha MEM culture medium (Gibco, USA) supplemented with 10% FBS and 1% antimycotic (Gibco, USA) and cultured in a similar manner as the G292 cells described above. For the experiments presented here, passages 5 & 6 were used.

Cell viability and apoptosis assay

Cell viability and apoptosis were assayed using an ApoLive-Glo™ Multiplex Assay (Promega) after treatment with various concentrations of verapamil (Sigma-Aldrich, Inc.). For the studies with the G292 cells, there were five groups: 1. untreated cells, 2. cells treated with 100 µg/ml (220 µM) verapamil, 3. cells treated with 50 µg/ml (110 µM) verapamil, 4. cells treated with 25 µg/ml (55 µM) verapamil and 5. cells treated with 12.5 µg/ml (27.5 µM) verapamil.

The protocol used for the cell viability and apoptosis combines assay chemistries so that viability and caspase activation (associated with apoptosis) can be assessed within a single assay well. In the first part of the assay the peptide substrate (glycylphenylalanyl-aminofluorocoumarin; GF-AFC) was added. It enters intact cells where it can be cleaved by the live-cell protease activity to generate a fluorescent signal proportional to the number of living cells. In the second part of the assay, the Caspase-Glo® 3/7 Reagent was added in an “add-mix-measure” format described in the manufacturer’s protocol; this results in cell lysis, followed by caspase cleavage of the substrate and generation of a “glow-type” luminescent signal produced by luciferase.

G292 cells of approximately 5000/well were seeded in a flat 96-well micro-plate as triplicates with the five groups mentioned above. Cells were incubated with verapamil for 7, 12, or 17 days with media changes every 3 days. At the end of the incubation period, the medium present was removed from the wells and replaced with 100 µl of medium. For the first part, 20 µl of viability reagent containing the GF-AFC substrate was added to each well, and briefly mixed by orbital shaking at 300-500 rpm for 30 seconds and then incubated at 37°C for 30-180 minutes. Fluorescence was measured at 485_{Ex}/520_{Em} (in order to determine viability) by using a fluorescent plate reader (Biotek). For the second part of the assay, 100 µl of Caspase-Glo 3/7 reagent was added to each well and briefly mixed by orbital shaking at 300-500 rpm for 30 seconds and then incubated at room temperature for 30-180 minutes. Luminescence that is proportional to the amount of caspase activity present as an assessment of apoptosis was measured using a Perkin Elmer Victor³™ V Wallac plate reader.

Activity Assessments with Verapamil Added Directly to Culture Media

Effect of verapamil on G292 osteosarcoma cell line and HOB normal cells

Cell activity was assessed with the MTT assay that has been well documented to measure mitochondrial enzymes as an indication of cell viability. In this assay, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) is converted into formazan crystals by

cellular mitochondrial activity. In most cell types, the total mitochondrial activity is related to the number of viable cells and hence this assay is also generally used as a viability assessment.

For the studies with either the G292 or HOB cells, there were five groups: 1. cells alone, 2. cells treated with 100 µg/ml verapamil, 3. cells with 50 µg/ml verapamil, 4. cells treated with 25 µg/ml verapamil and 5. cells treated with 12.5 µg/ml verapamil. The cells were seeded in complete media (alpha MEM, 10% FBS and 1% anti-mycotic) and after 80% confluence, the cells were treated with the varying concentrations of verapamil for the indicated time. The cells were then (without phenol red) 200 MEM µl MTT and 20 assay reagent (Sigma-Aldrich Inc.) for an additional 3 hrs and with 200 µl Dimethyl Sulfoxide (DMSO) at the end of the incubation period. Finally, the supernatants were transferred to a new 96 well plate for recording the absorbance at 540 nm using a 96-well plate reader.

Effect of calcium on G292 osteosarcoma cell line

For these experiments, cell activity was assessed using the MTT assay as described above with the following five sample groups: 1. G292 cells with 0 mM calcium (no calcium added) MEM media, 2. G292 cells with 1 mM calcium MEM media, 3. G292 cells with 0 mM calcium MEM media with verapamil, 4. G292 cells with 1 mM calcium media with verapamil and 5. G292 cells in complete media. Incubations under the various conditions were all for 48 hrs.

Effect of PDGF on G292 osteosarcoma cell line

A series of experiments to assess the effects of verapamil on growth factor stimulatory effects of G292 cells were also conducted using PDGF as well as Human Platelet Lysate (HPL). For the experiments with PDGF, PDGF-BB (human) recombinant protein purchased from Thermo Fisher (Rockford, IL, USA) was used. There were five groups: 1. G292 cells alone, 2. G292 cells with 125 ng/ml PDGF, 3. G292 cells with 125 ng/ml PDGF and 50 µg/ml verapamil, 4. G292 cells with 125 ng/ml PDGF induced for 24 hours followed by only 50 µg/ml verapamil for an additional 24 hours and 5. G292 cells with 50 µg/ml verapamil. In another series of experiments, HPL, derived from platelet rich plasma of pooled adult human donors and purchased from Zen-Bio, Inc., (Research Triangle Park, North Carolina, USA) was tested. There were six groups: 1. G292 cells alone, 2. G292 cells with 10% HPL, 3. G292 cells with 10% HPL and 50 µg/ml verapamil induced together at the same time, 4. G292 cells with 10% HPL induced for 24 hours, thereafter followed by only 50 µg/ml verapamil for 6 hours, 5. G292 cells with 50 µg/ml verapamil and 6. G292 cells with HPL and IGF-inhibitor (chroameceptin) (3 µM). Chroameceptin (Sigma -Aldrich, Inc.), is a benzochromene derivative that inhibits insulin-induced adipogenesis and selectively reduces the growth and viability of human hepatocellular carcinoma cells that over express IGF2 (Insulin-like Growth Factor-2) [18]. For both the PDGF and HPL experiments, the MTT assay was conducted as described above.

Studies with Verapamil Loaded in the nCS Scaffold

Preparation of the nCS/verapamil scaffold and determination of standard calibration curves

nCS was produced according to the method of Park et al., [19]. Briefly, a cryo-vacuum process was used to convert calcium sulfate dihydrate micro particles into calcium sulfate dihydrate nanocrystals and then subjected to oven drying to obtain Calcium Sulfate hemihydrate nanoparticles (nCS) as described previously. Verapamil

(600 µg, 300 µg and 150 µg) was incorporated into the nCS powder and was mixed with water in the ratio of 1:1 and small pellets of 1 mm in height and 3 mm in width were generated using a Polyvinyl Siloxane (PVS) mold. Sterilization was performed by Glow Discharge Treatment (GDT) for 15 minutes.

Standard calibration curves for verapamil were produced from common guidelines published by the National Measurement System Valid Analytical Measurement (VAM) program (United States Department of Trade and Industry) using a UV visible spectrophotometer. A set of standards containing a known amount of verapamil concentrations was prepared and the spectrophotometer response was measured for each standard to develop a calibration curve. The calibration curve was then used to determine the unknown drug concentration that was released in the test sample aliquots. Briefly, six standard calibration solutions with concentrations of (100, 50, 25, 12.5, 6.25 and 3.125 µg/ml) were prepared with Phosphate Buffered Saline (PBS) to similarly resemble the test sample aliquots. PBS media was used as a control blank. The standard calibration curves were then used to determine the unknown concentration of the verapamil in the sample aliquots.

Drug release detection from nCS samples

Verapamil release from nCS samples was conducted at 37 degrees celsius *in vitro* by placing test samples in a culture incubator. Test samples (600 µg, 300 µg and 150 µg) n=3 of verapamil were placed in 1 ml PBS in a 24 well culture plate. The PBS was then withdrawn completely and replaced with fresh buffer at predetermined time intervals of (1-10 days). Aliquots of unknown verapamil concentration were immediately analyzed by UV-Visible spectrophotometer (Beckman Coulter DU-800 spectrophotometer) at an absorbance value of 278 nm to determine the released amount from the sample pellet using standard calibration curves for the drug.

Preparation of drug loaded nCS samples for cell activity studies

In this experiment, G292 cells were incubated with nCS discs preloaded with various doses of verapamil and the MTT assay conducted as described above. There were five sample groups: 1. G292 cells with nCS alone (no added verapamil) disc, 2. G292 cells with 600 µg verapamil + nCS disc, 3. G292 cells with 300 µg verapamil + nCS disc, 4. G292 cells with 150 µg verapamil + nCS disc and 5. G292 cells alone.

Statistical Analyses

Statistical analysis was performed using SPSS-17.0 software. Where indicated, experimental data were reported as mean ± standard deviation of triplicate independent samples. Data were analyzed using Student's t-test and one-way analysis of variance and Tukey's HSD test was applied as a post hoc test if statistical significance was determined. A value of $p \leq 0.05$ was considered statistically significant.

Results

Verapamil can inhibit the activity of G292 cells

Incubation of the osteosarcoma cells at relatively high concentrations of verapamil was found to significantly decrease the % activity of the cells compared to control untreated levels, measured with the MTT assay. 50 µg/ml of verapamil significantly decreased the % activity of G292 osteosarcoma cells as compared to the other groups as represented by * ($p < 0.05$). 100 µg/ml of verapamil showed

significantly lower inhibitory effects on the cells as compared to the 50 µg/ml verapamil group but it showed significantly higher inhibitory effects as compared to the 25 µg/ml and 12.5 µg/ml groups of verapamil * ($p < 0.05$). There was no significant difference in the 25 µg/ml and 12.5 µg/ml groups of verapamil and these concentrations had no effect on the activity of the G292 cells compared to controls (Figure 1A).

The graph in figure 1B shows the results of an ApoLive-Glo™ Multiplex assay assessing the viability of G292 cells treated with verapamil. Viability levels increased in the control group as the incubation periods were extended to 17 days but the verapamil treatment group of 50 µg/ml showed significantly lower viability levels at all time periods as compared to the other groups as represented by * ($p < 0.05$) (Figure 1B). Accordingly, apoptosis levels were low in the controls and did not change over the 17 day period of incubation while the 50 µg/ml verapamil groups exhibited the highest level of apoptosis over the 17 day period showing a statistically significant greater increase ($p < 0.05$) compared to the other groups including the 100 µg/ml group that produced a lower level of apoptosis, although still significantly greater than controls (Figure 1C).

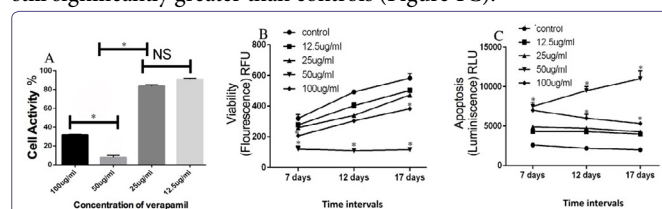


Figure 1: Inhibition of G292 cell activity by verapamil.

G292 osteosarcoma cells were treated with 12.5, 25, 50 & 100 µg/ml verapamil in MEM media and the effects of the drug were evaluated using the MTT and the ApoLive-Glo™ Multiplex Assay. (A) MTT assay: Results demonstrated that incubation for 2 days with doses of 50-100 µg/ml verapamil induced significantly greater inhibitory effects on cell activity than the lower doses of 25 & 12.5 µg/ml ($p < 0.05$). There was no difference in the inhibitory effects of 12.5-25 µg/ml ($p > 0.05$). (B,C) ApoLive-Glo™ Multiplex Assay: G292 cells were incubated for 7, 12 & 17 days with 12.5-100 µg/ml verapamil along with controls. The doses of 50 & 100 µg/ml induced significantly lower cell viability (B) and higher apoptosis (C) than the lower doses of 25 & 12.5 µg/ml ($p < 0.05$). There were no differences in these responses with verapamil treatment at doses of 12.5-25 µg/ml ($p > 0.05$).

Low and high concentration of verapamil has no effect on HOB osteoblasts cells

Only 25 µg/ml verapamil significantly inhibited the activity of the HOB cells as compared to the controls ($p < 0.05$). It should be noted, however, that this effect was relatively small, approximately a 33% decrease, compared to control values and not significantly different from the effects seen with the other concentrations of verapamil. Compared to control values, there was no inhibition of the osteoblastic cell activity due to the presence of verapamil at the other tested concentrations (50 µg/ml, 100 µg/ml & 12.5 µg/ml) ($p > 0.05$) (Figure 2).

Calcium-free conditions can inhibit G292 osteosarcoma cell activity

It was observed that incubation in calcium free media could significantly decrease cell activity. The group containing 0 mM Calcium (Ca) + 50 µg/ml Verapamil (0 mM Ca + VPM) demonstrated significantly higher inhibition of the activity of the G292 osteosarcoma cells. The group of 0 mM Ca showed significantly lower inhibitory activity effects as compared to the (0 mM Ca + VPM) group but significantly greater inhibition of activity in comparison to 1 mM

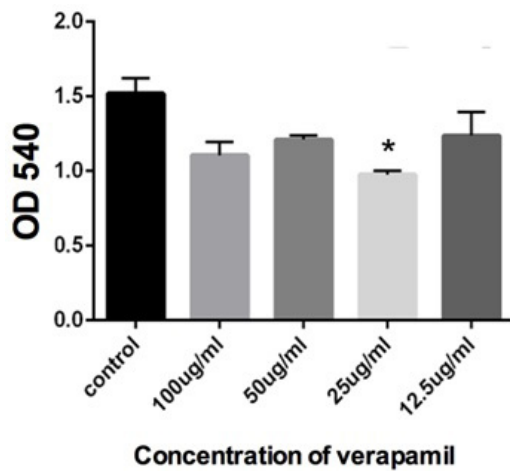


Figure 2: Effect of verapamil on HOB osteoblasts.

HOB osteoblasts cells were treated with varying doses of verapamil for 48 hours and the MTT assay performed to assess cell activity. The results did not reveal a significant difference between the drug concentrations of 12.5, 25, 50 & 100 µg/ml ($p > 0.05$) although 25 µg/ml did produce a small, but statistically significant effect compared to controls.

Ca + VPM group. The 1 mM Ca group demonstrated the lowest activity inhibitory effects. Furthermore, verapamil significantly inhibited activity of G292 cells in the presence of calcium free media ($p < 0.05$) as represented by * (Figure 3).

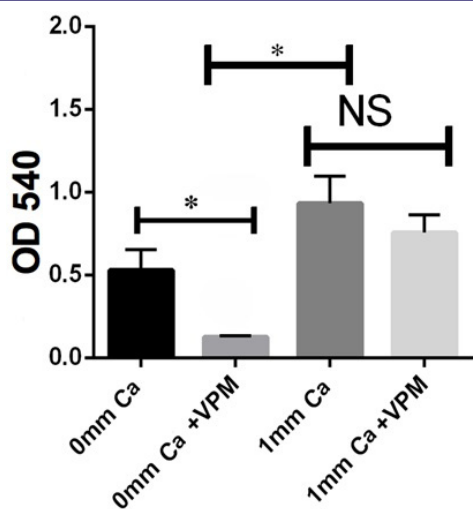


Figure 3: Effect of the absence of calcium on G292 cells.

G292 osteosarcoma cells were treated with or without calcium free media in combination with or without verapamil (50 µg/ml) for 48 hours and the MTT assay performed to assess cell activity. Verapamil significantly inhibited cell activity in the presence of calcium free media ($p < 0.05$).

Verapamil can inhibit PDGF and HPL-induced G292 osteosarcoma cell activity

It was observed that the G292 cells stimulated with PDGF for 24 hours followed by a 6 hours verapamil treatment demonstrated significantly greater inhibition as compared to cells treated simultaneously with PDGF and verapamil ($p < 0.05$). In the HPL stimulated cell groups, the G292 cells stimulated with 10% HPL for 24 hours followed by treatment with 50 µg/ml of verapamil demonstrated a significantly higher inhibition of cell growth which was comparable to the G292 cells treated group with HPL and chromeceptin (IGF-2 inhibitor)

group and the G292 cells treated with 50ug/ml verapamil group ($p < 0.05$) (Figure 4A & B).

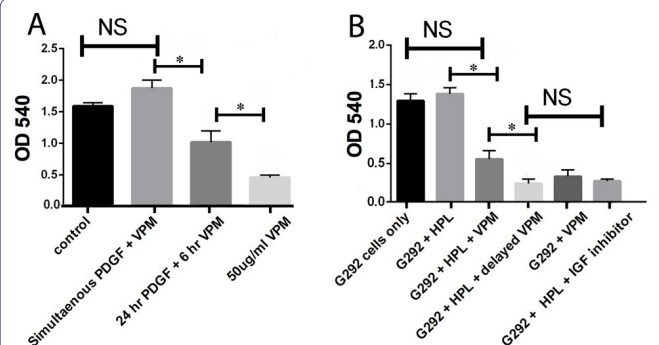


Figure 4: Effect of verapamil on HPL and PDGF induced activity of G292 cells.

(A) G292 osteosarcoma cells were treated with both PDGF and verapamil at the same time i.e., 0 hr as well as with PDGF for 24 hours followed by incubation with verapamil. Thereafter for 6 hours. Using the MTT assay, the groups of 24 hours PDGF+ 6 hour verapamil demonstrated significantly higher inhibition of cell activity compared to the other groups ($p < 0.05$). (B) G292 cells were treated with HPL and verapamil simultaneously; HPL for 24 hours followed by verapamil for 24 hours and HPL with IGF-I inhibitor. The 24 hours HPL+ 24 hours (50 µg/ml) verapamil group demonstrated significantly higher cell inhibition compared to other groups ($p < 0.05$). There was no difference between the groups of 24 hours HPL followed by 24 hours verapamil and the group of HPL and IGF-I inhibitor treatment.

Nanocalcium sulfate (nCS) permits the slow release of verapamil and maintain efficacy of the drug

The drug release study revealed that approximately 10% of the loaded verapamil was released from nCS on a daily basis and that equal quantities of the drug were released in a consistent manner (Figure 5A). The verapamil released was shown to be effective in inhibiting the activity of the G292 cells in a similar manner as to when the drug was added directly to the media. These data demonstrated a significantly lower inhibitory effect on G292 cell growth in the 600 µg verapamil + nCS group in comparison to the group treated with nCS containing 300 & 150 µg verapamil as represented by * ($p < 0.05$). There was no significant difference between the inhibitory effect on the cells in the 300 & 150 µg nCS + verapamil groups ($p > 0.05$) as represented by NS (Figure 5B).

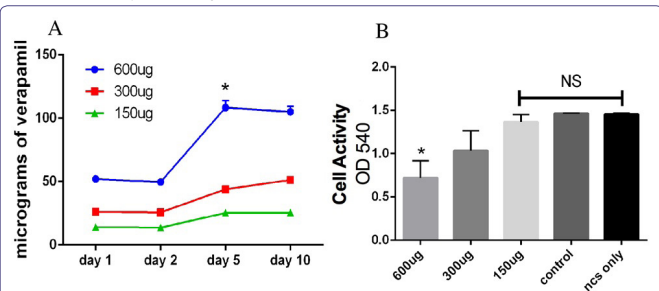


Figure 5: Studies with verapamil-loaded nano calcium sulfate.

(A) Release of the drug from nano calcium sulfate: Verapamil was incorporated into nCS at doses of 600, 300 & 150 µg and its release was studied on day 1, 2, 5 & 10 respectively using UV-Visible spectrophotometry. The release was significantly higher on the first 5 days and showed a decrease thereafter ($p < 0.05$). (B) G292 cells were treated with nCS loaded with verapamil at doses of 600, 300 & 150 µg. The 600 µg loaded group demonstrated significantly higher inhibition of cell activity compared to the other groups ($p < 0.05$).

Discussion

Several studies in the literature have suggested the role of verapamil, a Ca^{2+} channel antagonist, as an anti-tumor agent [20,21]. In this

report, we provide, for the first time, evidence demonstrating that verapamil has an inhibitory effect on G292 osteosarcoma cells. We found that verapamil could inhibit the activity of G292 osteosarcoma significantly in the absence of calcium. Furthermore, verapamil could also inhibit activity induced by PDGF and IGF on G292 osteosarcoma cells. We also found that nanocalcium sulfate could efficiently release verapamil in a consistent manner, which could help in the local delivery of verapamil to the affected site.

It has been shown that verapamil can reverse the resistance of cancer cells to some chemotherapeutic agents [20,21]. However, the mechanisms of verapamil anticancer activity are not fully understood. Due to the success of its use in the treatment of osteosarcoma in patients [22] as well as its ability to lead to apoptosis as a result of an increase in intracellular calcium in human colon cancer cells [23], we investigated the role of verapamil in growth suppression parameters of G292 osteosarcoma cells. In our study, it was observed that high doses of verapamil (50-100 $\mu\text{g/ml}$) significantly inhibited cellular activity whereas; lower doses (12.5- 25 $\mu\text{g/ml}$) had no apparent inhibitory effects on the osteosarcoma cells. In figure 1, the MTT assay results correlate well with the viability as well as apoptosis aspects of the ApoLive-Glo™ Multiplex assay. This suggests that a decrease in the MTT results (which are reflective of mitochondrial enzymatic activity) can be reflective of both decreases in cell viability (measured by membrane integrity) and increases in cell apoptosis (measured by caspase activity). This consistency of responses is interesting considering that with all three assays 50 $\mu\text{g/ml}$ verapamil produced the greatest response suggesting that the effect is not an assay procedural error and also that there can be a series of cellular events evoked by the drug that lead to a decrease in cell viability and cell death.

The lack of a linear dose response with verapamil in our studies can be due to the most probable complexity of mechanism of action of the drug involved in producing the effects on cellular responses. It has been reported that verapamil in other cell types can modulate intracellular calcium levels not only via ionic channels but also via intracellular calcium pools with subsequent effects on postreceptor signaling pathways involving several different mechanisms [24]. This multitude of effects each of which can be dose dependent can yield to end results on cellular viability that might be reflected in a nonlinear dose-response.

The complex nature of intracellular calcium regulation as well as potential actions of verapamil independent of a Ca^{2+} channel inhibitor can be responsible for its effects observed here in the absence of extracellularly added calcium. Since intracellular calcium is regulated not only by calcium channels but also by calcium pumps and ion exchangers in the plasma membrane as well as intracellular organelles, inhibition by verapamil can result even in the absence of added extracellular calcium since under this condition, the dynamics of calcium release by other mechanisms might lead to a net decrease in cytosolic calcium that results in changes in cell activity leading to decreased viability or increased apoptosis. Moreover, although verapamil is a potent and selective voltage dependent calcium channel antagonist at concentrations below 1 μM (0.45 $\mu\text{g/ml}$) it has been shown to have inhibitory effects on sodium or potassium conductances at higher concentrations in the 10-100 μM (4.5-45 $\mu\text{g/ml}$) range [25] where in the higher range we have seen significant effects in this present study. Verapamil has also been shown to act as a non-selective inhibitor, with a low binding affinity, of P-glycoprotein, a member of the ATP-Binding Cassette (ABC) transporter that functions as a physiological barrier to extrude toxins and xenobiotics

out of cells [26]. These effects can lead to actions of verapamil not dependent upon extracellular calcium. Since in this present study we were interested in the effects of verapamil as a possible therapeutic agent, we did not compare its results to those of another regulator of calcium such as a chelator like BAPTA.

We chose to study the G292 cell line because it is a human osteosarcoma cell line that has been fairly well studied with respect to its hormonal and growth factor responses as well as its calcium regulatory mechanisms. Since we were studying verapamil, an agent that might function via calcium channels, we surveyed previous literature on calcium channels in human osteosarcoma cell lines and found that there are apparently no major differences in the types of voltage-activated calcium channels in a number of cell lines studies, but normal human cells do differ from the osteosarcomas due to a absence of the alpha 1S isoform of the L-type channel [14]. Since to our knowledge there are not major differences in regulation of calcium among the osteosarcoma cell lines that are presently available, we did not repeat our studies in other cells lines for this present paper. We acknowledge, however, that studies with a variety of osteosarcoma cell lines might reveal some interesting differences in response to verapamil and should be pursued in future work. In particular osteosarcoma cells with differences in telomere maintenance such as by activation of telomerase and an Alternative mechanism of Telomere Lengthening (ALT) characterized by elongated, heterogeneous telomeres without the presence of telomerase activity should be further studied with respect to the effects of verapamil. G292 cells have been reported to be ALT+ so further studies in cell lines lacking ALT might reveal important information regarding possible differences in the mechanism of action of this agent in different osteosarcoma cell types [27].

Furthermore, we investigated if verapamil could affect normal osteoblastic cells and hence we treated the HOB osteoblasts cells with varying doses of verapamil (12.5 $\mu\text{g/ml}$ - 100 $\mu\text{g/ml}$). We found that high and low concentrations of verapamil failed to demonstrate a significant effect on the cellular growth activity of these normal human calvarial osteoblasts. Kim et al., demonstrated in their studies that in the absence of calcium in the media, verapamil could significantly inhibit cDNA synthesis in murine osteoblastic cells [28]. However, this phenomenon was reversed in the presence of calcium in the media.

Several studies have explored the role of platelet-derived growth factors in the proliferation and metastasis of osteosarcoma owing to their potential to induce platelet aggregation [29]. Additionally, there is positive correlation between the expression levels of platelet aggregation-inducing factors [30]. Apart from cell stimulation, these growth factors can also modulate cell migration and modulate matrix metalloprotease production. The interactions of tumor cells with platelets play a critical role in the progression of tumor malignancy [31]. Tumor cell-induced platelet aggregation enhances the rate of tumor embolization in the microvasculature and protects tumor cells from immunological assault and blood-shear stress [32]. Moreover, several factors such as transforming growth factor- β , insulin like growth factor, and Platelet -Derived Growth Factor (PDGF) are stored in platelet granules and are released during platelet aggregation. Such platelet-derived factors promote the epithelial-mesenchymal transition, tumor vascular angiogenesis and vascular permeability [33]. In our study, we observed increased growth of G292 upon incubation with PDGF and HPL. However, upon treatment with verapamil, a significant decrease in their activity was noticed. This could be attributed to the fact that verapamil at high doses can inhibit

platelet aggregation, which is in agreement with studies carried out by Kreiskott et al., [34]. The effect of verapamil on the HPL stimulation of G292 cells demonstrated similar results as compared to the IGF-inhibitor treatment on the HPL stimulation of these cells. However, verapamil was unable to inhibit cellular activity when administered concurrently with HPL and PDGF to the G292 cells; instead, the cells, when treated with verapamil, 24 hours after stimulating the cells with HPL and PDGF showed a remarkable decline in activity. This could be attributed to the fact that verapamil may not inhibit initial growth factor events such as PDGF and IGF receptor binding but can inhibit downstream PDGF and IGF effects.

More than 90% of verapamil is absorbed when given orally, but due to high first-pass metabolism, bioavailability is much lower (10-35%). Additionally, it takes 1 to 2 hours to reach peak plasma concentration after oral administration [35]. Acute overdose is often manifested by nausea, asthenia, bradycardia, dizziness, hypotension and cardiac arrhythmia [36]. Furthermore, verapamil, in a dose dependent manner, has been associated with an increased risk of cancer in the elderly, as reported in the Rotterdam study [37]. These statements provide important implications for a need to deliver this drug in a local manner. This may be particularly true for osteosarcomas since in our study, anti-growth activity of verapamil on G292 cells was noted at a relatively high concentration of 50 µg/ml (110 µM). Concentrations in this range may lead to undesirable reactions if administered clinically, which might make it imperative to deliver this drug to the affected site directly while trying to bypass the blood stream.

Nano-Calcium Sulfate (nCS) is a biocompatible, bioactive and biodegradable material with a compressive strength that is greater than that of cancellous bone and has been shown to release added growth factors in a sustained manner [19,38]. In our previous studies, we have successfully demonstrated how this nanomaterial can promote bone regeneration as well as release growth factors in a consistent manner [19,38]. Our data support the timely release of verapamil as well. In addition, the slow degradation rate of nCS can aid in delaying the release of the drug as well as prolong its efficacy. The potential efficacy of this delivery approach is evident from our data showing the release of verapamil in a sustained manner. This novel local delivery system could eliminate *in vivo* the first pass effect (hepatic effect) and prevented the drug from being absorbed into the blood stream, although studies will be required to substantiate this *in vivo*. Within the limitations of this *in vitro* study, verapamil at high concentrations appears to be a potent anti-growth agent for osteosarcomas having an inhibitory effect on basal as well as PDGF and HPL-induced cell activity in the G292 cell line.

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Conflicts of Interest

Dr. Rosemary Dziak is an inventor on a USA patent for nanocalcium particles use in bone regeneration. Dr. Gabriela Fernandes and Andrew Barone have no conflicts of interest with any aspects of the work described in this paper.

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