Cytotoxic Effect Of Verapamil On Human Embryonic Kidney Cell Line

Jamil L Ahmad, Chukwudi C Okebaram, Abdullahi Samaila

ABSTRACT: Introduction: The link between long term use of verapamil and cancer development has been suggested in literature many years back. However, there are numerous controversies surrounding this association with several epidemiological studies in the positive, negative and non-association between verapamil and cancer development. **Aim:** To investigate in mechanistic terms the link between chronic use of a calcium channel blocker (verapamil) and cancer development using human embryonic kidney (HEK293) cell line. **Method:** Trypan blue dye exclusion (cell counting) and 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT) assays were used to determine the proliferative as well as cytotoxic effects of verapamil. **Results:** Verapamil had a growth inhibitory rather than proliferative effect on HEK293 cells and the growth inhibition was found to be significant ($p<0.05$). **Conclusion:** The long term use of verapamil is associated with cellular growth inhibition and this possibly explained the rationale behind its use as part of combination chemotherapy for some human cancers.

Index Terms: Anti-hypertensives, cancer, growth inhibition, proliferative, verapamil

1 INTRODUCTION

The link between prolonged use of antihypertensive medicines and increased risk of cancer has been trailed with lots of controversies ever since the first report linking reserpine to development of cancer more than three decades ago. The past few decades have witnessed numerous reports associating different class and types of antihypertensive medicines to cancer. For instance, lung cancer has been linked to use of beta-blockers, general cancers linked to use of calcium channel blockers (CCBs) while cancers of the kidney and colon are linked to use of thiazide diuretics. In fact, antihypertensive agents are linked with increased risk of glioma (Volpe, M. 2011).

Calcium channel blockers (CCB)

CCB decreases heart rate, cardiac contractility and causes arterial dilatation and this is made possible by preventing the entry of calcium into cells of the heart and blood vessels (Elliott, William J 2011). Examples of CCB include amlodipine, nifedipine, diltiazem, nicardipine, felodipine, isradipine, bepridil, verapamil, nimodipine, nisoldipine, levamlodipine, lacidipine, clevidipine and lecarnidipine (Rang, Humphrey P. 2014).

- *Jamil L Ahmad - Department of Pharmacology and Therapeutics, Faculty of Basic Medical Sciences, College of Health Sciences, Usmanu Danfodiyo University, Sokoto Nigeria. Email: lukman.ahmadjamil.15@aberdeen.ac.uk*
- *Chukwudi C Okebaram - Department of Pharmacology and Therapeutics, Faculty of Basic Clinical Sciences, College of Medicine and Health Sciences, Abia State University, Uturu Nigeria. Email: c.okebaram.15@aberdeen.ac.uk*
- *Abdullahi Samaila – Department of Pharmacology, College of Medicine, Ummaru Musa Yar'adua University, Katsina, Nigeria. Email: abuuismaeel@gmail.com*

Link between anti-hypertensive agents and cancer development

The CCBs block the movement of extracellular calcium via ion-specific channels that traverse the cell wall. There are several types of the calcium channel but in humans the L-type calcium channel is inhibited by the CCBs. So, whenever the inward movement of calcium is prevented there will be relaxation of the vascular smooth muscle cells which ultimately leads to vasodilation and eventual reduction in blood pressure. In the heart muscle cells, there will be reduced cardiac contraction leading to the slowing down of the sinus pacemaker and atrioventricular conduction velocities (Taira, Norio 1987). The different types of dihydropyridine CCBs share similar chemical structure and pharmacologic action as a group which distinguished them from the other two non-dihyropyridine CCBs. Drugs belonging to these two subclasses act via binding to different sites on the L-type calcium channel, hence, explaining the pharmacologic rationale for their combination therapy, particularly diltiazem and any dihydropyridine (Opie, LH 1997). All CCBs have been approved for the treatment of hypertension either alone or in combination with the exception of nimodipine which is approved only for subarachnoid haemorrhage. CCBs are recommended as viable option as first-line therapy for hypertension. Nifedipine and nicardipine were discovered to induce gingival overgrowth among patients been treated with these medicines. In fact, they were found to induce an increase in DNA and type-1 collagen synthesis and enhanced proliferation rate of cells (Fujii, A 1994). It has been suggested that cancer cells proliferation can be halted through the modulation of calcium channels which promotes apoptosis along with modulation of cancer specific cytotoxicity of the immune cells (Bose, 2015). Patients who were exposed to CCBs were compared with two cohorts for comparison, that is, matched patients not exposed to CCB (non-CCB) and unmatched patients not exposed to CCB and at least any other antihypertensive (AHT) medication. The crude rates of cancer incidence per 1000 person – years were 16.51, 15.75 and 10.62 for the three cohorts, respectively. The adjusted HRs (confidence interval) for all cancers when compared with CCB, non-CCB and AHT cohorts were 0.88 (0.86 to 0.89) and 1.01 (0.98 to 1.04), respectively. When the duration of exposure to CCB were analyzed, excess risk was not observed (Grimaldi-Bensouda, 2016). An increased ductal and lobular breast cancer risk was discovered following long term use of CCB for greater than 10 years in women aged 55 – 74

years in the US. But diuretics, beta-blockers and angiotensin II antagonist use was not linked with increased risk of cancer (Li, 2013). Similarly, CCB was found not to enhance the growth of tumour cells in several experimental models (Mason, 1999). But however, on the contrary, inhibits the growth of tumour cells in certain neoplasia models and effectively served as adjuvant therapy in specific drug resistant cancer cells. Furthermore, evidence linking CCB to increased risk of cancer is lacking despite comprehensive epidemiologic studies which were conducted to hitherto prove an association (Mason, 1999). There was no link between CCB use and overall cancer risk and its use was not significantly linked with increased risk of cancers individually, in addition to the previously implicated cancers with the exception of kidney cancer [10] (Rosenberg, 1998). There was also no association between current use, use for five years or more and individual use of CCB with increased incidence of cancer. The use beta-blockers and ACEIs did not correlate with overall cancer or individual cancers but however both were linked with cancer of the kidney (Rosenberg, 1998). Nifedipine, a CCB was found to significantly increase the anti-tumor action of cisplatin against glioblastoma-1 (GB-1) cells (Kondo, 1995). Nifedipine increased cisplatin cytotoxicity in the absence of normal extracellular calcium ion. Furthermore, the synergistic anti-tumour effect of cisplatin and nifedipine was truncated by actinomycin D and cycloheximide, indicating reliance of such effect on synthesis of new RNA and protein. The non-cytotoxic nifedipine was confirmed to improved antitumour activity of cisplatin via a synergistic fashion on multidrug-resistant GB-1 cells devoid of calcium dependent endonuclease, which subsequently leads to apoptosis through interaction with another unidentified functional site apart from the calcium channel on GB-1 cells. The CCB flunarizine was found to be cytotoxic to Jurkat T-leukaemia cells and other haematological malignancies but not toxic to breast or colon cancer cells. Flunarizine treatment was associated with the activation of caspase-3, poly (ADP-ribose) polymerase cleavage and DNA fragments laddering, all these represent the hallmark of apoptosis (Conrad, David M. 2010). The CCB, that is, verapamil, amlodipine and diltiazem inhibited proliferation of HT-39 human breast cancer cell in a concentration-dependent fashion (Taylor, Simpson, 1992). The 50% inhibitory dose figures were 1.5 µM, 5 µM and, 10 µM for dihydropyridine amlodipine, benzothiazapine diltiazem and phenylalkylamine verapamil respectively. Similarly, verapamil was found to significantly retard the proliferation of the leukaemic U937 and THP1 cell lines in a dose and time dependent manner. The cytotoxic effect on U937 was observed at concentrations of ≥ 2, 0.2 and 0.1 mM after 24, 48 and 72-hour incubation period while that of THP1 cell line was ≥ 1, 1 and 0.2 mM. The fifty percent lethal dose (LD_{50}) was approximately 1.29 mM and 1.93 mM after 48-hour incubation period for U937 and THP1, respectively (Hajighasemi, Fatemeh 2012). Also, verapamil at a concentration of 70 µM had moderate cytotoxic effect on JK-6L cells but no effect on ARH-77 and RPMI 822 cell line (Meister, Silke 2010). Verapamil had no cytotoxic effect on monocytic cell line at less than 100 µM concentration (Meister, Silke 2010; Hajighasemi, Fatemeh 2012).

Aim of the study

To investigate in mechanistic term, the link between long term use of calcium channel blockers and cancer development using HEK293 cell line.

Specific objectives:

Using HEK293 cells to:

1. Determine the growth pattern of cells treated with verapamil
2. Determine cytotoxic effect of verapamil.

2 MATERIALS AND METHODS

Reagents

Minimum Essential eagle Medium (EMEM), Penicillin, Streptomycin, L-glutamine, Dimethyl Sulfoxide (DMSO), sterile ethanol, verapamil, fetal bovine serum (FBS), phosphate buffered saline (PBS), sodium chloride, trypsin and the human embryonic kidney (HEK293) cell line obtained from ECACC.

Verapamil preparation

Distilled water was used to dissolved verapamil and subsequently different concentrations of the drug were made using the prepared culture medium.

Cell line

The Human Embryonic Kidney (HEK) 293 cell line was used. This cells normally grow easily in tissue culture and transfected easily too. The HEK293 are immortalized human embryonic kidney cells that were obtained from ECACC. They are epithelial derived and SV40 transformed cells from a primary culture of foetal kidney. They have some unusual characteristics of having features of neuronal cells (Stepanenko, AA 2015). The cells were maintained in Minimum Essential Medium, alpha modification with 2 mM L-glutamine, 10% (v/v) foetal bovine serum (FBS), 5000 µg/ml Penicillin and 5000 U/ml Streptomycin. Cells were passed routinely every 3-4 days at a confluence with a split ratio of 1:3 to 1:5. Phase contrast microscopy was used to observe the monolayer and confluence determination. Cells were used for about 15 passages.

MTT Assay

The aim of 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT) assay is to access metabolic activity of cells. The MTT assay is dependent on the conversion of yellow water soluble MTT into some insoluble formazan purple crystals by living cells which eventually denotes activity of the mitochondria. MTT assay was performed on 96 well sterile cell culture plates. The flat-bottomed plates were used.

LD 50 determination

The concentration at which 50% of the cells died when compared with untreated cells was evaluated using the MTT based dose-response curve.

Statistical analysis

Statistical analysis was performed from three independent experiment carried out to ascertain the effect of verapamil on the viability of HEK293 cells. Analysis of variance (ANOVA) with Dunnet's post-test was applied for multiple drug exposures and results expressed as mean ± standard error of the mean (SEM). The Statistical Package for Social Science (SPSS) version 21 and Microsoft excel 2016 were used for statistical analysis and plotting of graph respectively.

3 RESULTS

The growth inhibitory/cytotoxic effect of verapamil on HEK293 cells in various concentrations and time interval are depicted in fig. 1 and 2. Fig. 1 and 2 represents the trypan blue dye exclusion and MTT assays respectively.

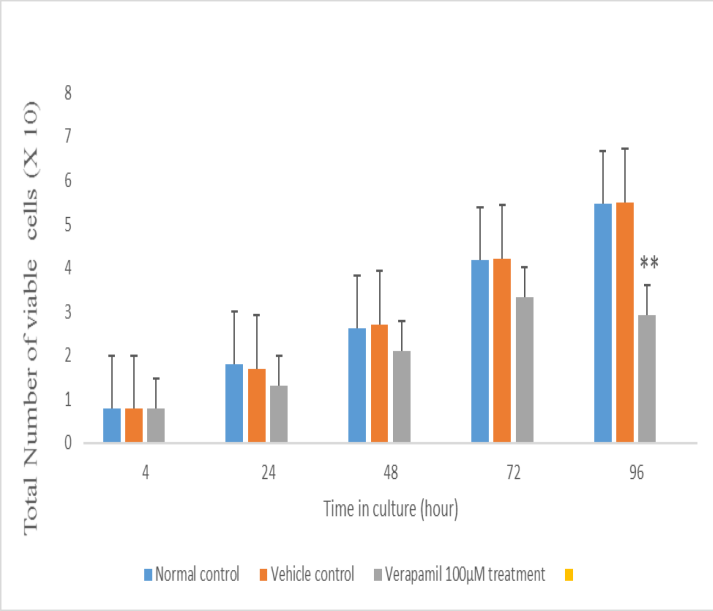

Fig. 1. *Trypan blue dye exclusion assay using 100 µM of verapamil. Cells were seeded at a density of 2.4 x 10^4 cells/cm^2 on 5 cm diameter tissue culture plates in duplicate. The cells were allowed to attach for a period of 4 hours at which point the first plates were harvested – time 0. Plates were harvested using the trypsin method and counted using a haemocytometer. Values are mean ± S.E.M. ** P< 0.05 one-way ANOVA with Dunnett's post-test. (n = 3 with 2 replicates per time point per experiment)*

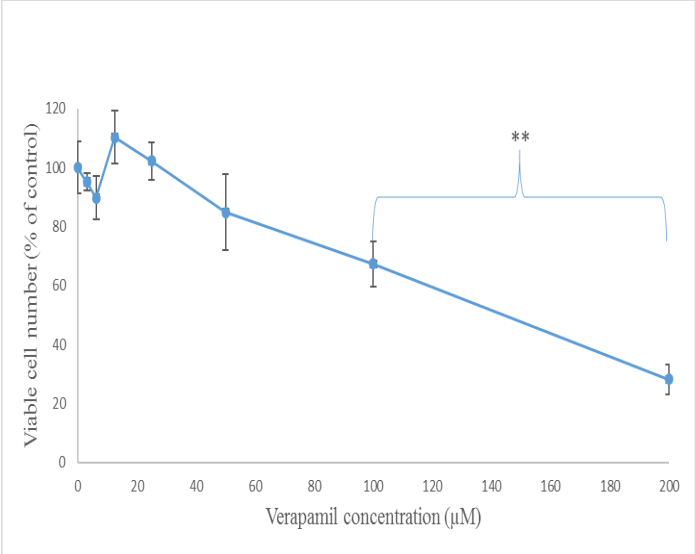

Fig. 2. *MTT assay. HEK293 cells were seeded at a density of 2.4 x 10^4 cells/cm^2 on 96 well micro titre plates and allowed to attach and grow for 48 hours. After 48 hours the medium was replaced with medium containing drug or vehicle. Plates were incubated for 48 hours and assayed based on the protocol of the MTT assay. Values are mean ± SD. ** P < 0.05 one-way ANOVA with Dunnett's post-test. (n = 3 with 6 replicates per experiment)*

Time (hour)	Normal control (%)	Vehicle control (%)	Verapamil treated cells (%)
24	98.90	97.98	98.95
48	98.25	98.77	98.82
72	98.34	98.25	97.65
96	83.80	90.91	94.93

Table 1. *Viability of HEK293 cells treated with 100 µM of verapamil. With the Trypan blue dye exclusion assay, cells were seeded at a density of 2.4 x 10^4 cells/cm^2 on 5 cm diameter tissue culture plates in duplicate. The cells were allowed to attach for a period of 4 hours at which point the first plates were harvested – time 0. Plates were harvested using the trypsin method and counted using a haemocytometer.*

The effect of verapamil on cell number and viability

Verapamil had a growth inhibitory effect rather than a cytotoxic effect on HEK293 cells (fig. 1). The viability of the cells remained above 94% in the majority of the experiments (table 1). Hence, verapamil appeared to cause a delayed exponential phase of HEK293 cell growth. The growth inhibitory effect on the cells was found to be significant ($p<0.01$) when compared with control (fig. 1). As the viability remained persistently high throughout the experiment, this will suggest that the cells were not subject to cell death by necrosis. High viability would have suggested that the cells are not going through necrosis due to verapamil treatment.

Effect of verapamil on HEK293 cell line

Using the MTT assay the extent of the effect of low concentrations of the calcium channel blocker verapamil was determined (fig. 2). The concentrations used were 3.13, 6.25, 12.5, 25.0, 50.0, 100.0 and 200 µM. The growth of the cells was inhibited in a dose dependent manner indicating a cytotoxic effect of verapamil. Hence, there was increased cytotoxicity with increased concentrations of verapamil and a sigmoidal curve was produced in the process. The maximum response observed was that 50% of the cell population remained viable at 140 µM and only about 30% remained viable at the highest concentration of 200 µM (fig. 2).

4 DISCUSSION

Interestingly, while some CCBs such as nifedipine and diltiazem enhanced proliferation of cells verapamil on the other hand inhibit growth of cells based on our findings. Based on the pharmacokinetic properties of verapamil, the normal 80 – 160 mg of oral verapamil produces a peak plasma concentrations of 336 µg/L within 1 – 2 h of administration (McTavish, Donna 1989). A single dose of 80 or 120 mg yielded a mean peak plasma concentration of 168 µg/L after 1.2 hours of administration and 120 – 240 mg yielded 753 µg/L after four weeks of twice daily intake (McTavish, Donna 1989). Verapamil is distributed widely throughout the human body with apparent volume of distribution of 113 – 418L and 257 – 406L after intravenous and oral administration, respectively. There is more increased volume of distribution in disease conditions such as hepatic cirrhosis. Verapamil is about 90% plasma protein bound and binding is not dependent on plasma concentration over 35 – 1557 µg/L range (McTavish, Donna 1989). Hence, it implies that 1, 2 and 100 µM of verapamil will produce peak plasma concentrations of approximately 336, 753 and 34,250 µg/L, respectively. And verapamil did inhibited growth of the cells at far lower concentrations based on our findings. While our research found that HEK293 cell

proliferation was significantly inhibited at 100 µM which is different from a study which discovered that verapamil at 200 µM significantly decreased the proliferation of the human monocytic U937 after 48-hour incubation while that of THP1 cells occurred after 72-hour incubation (Hajighasemi, Fatemeh 2012). The difference observed might be due to the different cell lines used because Hajighasemi, Fatemeh 2012 used leukaemic monocytic cell line and we used human embryonic kidney cells. That explains the high sensitivity of our cells to verapamil. Also, our finding contradicted that of Meister & Silke who discovered that verapamil had no cytotoxic effect on the ARH-77 and RPMI 8226 cell lines. The difference between our findings might be attributed to difference in the number and type of cells and method used for determination of viability. While Meister & Silke used 1 x 10^5 cell/well of multiple myeloma cell line, we used 2.4 x 10^4 cell/well of human embryonic kidney cell line. Multiple myeloma and human embryonic kidney showed different sensitivities to the same drug (Khwaja, Asim, 2010). Furthermore, they used alamar blue assay for the evaluation of cell viability whereas we used MTT assay and trypan blue dye exclusion assay for estimation of cell viability. Similarly, pre-treatment of the highly metastatic 4T1 breast cancer cell line with verapamil was found to significantly enhance the cytotoxic effect of doxorubicin in hitherto doxorubicin-resistant 4T1-R breast cancer cells (Bao, Lili 2011). Also, the anti-tumour or cytotoxic effect of tamoxifen was significantly enhanced with addition of verapamil to breast cancer cell line positive for oestrogen receptor. Verapamil was also found to increase the cytotoxic effect of tamoxifen (Gupta, Vicram 1994). A number of studies have discovered the anti-tumour and cytotoxic effect of calcium channel blockers which have since found practical and clinical application in cancer chemotherapy, (Berridge, Michael J 2000; Perez-Reyes, E. 2003; Jang, Sun Jeong 2013; Dziegielewska, Barbara 2014). Furthermore, verapamil, amlodipine and diltiazem were found to inhibit the proliferation of HT-39 human breast cancer cell in a concentration-dependent manner (Taylor, Simpson, 1992). The IC_{50} were 1.5 µM, 5 µM and, 10 µM for dihydropyridine amlodipine, benzothiazapine diltiazem and phenylalkylamine verapamil, respectively. There was rapid reduction of intracellular calcium concentration in the HT-39 cell line following amlodipine administration in a concentration-dependent manner. There was no effect on the level of intracellular calcium when 1 µM of amlodipine was added, 13.7% reduction was observed with 3 µM and 33.2% reduction was observed with 10 µM in the HT-39 cell lines (Taylor, Simpson, 1992).

CONCLUSION

The long term use of verapamil is associated with cellular growth inhibition and this possibly explained the rationale behind its use as part of combination chemotherapy for some human cancer.

ACKNOWLEDGMENT

Special gratitude to all our teachers and Professors for impacting great knowledge into us. We sincerely declare that we receive no any grant towards the production of this work and no conflicting interest.

REFERENCES

[1] BAO, L., HAQUE, A., JACKSON, K., HAZARI, S., MOROZ, K., JETLY, R. and DASH, S., 2011. Increased expression of P-glycoprotein is associated with doxorubicin chemoresistance in the metastatic 4T1 breast cancer model. The American journal of pathology, **178**(2), pp. 838-852.

[2] BERRIDGE, M.J., LIPP, P. and BOOTMAN, M.D., 2000. The versatility and universality of calcium signalling. Nature reviews Molecular cell biology, **1**(1), pp. 11-21.

[3] BOSE, T., CIEŚLAR-POBUDA, A. and WIECHEC, E., 2015. Role of ion channels in regulating Ca2+ homeostasis during the interplay between immune and cancer cells. Cell death & disease, **6**(2), pp. e1648.

[4] CONRAD, D.M., FURLONG, S.J., DOUCETTE, C.D., WEST, K.A. and HOSKIN, D.W., 2010. The Ca2 channel blocker flunarizine induces caspase-10-dependent apoptosis in Jurkat T-leukemia cells. Apoptosis, **15**(5), pp. 597-607.

[5] DZIEGIELEWSKA, B., GRAY, L.S. and DZIEGIELEWSKI, J., 2014. T-type calcium channels blockers as new tools in cancer therapies. Pflügers Archiv-European Journal of Physiology, **466**(4), pp. 801-810.

[6] ELLIOTT, W.J. and RAM, C.V.S., 2011. Calcium channel blockers. The Journal of Clinical Hypertension, **13**(9), pp. 687-689.

[7] FUJII, A., MATSUMOTO, H., NAKAO, S., TESHIGAWARA, H. and AKIMOTO, Y., 1994. Effect of calcium-channel blockers on cell proliferation, DNA synthesis and collagen synthesis of cultured gingival fibroblasts derived from human nifedipine responders and non-responders. Archives of Oral Biology, **39**(2), pp. 99-104.

[8] GRIMALDI-BENSOUDA, L., KLUNGEL, O., KURZ, X., DE GROOT, M.C., AFONSO, A.S.M., DE BRUIN, M.L., REYNOLDS, R. and ROSSIGNOL, M., 2016. Calcium channel blockers and cancer: a risk analysis using the UK Clinical Practice Research Datalink (CPRD). BMJ open, **6**(1), pp. e009147.

[9] GUPTA, V., KAMATH, N., TKALCEVIC, G.T. and SINGH, S.V., 1994. Potentiation of tamoxifen activity by verapamil in a human breast cancer cell line. Biochemical pharmacology, **47**(9), pp. 1701-1704.

[10] HAJIGHASEMI, F. and KAKADEZFULI, N., 2012. Sensitivity of monocytic cell lines to verapamil in vitro. Research Journal of Biological Sciences, **7**(5), pp. 209-214.

[11] JANG, S.J., CHOI, H.W., CHOI, D.L., CHO, S., RIM, H., CHOI, H., KIM, K., HUANG, M., RHIM, H. and

LEE, K., 2013. In vitro cytotoxicity on human ovarian cancer cells by T-type calcium channel blockers. Bioorganic & medicinal chemistry letters, **23**(24), pp. 6656-6662.

[12] KHWAJA, A., 2010. PI3K as a target for therapy in haematological malignancies. Phosphoinositide 3-kinase in Health and Disease. Springer, pp. 169-188.

[13] LI, C.I., DALING, J.R., TANG, M.C., HAUGEN, K.L., PORTER, P.L. and MALONE, K.E., 2013. Use of antihypertensive medications and breast cancer risk among women aged 55 to 74 years. JAMA internal medicine, **173**(17), pp. 1629-1637.

[14] MASON, R.P., 1999. Calcium channel blockers, apoptosis and cancer: is there a biologic relationship? Journal of the American College of Cardiology, **34**(7), pp. 1857-1866.

[15] MCTAVISH, D. and SORKIN, E.M., 1989. Verapamil. Drugs, **38**(1), pp. 19-76.

[16] MEISTER, S., FREY, B., LANG, V.R., GAIPL, U.S., SCHETT, G., SCHLÖTZER-SCHREHARDT, U. and VOLL, R.E., 2010. Calcium Channel Blocker Verapamil Enhances Reticulum Stress and Death Induced by Proteasome Inhibition in Myeloma Cells. Neoplasia, **12**(7), pp. 550-IN3.

[17] OPIE, L., 1997. Pharmacological differences between calcium antagonists. European heart journal, **18** (suppl A), pp. 71-79.

[18] PEREZ-REYES, E., 2003. Molecular physiology of low-voltage-activated t-type calcium channels. Physiological Reviews, **83**(1), pp. 117-161.

[19] RANG, H.P., RITTER, J.M., FLOWER, R.J. and HENDERSON, G., 2014. Rang & Dale's Pharmacology: with STUDENT CONSULT Online Access. Elsevier Health Sciences.

[20] STEPANENKO, A. and DMITRENKO, V., 2015. HEK293 in cell biology and cancer research: phenotype, karyotype, tumorigenicity, and stress-induced genome-phenotype evolution. Gene, **569**(2), pp. 182-190.

[21] TAIRA, N., 1987. Differences in cardiovascular profile among calcium antagonists. The American Journal of Cardiology, **59**(3), pp. B24-B29.

[22] VOLPE, M., AZIZI, M., DANSER, A.H., NGUYEN, G. and RUILOPE, L.M., 2011. Twisting arms to angiotensin receptor blockers/antagonists: the turn of cancer. European heart journal, **32**(1), pp. 19-22.