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Sensitivity of Monocytic Cell Lines to Verapamil *in vitro*

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Abstract: Verapamil as a calcium channel blocker broadly used in therapy of many cardiovascular diseases such as hypertension and arrhythmia. Moreover, the anti-tumor effect of several chemotherapeutic agents has been increased by verapamil. Also, the inhibitory effect of some calcium channel blockers on tumor cell growth and invasion has been reported. Moreover, induction of apoptosis in leukemia cells by a Ca^{2+} channel blocker has been revealed. The present study was conducted to evaluate the verapamil cytotoxicity in two human monocytic cell lines *in vitro*. The human monocytic U937 and THP1 cells were cultured in complete RPMI medium. Then, the cells at logarhytmic growth phase were incubated with different concentrations of verapamil (0.01-2 mM) for 24, 48 and 72 h periods. Next, the cell viability was assessed with trypan blue dye exclusion and MTT 3-(4, 5-dimethyl thiazol-2, 5-diphenyltetrazoliumbromide) methods. Verapamil significantly decreased the proliferation of U937 and THP1 cells dose and time dependently. This cytotoxic effect after 24, 48 and 72 h incubation time was shown at ≥1, 0.2 and 0.1 mM concentration for U937 and at ≥1, 1 and 0.2 mM concentration for THP1 cells, respectively. In U937 cells verapamil cytotoxicity at 0.2 mM concentration, significantly increased with time in this order 72>48 h ($p<0.05$). According to the results of this study, verapamil showed a dose and time-dependent cytotoxic effect on human U937 and THP1 cells. Moreover, the sensitivity of U937 and THP1 cells to verapamil was somewhat different. So, the anti-tumor effects of verapamil reported by several investigations may be in part due to its direct cytotoxic effects. Thus, verapamil with potential inhibitory effect on leukemic U937 and THP1 cells growth might be useful as an anti-proliferative agent in leukemia.

Key words: Cytotoxicity, verapamil, monocytes, leukemia, anti-proliferative, cytotoxic effect

INTRODUCTION

Verapamil is a calcium channel blocker widely used in treatment of many cardiovascular diseases such as hypertension and arrhythmia (Iskenderov *et al*., 2011; Kostis *et al*., 2011). The efficacy of verapamil to increase the anti-tumor effect of numerous chemotherapeutic agents has been shown (Liu *et al*., 2011a; Kameda *et al*., 2001; Gupta *et al*., 1994). Accordingly, verapamil has increased the treatment efficacy of chemotherapeutic agents in metastatic colon cancer patients (Liu *et al*., 2011b). In addition, enhancement of hyperthermia-induced apoptosis by verapamil in U937 cells has been indicated (Kameda *et al*., 2001). Furthermore, verapamil enhanced the apoptotic effect of BCL-2 inhibitors in B-cell lymphoma (Vogler *et al*., 2011). Also, combinations of vincristin sulfate and verapamil increased the therapeutic effectiveness of the drug in hepatocellular carcinoma (Song *et al*., 2010). Moreover, the reversal effect of verapamil on Multi Drug Resistance (MDR) capacity of cancer cells has been reported (Koo *et al*., 2008; Kim *et al*., 2008). One of major causes of fail in anti-tumor chemotherapy is drug resistance. One reason of multidrug resistance is overexpression of P-glycoprotein (PgP) on the cancer cells (Wang *et al*., 2011). Verapamil is a well-known PgP inhibitor (Liu *et al*., 2010) and enhances the accumulation and cellular distribution of drugs in some drug-resistant cell lines (Wang *et al*., 2011). In this regard, improvement of chemotherapy by targeted intra-arterial verapamil combination in primary liver cancer has been shown (Pingsheng *et al*., 2012). In addition, pretreatment of drug-resistant metastatic breast cancer cells with verapamil enhanced nuclear translocation of doxorubicin and cytotoxicity (Bao *et al*., 2011). Furthermore, reducing effect of verapamil on T-cytotoxic lymphocyte proliferation has been demonstrated (Koo *et al*., 2008). Moreover, beneficial effect of verapamil in chemotherapy of hematologic malignancies has been reported (Wang *et al*., 2006; Meister *et al*., 2010). Accordingly, verapamil combined to bortezomib decreased the myeloma cells viability through inducing cell death. By the way current therapeutic strategies for hematologic malignancies have not been very successful and some had shown sever side-effects (Dressel *et al*.,

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2011). The inhibitory effect of some calcium channel blockers on tumor cell growth and invasion has also been reported (Conrad *et al*., 2010; Zhang *et al*., 2007; Li *et al*., 2011). Moreover, induction of apoptosis in leukemia cells by a Ca^{2+} channel blocker has been revealed (Yanamandra *et al*., 2011). Besides the anti-inflammatory effects of verapamil has been shown (Skal'skii, 2010; Liu *et al*., 2011a, b). The therapeutic properties of a Ca^{2+} channel blocker in inflammatory bowel disease has also been described (Di Sabatino *et al*., 2009).

Regarding that verapamil increases the sensitivity of cancer cells to chemotherapeutic drugs (Xia *et al*., 2010) and also enhances apoptosis of tumor cells in combined drug therapy (Meister *et al*., 2010), in the present study, the effect of verapamil on viability of two human monocytic cell lines has been evaluated *in vitro*.

MATERIALS AND METHODS

Reagents: RPMI-1640 medium, penicillin, streptomycin and Trypan Blue (TB) were from sigma (USA). MTT (3-(4, 5-dimethyl thiazol-2, 5-diphenyltetrazoliumbromide)) were purchased from Merck (Germany). Fetal Calf Serum (FCS) was from Gibco (USA). Verapamil was purchased from Sobhandarou Pvt. Co. Ltd (Tehran, Iran). Microtiter plates, flasks and tubes were from Nunc (Falcon, USA).

Preparation of verapamil: Verapamil was dissolved in distilled water and stored as a stock at 20°C until use. The stock was diluted in culture medium to prepare appropriate concentrations before use.

Cell lines: Human monocytic cells (U937 (NCBI C130) and THP1 (NCBI C563)) were obtained from NCBI (National Cell Bank of Iran, Pasteur Inst. of Iran, Tehran). The cells were maintained in RPMI-1640 medium supplemented with 10% FCS in 5% CO_2 at 37°C.

Cell culture and treatment: The method has been described in detail elsewhere (Hajighasemi and Mirshafiey, 2010). Briefly, the cells were cultured in RPMI-1640 medium supplemented with 10% FCS, penicillin (100 IU mL^{-1}) and streptomycin (100 μg mL^{-1}) at 37°C in 5% CO_2. The cells were seeded at a density of 4×10^4 cell/well and then incubated with different concentrations of verapamil (0.01-2 mM) for 24, 48 and 72 h. Cytotoxicity was explained as the percentage of viable cells at different doses of verapamil. All experiments were done in triplicate.

Cell proliferation assays: To evaluate the effect of different concentrations of verapamil on viability of monocytic cell lines, researchers used Trypan Blue dye exclusion (TB test) (Moldeus *et al*., 1978) and MTT assay (Mosmann, 1983).

Trypan blue dye exclusion test: Principle of trypan blue dye exclusion test is exclusion of dye by viable cells and taking it up by dead cells. Viability is evaluated by direct counting of viable and dead cells. Percentage of the number of viable cells to the total number of cells is considered as viability percentage.

MTT assay: In MTT assay, the conversion of yellow water soluble MTT to a blue-insoluble formazon was assessed according to the method developed by Mosmann (1983). At the end of incubation time, 20 μL of MTT solution (5 mg mL^{-1} in PBS) was added to each well and incubated at 37°C for 4 h. Subsequently, the medium was depleted and then 100 μL of the isopropanol-HCl solution (0.04 N) was added to each well. So, the insoluble formazon derivative was dissolved and absorbance at 570 nm was measured using a microplate reader (ICN Flow Titertech Multiscan plus, USA). The results were expressed as cell numbers per control.

LD 50 determination: The 50% Lethal Dose (LD (50)) was calculated by MTT based dose-response curve as the concentration at which 50% cell death rate occurred relative to the untreated cells.

Statistical analysis: The effect of verapamil on viability of monocytic cell lines was assessed in three independent experiments and the results were expressed as mean±SEM. Statistical comparisons between groups were made by Analysis of Variance (ANOVA). The $p<0.05$ was considered significant. Test of multiple comparison of Tukey was applied (5%) for statistically significant differences. The software SPSS 11.5 and Excel 2003 were used for statistical analysis and graph making, respectively.

RESULTS

Cytotoxicity of verapamil on human leukemic U937 and THP1 cells in different concentrations and time intervals are shown in Fig. 1 and 2. In Fig. 1 and 2, the a and b represent the results of trypan blue dye exclusion and MTT assays, respectively.

Cytotoxic effect of verapamil on U937 cells: Verapamil significantly decreased the proliferation of U937 cells in both staining methods at all time intervals dos dependently (Fig. 1a, b). The results depicted in Fig. 1a, b

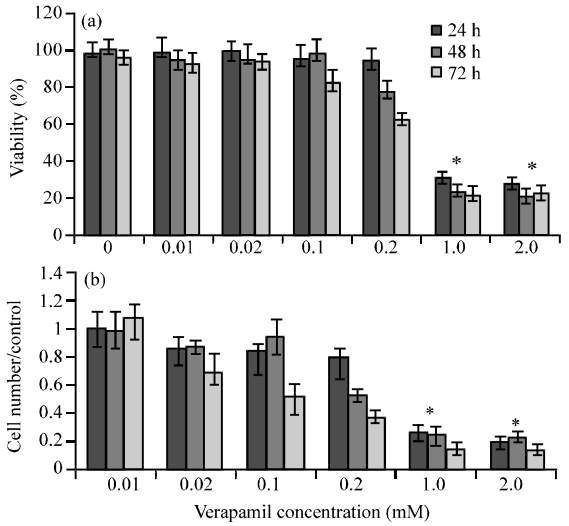

Fig. 1: Effect of verapamil on proliferative responses of human leukemic U937 cell line. The U937 cells were treated with different concentrations of verapamil (0.01-2 mM) for 24, 48 and 72 h. The results are presented as % of viability demonstrated by: a) Trypan blue dye exclusion (TB) test; b) Cell number/control demonstrated by MTT assay. Data are mean±SEM of triplicate cultures; n = 3; *p<0.05 was considered significant

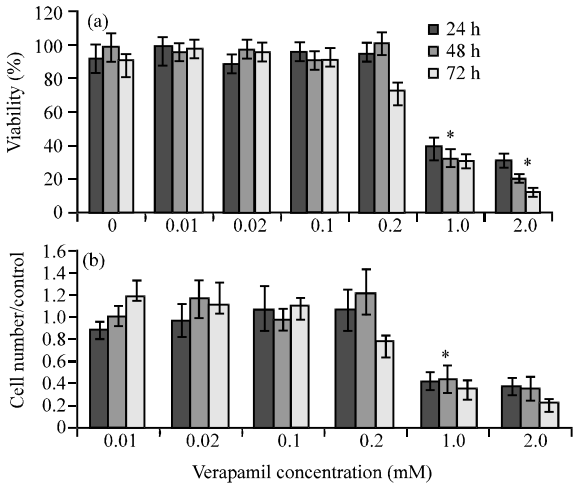

Fig. 2: Effect of verapamil on proliferative responses of human leukemic THP1 cell line. The THP1 cells were treated with different concentrations of verapamil (0.01-2 mM) for 24, 48 and 72 h. The results are presented as % of viability demonstrated by: a) Trypan blue dye exclusion (TB) test; b) Cell number/control demonstrated by MTT assay. Data are mean±SEM of triplicate cultures. n = 3; *p<0.05 was considered significant

showed that after 24, 48 and 72 h incubation, verapamil significantly decreased the proliferation of U937 cells at ≥1, 0.2 and 0.1 mM concentrations, respectively compared with untreated control cells ($p<0.05$).

Verapamil cytotoxicity at 0.2 mM concentration significantly increased with time in this order 72>48 h in U937 cells ($p<0.05$) (Fig. 1).

MTT assay represented a typical dose-response curve with LD50s of 1.028, 1.285 and 1.002 mM after 24, 48 and 72 h treatment of U937 cells, respectively.

Cytotoxic effect of verapamil on THP1 cells: Verapamil significantly decreased the proliferation of THP1 cells in both staining methods at all time intervals dose dependently (Fig. 2a, b).

According to Fig. 2a, b, after 24 and 48 h verapamil significantly decreased the proliferation of THP1 cells at ≥1 mM concentrations compared with untreated control cells ($p<0.05$).

However after 72 h incubation, verapamil significantly decreased the proliferation of THP1 cells at ≥0.2 mM concentrations compared with untreated control cells ($p<0.05$).

MTT assay represented a typical dose-response curve with LD50s of 1.519, 1.939 and 1.290 mM after 24, 48 and 72 h treatment of THP-1 cells, respectively.

DISCUSSION

In the present study, the effects of verapamil on proliferation of two human monocytic cell lines were evaluated. The results of this study determined that verapamil had a cytotoxic effect on the monocytic cells used in this study dose and time-dependently. This cytotoxicity for U937 cells was shown at ≥1, 0.2 and 0.1 mM concentrations of the drug after 24, 48 and 72 h incubation time respectively. Moreover, the cytotoxic effect of verapamil on THP1 cells was revealed at ≥1 mM concentration of drug after 24 and 48 h incubation time and at ≥0.2 mM after 72 h incubation time. According to the results, U937 and THP1 leukemic cells have different sensitivity to verapamil as the cytotoxicity of verapamil at ≥0.2 mM concentrations on U937 cells was shown after 48 h incubation time whereas on THP1 cells was revealed after 72 h incubation time. So, it seems that the U937 cells are more sensitive to verapamil than THP1 cells.

Similar to this, Meister *et al.* (2010) demonstrated that verapamil displayed a slight cytotoxic effect on JK-6L cells and no effect on ARH-77 and RPMI 8226 cells at 70 μM (0.07 mM) concentration. In the study, consistent to Meister *et al.* (2010), verapamil did not show any cytotoxicity at <0.1 mM (100 μM) concentration on the

monocytic cells used in this study. Also, in Meister *et al.* (2010)'s study, different cells showed different sensitivity to verapamil (similar to this study). In Meister *et al.* (2010)'s study, verapamil caused a little cytotoxic effect on JK-6L cells at 70 μM. This slight difference between the results and Meister may be in part due to the type, number of cells and the methods used for assessment of cell viability. Meister *et al.* (2010) used 1×10^5 cells/well of multiple myeloma origin while we used 4×10^4cell/well of human monocytic leukemia cells. Leukemia and multiple myeloma cells display variable sensitivities to a single drug (Khwaja, 2010). Furthermore, used alamar blue assay for determination of cell viability and Annexin V-fluorescein isothiocyanate/propidium iodide staining to measure cell apoptosis while we used MTT and trypan blue dye exclusion methods for cell viability and proliferation measurements.

In this study, the LD50s of verapamil for U937 cells at 24, 48 and 72 h were 1.028, 1.285 and 1.002 mM and for THP1 cells were 1.519, 1.939 and 1.290 mM, respectively. According to these data, the LD50s of verapamil for U937 were found to be lower than THP1 cells. It might indicate that possibly U937 cells are more sensitive to verapamil than THP1 cells and once again point out that different cells have different sensitivities to verapamil.

Inhibition of murine mammary carcinoma cells invasion and metastasis by verapamil has been reported *in vivo* (Farias *et al.*, 1998). Moreover, consistent to this results, Farias showed an extensive cytostatic effect of verapamil on mammary tumor cell proliferation *in vitro*. In Farias's study, no cytotoxicity was shown at ≤100 μM concentrations of the verapamil (similar to this study) as was detected by morphological cytotoxic study, metabolic titer assay and trypan blue dye exclusion test. Furthermore in Farias *et al.* (1998)'s study, verapamil was highly cytotoxic at >250 μM concentrations. Similarly, researchers did not detect any cytotoxicity at <200 μM concentrations of verapamil and determined a high cytotoxicity at ≥200 and 1000 μM concentrations of verapamil for U937 and THP1 cells, respectively. In another study, verapamil inhibited the proliferation of Con-A stimulated mice spelenic lymphocytes at 100 μM concentration *ex vivo* (Satoh and Wasaki, 2011). In this study, verapamil showed cytotoxicity at higher concentrations than that in Satoh and Wasaki (2011)'s study. However, researchers did not assessed the verapamil effect at exact 100 μM concentration. Also in the study, the origin and type of the cells and condition of the study were different from that of Satoh and Wasaki (2011)'s study. Satoh and Wasaki studied on mouse spelenic lymphocytes *ex vivo* while researchers used human monocytic leukemia cells *in vitro*. This may suggest that normal cells may be more sensitive to verapamil than tumor cells. In addition, enhancement of hyperthermia-induced apoptosis by verapamil in leukemia U937 cells has been indicated (Kameda *et al.*, 2001). Moreover, the antiproliferative effect of verapamil (at 10 μM concentration) on U937 cells in the presence of Bestatin and actinonin has been reported (Grujic and Renko, 2002) which is lower than cytotoxic dose of verapamil in this study. The discrepancy between the results of this study and Grujic and Renko (2002) may be in part due to that we used verapamil alone while they used a combination of verapamil, bestatin and actinonin. The enhancement of anti-tumor effects of several chemotherapeutic agents by verapamil in various cancers such as metastatic colon cancer, B-cell lymphoma and hepatocellular carcinoma has been reported (Liu *et al.*, 2011a, b; Vogler *et al.*, 2011; Song *et al.*, 2010). The amplification effect of verapamil in chemotherapy of cancers has been attributed to its reversal effect on Multi Drug Resistance (MDR) capacity of cancerous cells (Koo *et al.*, 2008; Kim *et al.*, 2008; Liu *et al.*, 2010). In this regard, verapamil enhanced the accumulation and cellular distribution of drugs in some drug-resistant cell lines (Wang *et al.*, 2011). The results along with other studies (Satoh and Wasaki 2011; Grujiæ *et al.*, 2002) revealed that verapamil alone could exert a remarkable cytotoxicity at different concentrations in different cells. Accordingly, the anti-tumor effects of verapamil reported by several investigations (Liu *et al.*, 2011a, b; Kameda *et al.*, 2001; Gupta *et al.*, 1994 ; Vogler *et al.*, 2011; Song *et al.*, 2010) may be in part due to its direct cytotoxic effects (the results) other than its reversal effect on Multi Drug Resistance (MDR) capacity of tumor cells. The anti-tumor effects of some calcium channel blockers by induction of apoptosis in leukemia and multiple myeloma (Conrad *et al.*, 2010; Yanamandra *et al.*, 2011) and inhibition of ovarian cancer cell proliferation (Li *et al.*, 2011) has been reported.

Meanwhile, the dose-dependent anti-inflammatory effects of verapamil through inhibition of LPS-induced pro-inflammatory cytokines production and NF-KB activation has been shown *in vivo* (Li *et al.*, 2006). Also in another study, verapamil suppressed the pro-inflammatory cytokines production (Matsumori *et al.*, 2010). Besides suppressive effect of verapamil on LPS-induced release of pro-inflammatory factors such as superoxide and Nitric Oxide (NO) production through a calcium channel independent pathway has been revealed (Liu *et al.*, 2011a, b). In addition inhibitory effect of verapamil on MMP-9 activity in murine mammary tumor cells has been shown (Farias *et al.*, 1998). The important role of monocytes/macrophages in inflammation, make it

probable that anti-inflammatory effects of verapamil may be in part due to its cytotoxic effects on monocytes (the results of this study).

Taken together the findings in company with others' studies suggest that verapamil along with its calcium channel blocker effect, might be a cytotoxic agent for tumor cells and different cells have different sensitivity to verapamil. Although, the anti-tumoral effects of verapamil on a number of cancers have been reported (Liu *et al*., 2011a, b; Kameda *et al*., 2001; Gupta *et al*., 1994; Vogler *et al*., 2011; Song *et al*., 2010) its toxicity on normal cells, at its anti-tumor concentrations has not been declared yet. Thus, it is noteworthy to investigate the verapamil toxicity on normal as well as cancer cells in various time intervals *in vivo* to find out the optimum anti-cancer concentration of the drug at its least cytotoxicity on normal cells. Altogether, the results of the present study offer that verapamil might be a useful candidate in development of chemotherapeutic strategies for leukemia patients as well as other malignancies.

CONCLUSION

In this study, verapamil showed a cytotoxic effect on the used leukemic cells dose and time-dependently. Verapamil along with its calcium channel blocker effect might be a cytotoxic agent for tumor cells. The anti-tumor effects of verapamil reported by several investigations may be in part due to its cytotoxic effects. Therefore, verapamil could be a useful candidate in development of chemotherapeutic strategies for leukemia patients as well as other malignancies.

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