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Verapamil Enhanced in Vitro Chemosensitivity of a Murine Bladder Carcinoma, FCB*

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Summary. The in vitro enhancement of chemotherapeutic efficacy by verapamil, a calcium antagonist, was assessed using FCB, a transplantable murine transitional cell carcinoma. Exponentially growing FCB cells were partially resistant to treatment with both thiotepa (10^{-4} M) and Adriamycin (10^{-5} M), however, there was a significant reduction in cell growth when either agent was administered in combination with verapamil (10^{-5} M); the effect was evident over a wide range of drug concentrations (10^{-4}–10^{-9} M). There was also a pronounced inhibition of DNA precursor incorporation when verapamil was used in combination with either agent. Fluorometric analysis of Adriamycin uptake indicated that verapamil caused an increase in the intracellular concentration of the agent. The data presented are consistent with the postulate that verapamil enhances chemotherapeutic efficacy by altering cellular permeability to the cytotoxic agents. Our study indicates that the use of verapamil in combination with cytotoxic agents for intravesical chemotherapy of bladder tumors may prove to be beneficial in human patients.

Key words: Verapamil, Adriamycin, Thiotepa, Bladder cancer, Calcium channel blockers.

Introduction

Intravesical chemotherapy has become a common adjuvant to endoscopic surgery, however, bladder tumors are often not chemoresponsive. Thiotepa (THT), a polyfunctional alkylating agent which binds DNA, has been widely used in intravesical chemotherapy but with only limited success. It has been reported to be clinically ineffective in approximately one-third of the patients treated [1, 13, 20, 32]. Additionally, adverse systemic effects have been shown to occur with high intravesical dosages of THT since it is readily absorbed from the bladder [21, 22, 33]. The use of an anthracycline antibiotic, Adriamycin (ADM), has yielded better clinical results. Adriamycin has shown an 86% response rate in noninvasive bladder tumors [16, 17] and as high as an 14–17% response rate in metastatic urothelial tract cancer [27, 29], while exhibiting less systemic toxicity [3, 5, 12]; presumably due to its limited absorption by the urothelial lining [18, 21]. Improvements in chemotherapeutic efficacy are clearly desirable, since many patients fail to respond to these and other agents. To this end, a calcium channel blocker, verapamil, is being investigated for its ability to increase the efficacy of these two clinically used antineoplastic agents against a murine bladder tumor.

Verapamil has been shown to block the transmembrane flux of calcium in cardiac muscle [6, 15] and has been used clinically in the treatment of hypertension [9], cardiac arrhythmias [8] and angina [19]. More recently, verapamil has been shown to reduce drug resistance of P388 leukemia cells and Ehrlich ascites carcinoma cells. Tsuruo et al. [30] demonstrated that verapamil, while not cytotoxic itself, enhanced vincristine and vinblastine cytotoxicity in both drug-sensitive and drug-resistant P388 leukemia cells. When combined with verapamil, vincristine resistance was overcome both in vitro and in vivo as the result of a ten-fold increase in the intracellular drug level. Verapamil restored daunorubicin sensitivity of a drug-resistant Ehrlich ascites carcinoma, substantially reducing the drug concentration required to inhibit 50% of DNA and RNA synthesis [26]. The calcium blocker also facilitated Adriamycin accumulation in P388 leukemia cells [31].

Recently we demonstrated that verapamil enhanced the chemotherapeutic response of the T-24 human bladder cancer cell line to Adriamycin [25]. In this study we used a transplantable murine transitional cell carcinoma (FCB) to explore the value of verapamil in the management of primary cultures of urologic tumors.

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Materials and Methods

Drug Preparation

Thiotepa (American Cyanamid Corp., Pearl River, NY) and Adriamycin (Adria Laboratories, Columbus, OH) were dissolved in Eagle's minimum essential medium (EMEM; M.A. Bioproducts, Walkersville, MD) at a concentration of 10^{-3} M. A stock solution of verapamil (NSC 272,306-NA) was made in absolute ethanol and diluted with EMEM to 10^{-5} M. The final concentration of ethanol was less than 0.01%.

Tumor Cell Preparation

The transplantable murine bladder tumor FCB (Mason Research Institute, Worcester, MA) was maintained by subcutaneous passage in syngeneic C57BL mice (Laboratory Supply, Indianapolis, IN). All mice were maintained on a 12 h light-dark cycle and received food (Purina Mouse Chow) and water ad lib. Under light ether anesthesia animals were inoculated subcutaneously with 0.2 cc of freshly minced FCB tumor in the suprascapular region. Approximately 12 days later tumors were aseptically excised and viable tumor tissue freed of any necrotic areas and visible connective tissue. Tumor tissue was finely minced and chopped into 0.5 mm^3 blocks with a McIlwain tissue chopper (Brinkmann Instruments, Westbury NY). The tumor bits were pipetted vigorously to produce a suspension of single cells and passed through sterile 110 and 41 μm Nitex filters (Tetko Co., Elmoford, NY). The single-cell suspension was washed twice in EMEM and plated in 24-well multiwell polystyrene culture plates (Falcon Laboratories, Oxnard, CA) at a density of 1.0×10^5 cells per well in 0.5 ml of EMEM supplemented with 10% fetal calf serum, 1.0 μg/ml corticosterone, 1.0 μg/ml ovine prolactin, 0.125 U/ml insulin, and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml Fungizone). Monolayer cultures were maintained in an atmosphere of 95% air-5% CO_2 and 95% relative humidity.

Dose Response Curves

Dose response curves were obtained by exposing established cultures in supplemented EMEM to either THT or ADM for 24 h at concentrations ranging from 10^{-3} to 10^{-9} M. Cells were washed three times with a Ca^{++} -Mg^{++} -free phosphate-buffered saline solution and the remaining cells were removed from the substrate using a trypsin-Versene mixture (M.A. Bioproducts, Walkersville, MD). Cell number was determined by use of a Coulter counter (Coulter Electronics, Hialeah, FL) and cell viability was evaluated by vital dye exclusion. All results were expressed as a percentage of the control values. Evaluation of verapamil toxicity was preformed by incubating cultures in verapamil (10^{-5} M) containing media for 72 h after which the number of attached and viable cells remaining was compared to control cultures. Viability of floating cells in the treatment media before trypsinization as well as cells in the trypsinized cell suspension were assessed by use of trypan blue. In all cultures tested the viability of cells in the treatment media was less than 5% and the viability of cells in the trypsinized cell suspension was greater than 95%.

Chemotherapeutic Enhancement

Verapamil's potentiation of drug sensitivity was evaluated using a fixed concentration of verapamil (10^{-5} M) and a wide range of drug concentrations (THT 10^{-8}, 10^{-6}, 10^{-4} M; ADM 10^{-9}, 10^{-7}, 10^{-5} M). To assess cell growth in each culture plate, cells from four control wells were determined at the beginning and at the end of each test period. At the end of the test period all treated cultures were rinsed three times with a Ca^{++} -Mg^{++} free phosphate-buffered saline and the remaining attached viable cells were removed from the substrate by use of a trypsin-Versene mixture (M.A. Bioproducts, Walkersville, MD) and counted by use of a Coulter counter (Coulter Electronics, Hialeah, FL). Cell growth was determined using the equation:

$$\% \text{ Growth} = (T - C_0)/(C - C_0) \times 100$$

where C_0 represents the cell number at onset of treatment, T is the cell number after drug treatment, and C is the cell number in untreated controls at harvest [34].

Time dependent verapamil enhancement of chemosensitivity was tested by first exposing FCB cells to either THT (10^{-4} M) or ADM (10^{-5} M) for 1, 6, or 24 h. At the end of the treatment period the drug-containing medium was removed, the cells washed twice and refed drug-free supplemented EMEM for the remainder of the 72 h test period. Other cultures were treated with a combination of the cytotoxic agent and verapamil (10^{-5} M) for the first 1, 6, or 24 h followed by washing and refeeding with verapamil containing media for the remainder of the 72 h test period. In addition, other cultures were exposed to either the cytotoxic agents alone or to a combination of verapamil and the cytotoxic agents for the entire 72 h. To assess cell growth in each treatment group, cultures phosphate-buffered saline and attached viable cells were removed by use of trypsin-Versene (M.A. Bioproducts, Walkersville, MD). Cell numbers and viability were determined as described earlier and cell growth was determined by the above equation. Again in all cultures tested the viability of cells in the treatment media was $< 5\%$ and the viability of cells in the trypsinized cell suspension was $> 95\%$.

Thymidine Labeling Indices

Thymidine labeling indices (number of S-phase cells) were determined using the above protocol and after a 30 min pulse with 1.0 μCi/ml [^{3}H]thymidine (6.2 Ci/mM; New England Nuclear). Cultures were rinsed three times with a Ca^{++} -Mg^{++} -free phosphate-buffered saline to remove any unincorporated label and fixed in 10% neutral buffered formalin. Cells were dehydrated through ascending concentrations of ethanol (30–100%) and dipped in NTB-3 nuclear track emulsion (Eastman Kodak, Rockchester, NY). The plates were exposed at 4 °C for 7–9 days and developed in Dektol (diluted 1:1 with distilled water) developer [2]. A labeled cell was defined as one displaying 5 or more grains in the emulsion overlying the nucleus. Average background was approximately 7–10 grains per 50 μm^2 [24]. Results were determined after counting a minimum of 3,000 cells per treatment group and expressed as a percentage of control values.

Intracellular Drug Concentration

Intracellular Adriamycin was determined after incubating FCB cells with media containing 10^{-5} M ADM alone or in combination with 10^{-5} M verapamil. Triplicate aliquots of 1×10^6 cells were removed after 3 h of exposure at 37 °C, centrifuged, washed three times with cold 0.85% sodium chloride solution, extracted with 50% ethanol-0.3 N hydrochloric acid and recentrifuged [7]. ADM fluorescence was determined in the supernatant at excitation and emission wavelengths of 470 and 585 nm in an Aminco-Bowmam spectrophotometer (American Instrument Co., Silver Spring, MD). ADM content was extrapolated from a standard curve prepared with known concentrations of ADM hydrochloride.

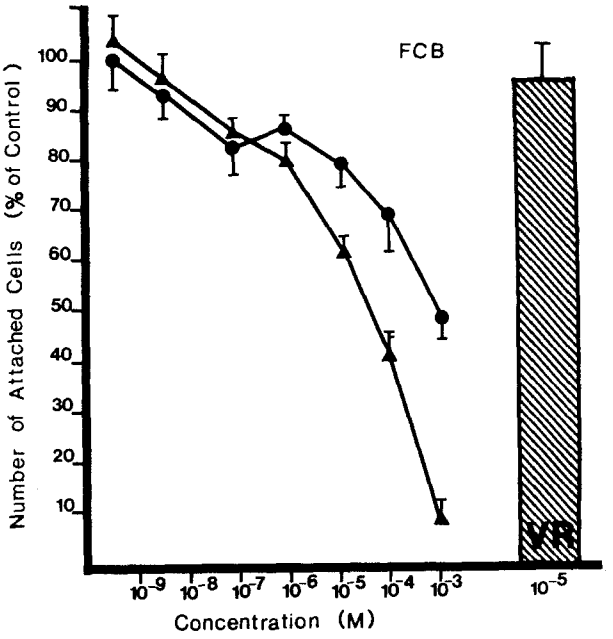

Fig. 1. Dose response curve of primary cultured cells from a transitional cell carcinoma, FCB. Cultures were treated for 24 h with various concentrations of either ADM (▲) or THT (●) and the number of attached viable cells was determined. Each data point represents the average of triplicate plates from three seperate tumors. Bar graph represents the number of attached cells after 72 h of treatment with verapamil

Results

Subcutaneous inoculation of adult female C57BL mice with primary FCB tumor explants produced palpable growths within seven to ten days in 85.7% of the animals. Enclosed by a connective tissue capsule, the friable tumor mass was easily dispersed as a single-cell suspension. When plated at 1.0×10^5 cells per well in 0.5 ml of supplemented EMEM, monolayer cultures grew exponentially over the 72 h experimental period.

The 24-h dose response study revealed that these bladder tumor cells were partially resistant to THT at concentrations as high as 10^{-4} M. The ID_{50} concentration was achieved only after exposure to 10^{-3} M THT (Fig. 1). Adriamycin was more effective at all concentrations tested; ID_{50} was at 5×10^{-4} M (Fig. 1). Verapamil at concentrations as high as 10^{-5} M showed no growth inhibiting effects even after 72 h of continuous exposure (Bar Graph; Fig. 1). Combining verapamil with various concentrations of ADM or THT generally resulted in an enhanced inhibition of cell growth. An improvement of approximately 80% was observed at all concentrations of THT tested when combined with 10^{-5} verapamil while an increase of approximately 70% was observed with a ADM-verapamil combination (Table 1). In all cases, the effects were significantly greater than those demonstrated by the cytotoxic agent alone, and were proportional to the concentration of the cytotoxic agent utilized. As can be seen in Table 1, the cytotoxic drug concentration which results in an ID_{50} level was much decreased when the agent was combined with a nontoxic dose of verapamil.

FCB cells were resistant to 10^{-4} M THT at all intervals tested, however, simultanous administration of THT and verapamil significantly reduced cell growth at each treatment interval tested (Table 2). Similarly, the addition of verapamil to ADM caused cell growth to fall from 71.9% to 16.5% at the one hour interval. This effect was even more pronounced at longer treatment intervals; with 72 hours of treatment not only showing a decrease in cell growth but exhibited cytotoxic growth characteristics (Table 2).

Verapamil (10^{-5} M) alone exerted little effect on the thymidine labeling index of FCB cells (labeled cells were 95.2% of untreated controls). However, as little as a one

Table 1. Effects on FCB cell growth after 24 h exposures to Adriamycin or thiotepa alone or in combination with verapamil[a]

Concentration	Number of attached cells[b] (% of control)		% cell growth[b, c]	
	THT	THT + VR	THT	THT + VR
10^{-8} M	102.2 ± 6.5	50.4 ± 4.7[d]	103.3 ± 9.8	24.8 ± 7.1[d]
10^{-6} M	89.4 ± 10.9	44.1 ± 3.0[d]	82.1 ± 13.8	15.3 ± 4.6[d]
10^{-4} M	69.7 ± 11.4	40.5 ± 3.3[d]	54.1 ± 17.2	9.8 ± 5.1[d]
	ADM	ADM + VR	ADM	ADM + VR
10^{-9} M	97.7 ± 2.1	44.6 ± 6.7[d]	97.1 ± 2.6	29.9 ± 4.8[d]
10^{-7} M	87.8 ± 10.2	36.4 ± 3.7[d]	84.6 ± 12.9	21.8 ± 7.9[d]
10^{-5} M	60.3 ± 3.5	31.1 ± 3.9[d]	49.7 ± 4.5	12.7 ± 4.9[d]

[a] FCB cultures treated with verapamil at 10^{-5} M

[b] Mean ± S.E. from triplicate experiments

[c] A positive % cell growth represents cellular proliferation with 100% cell growth equating to the growth of the nontreated cultures. A negative % cell growth equates to a loss of cells

[d] Significantly different ($p < 0.05$) from cytotoxic agent alone

Table 2. Effects of Adriamycin (10^{-5} M) and thiotepa (10^{-4} M) alone or in combination with verapamil (10^{-5} M) on cell growth and thymidine incorporation into DNA in cultured FCB cells

Treatment intervals		% Cell growth[a, b]		Number of S-phase cells[a] (% of control)	
Exposure	Drug-free	THT	THT + VR	THT	THT + VR
1 h	71 h	55.0 ± 10.1	−2.9 ± 4.6[c]	110	5.5[c]
6 h	56 h	60.0 ± 26.8	−1.2 ± 15.3[c]	187	10.2[c]
24 h	48 h	52.3 ± 2.0	−0.1 ± 17.0[c]	183	3.4[c]
72 h	0 h	53.8 ± 5.3	−1.9 ± 24.7[c]		
		ADM	ADM + VR	ADM	ADM + VR
1 h	71 h	71.9 ± 24.5	16.5 ± 11.7[c]	160	61[c]
6 h	56 h	55.1 ± 14.5	11.8 ± 16.5[c]	194	82[c]
24 h	48 h	50.0 ± 8.9	5.6 ± 10.6[c]	110	53[c]
72 h	0 h	42.8 ± 14.7	−6.9 ± 14.6[c]		

[a] Mean ± S.E. from triplicate experiments
[b] A positive % cell growth represents cellular proliferation with 100% cell growth equating to the growth of the nontreated cultures. A negative % cell growth equates to a loss of cells
[c] Significantly different ($p < 0.05$) from cytotoxic agent alone

hour of combined verapamil-THT treatment resulted in significant reductions in the number of S-phase cells (Table 2). For ADM, the combined exposure with verapamil also led to a substantial reduction in DNA synthesis even after one hour of treatment. The greater than 100% rate seen in the verapamil-free drug treatment groups represent an initial decrease in the cell number during the treatment period and an effort of the cultures to grow and repair during the drug-free period.

Cytofluorometric data of cells exposed to it either ADM alone or in combination with verapamil, are presented in Table 3. The intracellular ADM concentration after 3 h of exposure was 385 ng/10^6 cells while the combination of ADM and verapamil showed a statistically significant increase (250%) in intracellular ADM.

Table 3. Effect of 3-h treatment with Adrimycin alone or in combination with verapamil on intracellular drug accumulation

Treatment	ng ADM/10^6 Cells[c]
ADM[a]	385 ± 45
ADM + VR[b]	963 ± 185[d]

[a] FCB cultures treated with ADM at 10^{-5} M
[b] FCB cultures treated with ADM (10^{-5} M) and verapamil at 10^{-5} M
[c] Mean ± S.E. from triplicate experiments
[d] Significantly different ($p < 0.05$) from cytotoxic agent alone

Discussion

The evidence presented indicates that verapamil enhances the in vitro efficacy of the chemotherapeutic agents THT and ADM against the murine bladder cancer FCB. The growth inhibiting effects of THT and ADM on FCB cells were significantly increased with simultaneous administration of nontoxic levels of verapamil. In both cases, cell growth was significantly retarded (Table 1 and 2) and the ID_{50} dose was achieved with lower cytotoxic drug concentrations (Table 1) when verapamil was included in the treatment regime for as brief a period as one hour. In addition, thymidine labeling indices, which provides an accurate indicator of chemosensitivity [4, 14, 28], correlated well with cell survival data. These findings corroborate our earlier report that verapamil is effective in enhancing the in vitro response of bladder cancer cells to anticancer drugs [25].

Although 24 to 72 h drug exposures are commonly used to assess in vitro drug responses of monolayer cell cultures, in the present study exposures as brief as a one hour were sufficient to distinguish responding from nonresponding cultures. Data obtained through continuous exposure of long duration tends to reflect direct cytotoxicity rather than a lasting influence on the reproductive potential of the tumor cells. Interestingly, the stem cell assays as described by Hamburger et al. [10] also utilize a one hour treatment period and in contrast to the stem cell assay the laborious process of colony counting was avoided in our procedure, where accurate and rapid quantitation was obtained using a Coulter counter. It should be pointed out that the one hour drug exposure also approximates the duration of treatment for intravesical chemotherapy and that in vitro monolayer test systems resembled the intracavitational growth of the superficial bladder cancer. However,

further testing using models such as transplantable tumors (i.e., FCB or MBT tumors) or primary cultures of human biopsies are necessary, to assess the correlation between in vitro and in vivo responses.

While the mechanism by which verapamil enhances chemotherapeutic efficacy has yet to be elucidated, it would appear to be linked either to the fact that calcium channels are directly involved in regulating entry of chemotherapeutic agents or that their entry into tumor cells is mediated through a change in the intracellular calcium environment. Studies using calcium blockers and calmodulin inhibitors suggest that the mechanism of therapeutic enhancement centers around the level of available intracellular calcium [7, 11, 30, 31]. Although the intracellular drug concentration of THT was not determined in this study, quantitation of intracellular ADM revealed more than a two-fold elevation in ADM levels when verapamil was coadministered. Our data thus support the concept that verapamil alters membrane permeability, thus allowing higher intracellular drug concentrations.

The data shows that verapamil enhances the efficacy of both thiotepa and Adriamycin on primary cultures of bladder tumors. Should this finding also be found to apply to other primary cultures of animal and human origin, verapamil or other calcium antagonists may well be useful in the intravesical treatment of human bladder cancer. Possible advantages could be in improving the efficacy of the standard treatment regimen, reducing the amount of cytotoxic agent administered, or in reducing the duration of treatment in patients for whom prolonged treatment may be detrimental.

A transplantable transitional cell carcinoma such as FCB permits comparison of in vitro and in vivo assessments of chemotherapeutic efficacy. Intravesical implantation of FCB cells using methods established by Shapiro et al. [23] allows in vivo evaluation of combined verapamil chemotherapeutic management of superficial bladder carcinomas. Should our in vitro findings be substantiated by such in vivo trials, then verapamil combined chemotherapy may offer the prospect of an improvement in the management of bladder cancer patients.

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