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Muscarinic Activation Enhances the Anti-proliferative Effect of Paclitaxel in Murine Breast Tumor Cells

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Abstract: Muscarinic acetylcholine receptors (mAChR) are expressed in cells without nervous origin. mAChR are up-regulated in tumor cells and their stimulation can modulate tumor growth. In this work we investigated the ability of mAChR activation to induce tumor cell death. We studied the action of a combination of low doses of the muscarinic agonist carbachol plus paclitaxel, a chemotherapeutic agent frequently used in breast cancer treatment, in terms of effectiveness. Long term treatment with carbachol exerted anti-proliferative actions on LM2 and LM3 murine mammary adenocarcinoma cells, similarly to paclitaxel. The combination of carbachol with paclitaxel at submaximal concentrations, added during 20 h decreased tumor cell proliferation in a more potent manner than each drug added separately. This effect was reverted by the muscarinic antagonist atropine, and was due to a potentiation of tumor cell apoptosis tested by TUNEL assay. This treatment did not affect the proliferation of the non tumorigenic mammary cell line NMuMG. In conclusion, the combination of a muscarinic agonist plus paclitaxel should be tested as a useful therapeutic tool in breast cancer treatment.

Keywords: Apoptosis - breast cancer cells - muscarinic receptors - paclitaxel.

INTRODUCTION

G protein-coupled receptors (GPCR) constitute the largest family of cell surface receptors involved in signal transduction. This family includes among others, muscarinic acetylcholine receptors (mAChR), which can be activated by parasympathetic nervous system firing. In the last years, it has been reported that mAChR, acethylcholine, the enzymes that synthesize and degrade it (choline acetyltransferase and acetylcholinesterase, respectively) and also nicotinic receptors are expressed in non neuronal cells, and constitute the non neuronal cholinergic system [1]. Five subtypes of mAChR have been identified by molecular cloning: M_1-M_5 [2]. mAChR signaling has been extensively reviewed and large amounts of knowledge have been accumulated concerning to their distribution and function in different tissues [2]. These receptors play key physiological roles and changes in their expression and/or function can be involved in several diseases like cancer. We have previously described that cholinergic short-time stimulation with the synthetic agonist carbachol promotes tumor progression in LM2, LM3 and LMM3 tumors [3-5]. Paclitaxel is one of the cytostatic agents most widely used for treatment of breast cancer. Although this drug efficacy has been extensively proved, different tumors, including breast tumors have developed resistance to its usage [6]. For this reason, combinations of low doses of paclitaxel with other chemotherapeutic agents, that increase anti-tumor efficacy and reduce side effects, have been tested [7-9]. It is well known that anti-tumor therapies can inhibit proliferation and/or induce cell death mainly by two mechanisms apoptosis or necrosis. Apoptosis is low flogistic, since intracellular content is not released to the extracellular media, while in necrosis the liberation of intracellular material promotes inflammation that could worsen tumor progression.

Since other authors have postulated that mAChR activation can either stimulate or inhibit cellular growth depending on prior levels of cellular activity [10], in this paper we investigated the ability of a combination of paclitaxel plus carbachol to induce cell death in LM2 and LM3 murine mammary tumors *via* mAChR activation.

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MATERIALS AND METHODS

Cell Culture

Tumor cell lines, LM2 and LM3 derived from two different spontaneous murine mammary tumors (M2 and M3 respectively) were established and characterized in the A.H. Roffo Oncology Institute from the University of Buenos Aires [11-12]. LM2 and LM3 cells were maintained in F12 medium (Gibco, Grand Island, NY) with 3 mM L-glutamine, 80 µg/ml gentamycin, supplemented with 5% heat inactivated fetal bovine serum (FBS) (PAA Laboratories, Pasching, Austria) and were cultured at 37°C in a humidified 5% CO_2 air atmosphere. Serial passages were performed by detaching tumor cells with phosphate buffered saline (PBS) or with 0.25% trypsin and 0.02% ethylenediamine tetraacetic acid (EDTA) in Ca^{++} and Mg^{++} free PBS of confluent monolayers. The non-tumorigenic epithelial cell line derived from murine mammary gland, NMuMG (CRL-1636 ATCC, Manassas, VI) was maintained in F12 medium with 5% FBS and 40 U/ml insulin. Cell viability was evaluated by Trypan blue exclusion test and the absence of mycoplasma was determined by Hoechst method [13].

Cell Proliferation Assay

Cell proliferation was evaluated by using the soluble tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (MTT) colorimetric assay (Cell Titer 96TM Aqueous Non-Radioactive Cell Proliferation Assay) (Promega, Madison, WI). MTT couples to phenazine methosulfate and is reduced to formazan. Cells were seeded in 96-well plates at a density of 10^4 cells per well in triplicate in F12 medium supplemented with 5% FBS and were left to adhere overnight. Subconfluent conditions, about 60%–70%, were chosen to allow detection of maximal growth. Then, cells were deprived of FBS 24 h previous to the assay, to induce quiescence, and were treated with increasing concentrations of the synthetic cholinergic agonist carbachol or paclitaxel during different periods of time alone or in combination during 20 h. Effects were tested in the absence or presence of the non selective muscarinic antagonist, atropine (10^{-8} M). After treatment, medium was replaced by fresh medium free of FBS and cells were cultured during 24 h. Supernatants were discarded, and viable cells were detected by adding 20 µl MTT:PMS (20:1) to each well, and the production of formazan was evaluated by measuring the absorbance at 540 nm with an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Rad Laboratories Inc., Oakland, CA) after 4 h at 37°C.

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Values are mean ± S.E.M. and results were expressed as percentage of inhibition in relation to control (cells without treatment).

Radioligand Binding Assay

Binding assays were performed using the non-selective tritiated muscarinic antagonist quinuclidinyl benzilate ([^{3}H]-QNB) (specific activity: 42 Ci/mmol) (NEN, Life Sci. Prod. Boston, MA). For specific binding assays LM2, LM3 or NMuMG cells were plated in F12 medium with 5% FBS in 24-well plates ($5x10^4$ cells per well). Cells were incubated in a final volume of 200 µl with 1 nM of [^{3}H]-QNB at 25°C for 90 min in the absence or presence of increasing concentrations of atropine or paclitaxel. After 3 washes with 300 µl of F12 medium at 4°C, the reaction was stopped by the addition of NaOH 0.2 M. The content of each well was added to 3 ml of biodegradable scintillation solution (Perkin Elmer Life Science, Turku, Finland). The radioactivity was quantified in a manual counter (Triathler Hidex, Turku, Finland) with efficiency (η) of 50% [3]. Results were expressed as percent of [^{3}H]-QNB bound respect to the radioactive ligand bound in the absence of atropine or paclitaxel (100% of binding). pD2 values were calculated as –log of IC50 expressed in molar concentrations obtained from competition experiments.

Detection of Apoptotic and Necrotic Cells

Apoptosis and necrosis were evaluated by a modified terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay [14]. Briefly, cells were mounted on 3-aminotesopropyltrietoxysilane (Sigma Chemical Co., St Louis, MO) coated slides. Then, cells were incubated with 20 µg/ml proteinase K (Promega, Madison, WI) in PBS for 15 min at room temperature. Endogenous activity was quenched by incubation with 2% H_2O_2 in PBS for 5 min at room temperature. Cells were treated with 10% bovine serum albumine (BSA) for 30 min at room temperature. Then, the biotin-conjugated mouse monoclonal anti-digoxin antibody (Sigma Chemical Co., St Louis, MO) diluted 1:750 in PBS was added at 4ºC, overnight. After rinsing with compensation buffer: 80 mM Tris-HCl, 20 mM Tris (hydroxymethyl)-aminomethane and 0.075% BRIJ 35 (v/v), pH 7.5 for 10 min at room temperature, cells were incubated with a biotinylated rabbit anti-mouse IgG (DAKO, Carpinteria, CA) diluted 1:100, for 45 min at room temperature. Then, cells were washed with compensation buffer for 15 min at room temperature and incubated with peroxidase-labeled streptavidin (DAKO, Carpinteria, CA) diluted 1:100 for 45 min. After washing, cells were incubated for 5 min with 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride / 0.01% H_2O_2. Cells were counterstained with 0.5% methyl green to reveal nuclei, and the slides were observed with an IM35 Carl Zeiss microscope (Oberkochen, Germany) to identify apoptotic and necrotic cells (with or without DNA clusters of dark brown) or live cells (stained in green).

Chemicals

All drugs were purchased from Sigma Chemical Co. (St. Louis, MI) unless otherwise stated. Solutions were prepared fresh daily.

Statistical Analysis

Results were expressed as mean ± S.E.M. of at least five experiments. A GraphPad Prism computer program one-way ANOVA analysis for paired samples was used to determine the significance of differences between mean values in all control and test samples. The analysis was complemented by using a Tukey test to compare among mean values. Differences between means were considered significant if $p<0.05$.

RESULTS

Effect of Carbachol on Cell Proliferation

We have previously demonstrated that LM2 and LM3 cells express the five subtypes of mAChR while NMuMG cells did not [15]. In addition, we observed that the treatment with carbachol, stimulated tumor cell proliferation in a concentration-dependent manner, when it was added during short periods of time ranging from 15 min to 60 min. Here we investigated the action of

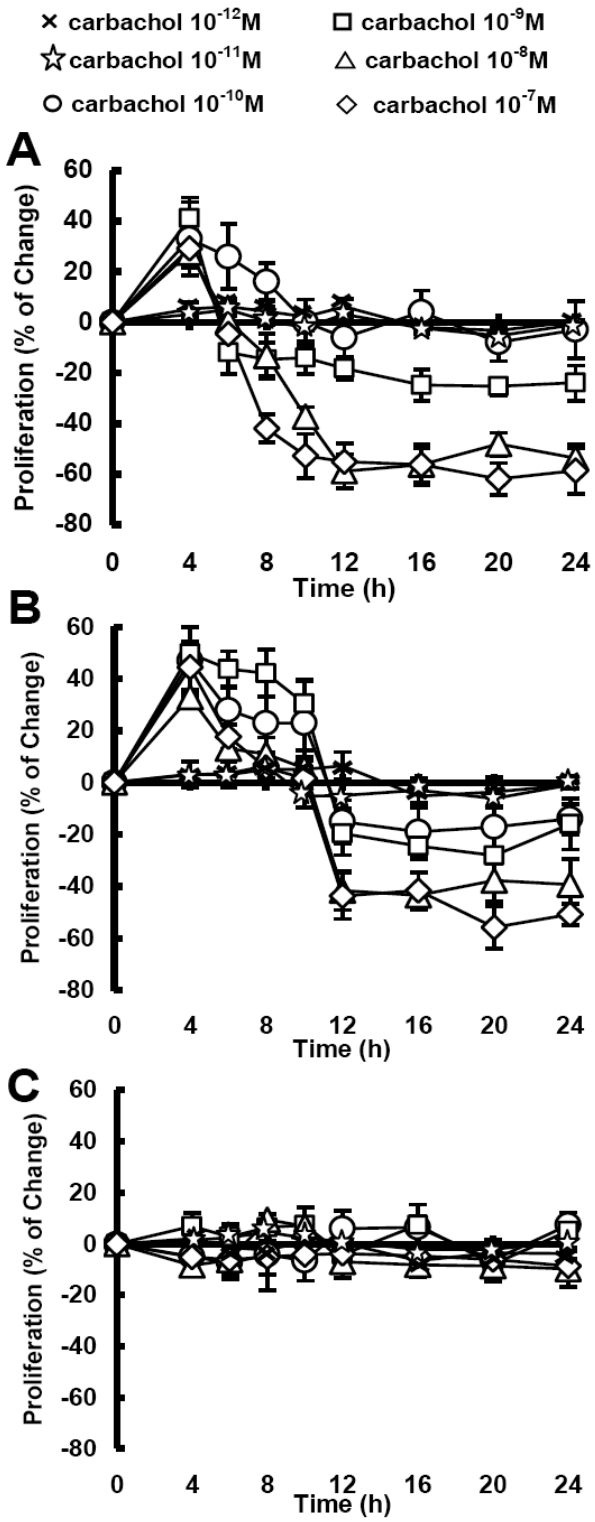

Fig. (1). Effect of carbachol on tumor cell proliferation. Time-response curves of carbachol added at different concentrations to **A**) LM2 cells **B**) LM3 cells and **C**) NMuMG cells. Results were expressed as percent of change respect to control (cells without treatment). Values are mean ± S.E.M. of 6 experiments performed in duplicate.

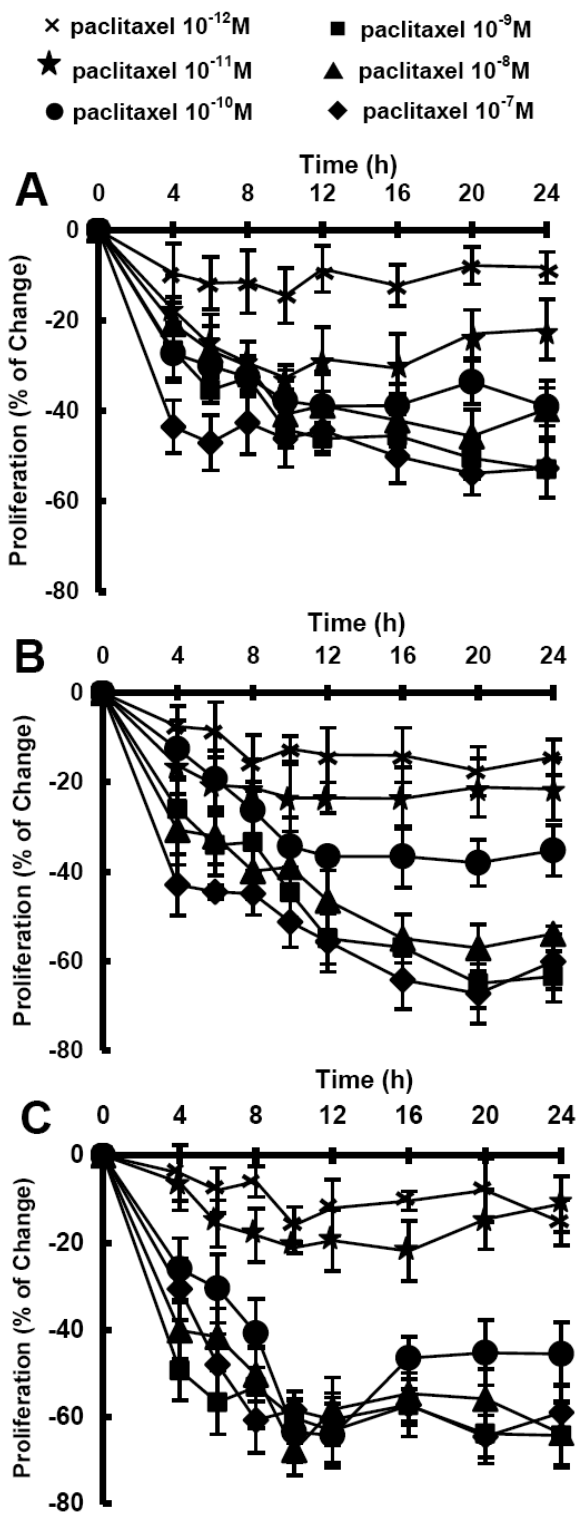

Fig. (2). Effect of paclitaxel on tumor cell proliferation. Time-response curves of paclitaxel added at different concentrations to **A**) LM2 cells **B**) LM3 cells or **C**) NMuMG cells. Results were expressed as percent of change respect to control (cells without treatment). Values are mean ± S.E.M. of 6 experiments performed in duplicate.

carbachol on LM2 and LM3 cell proliferation when it was added for longer periods of time. When carbachol was added to tumor cell cultures for 10 h or less, cell proliferation was observed ($p<0.001$ vs. control: cells without treatment) (Fig. (**1** panels **A** and **B**)). On the contrary, the addition of carbachol for longer periods of time exerted an inhibitory effect on tumor cell proliferation ($p<0.001$ vs. control: cells without treatment) (Fig. (**1** panels **A** and **B**)). The cholinergic agonist reduced LM2 cell proliferation by 62±6%, and by 56±8% LM3 cell proliferation ($p<0.001$ vs. control) (Fig. (**1** panels **A** and **B**)); the maximal effective concentration (CEmax) of the agonist that exerted these effects was 10^{-7} M added during 20 h for both cell types. Carbachol did not alter NMuMG cell proliferation at any of the concentrations used or tested times (Fig. (**1C**)).

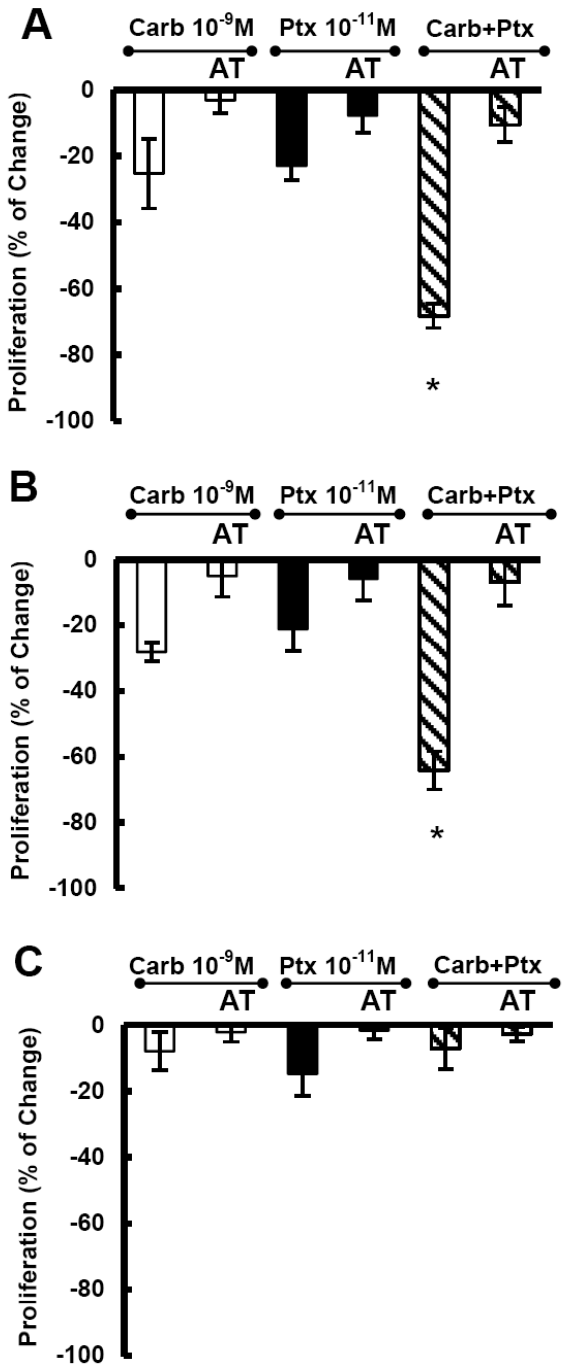

Fig. (3). Effect of carbachol and paclitaxel on tumor cell proliferation. Carbachol (Carb) (10^{-9}M) and paclitaxel (Ptx) (10^{-11}M) were added separately or together for 20 h to **A**) LM2 cells, **B**) LM3 cells or **C**) NMuMG cells in the absence or presence of 10^{-8}M atropine (AT). Results were expressed as percent of change respect to control (cells without treatment). Values are mean ± S.E.M. of 6 experiments performed in duplicate. *$p<0.001$ vs. control.

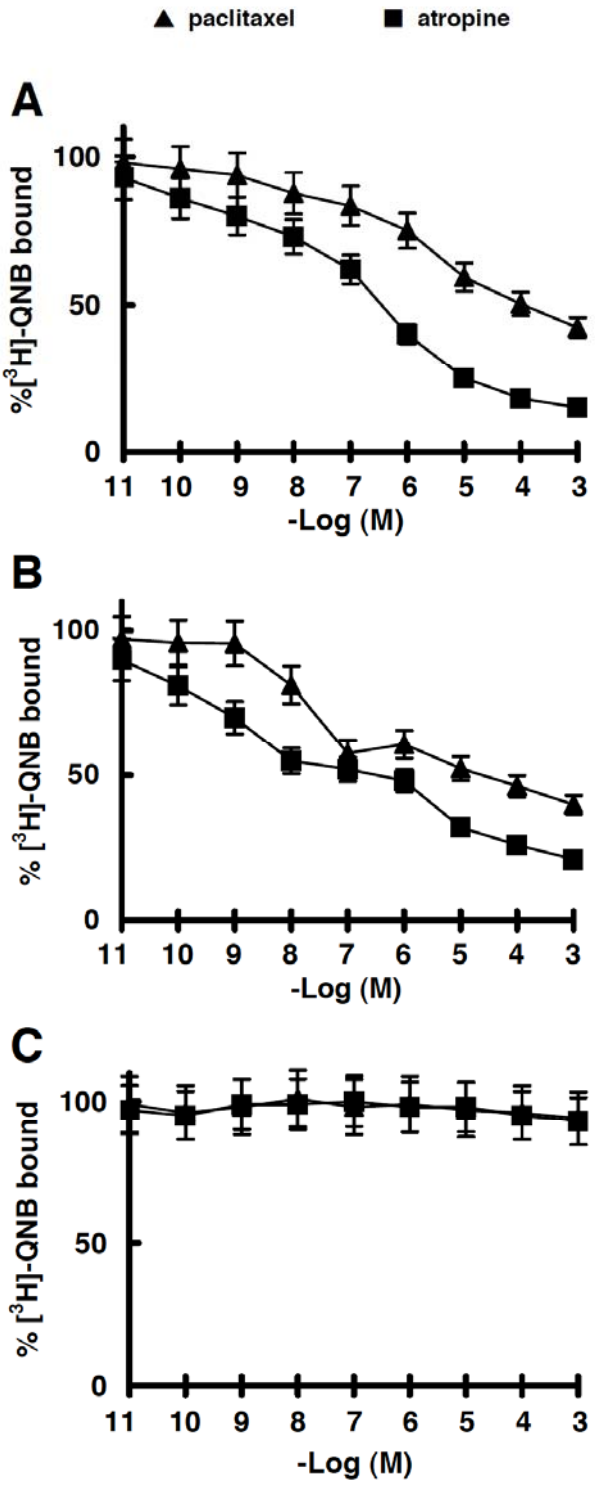

Fig. (4). Competition curves of tritiated quinuclidinyl benzilate ([^{3}H]QNB) binding to tumor cells. [^{3}H]-QNB (1 nM) was added to **A**) LM2 cells, **B**) LM3 cells or **C**) NMuMG cells in the absence (100%) or presence of increasing concentrations of paclitaxel (▲) or atropine (■). Values are mean ± S.E.M. of 5 independent experiments performed in duplicate.

Effect of Paclitaxel on Cell Proliferation

Paclitaxel is a chemotherapeutic agent widely used to treat breast cancer. As shown in Fig. (**2A** and **B**), paclitaxel decreased LM2 and LM3 cell proliferation in a concentration-dependent manner from 10^{-11}M to 10^{-7}M ($p<0.001$ vs. control: cells without treatment). Although, the CEmax of paclitaxel to inhibit proliferation was 10^{-7}M for both tumor cell lines (LM2: -54±4% and LM3: -67±6%) ($p<0.001$ vs. control) when it was added for 20 h, this concentration induced NMuMG cell death in the same magnitude (Fig. (**2C**)). Considering that the optimum conditions for cancer treatment should be the usage of chemotherapeutic agents at concentrations effective to kill tumor cells with minimal effects on normal cells, we used a combination of paclitaxel (10^{-11}M) and carbachol (10^{-9}M) both at submaximal concentrations, added during 20 h. As shown in Fig. (**3**), this combination reduced tumor cell proliferation in a more potent manner than each drug separately added, without affecting non-tumorigenic mammary cells. In LM2 cells carbachol plus paclitaxel decreased proliferation by 68±5% potentiating the action of each drug separately added (carbachol: -25±4%; paclitaxel: -23±5%) ($p<0.001$ carbachol plus paclitaxel vs. carbachol or paclitaxel). Similar effects were observed in LM3 cells (carbachol plus paclitaxel: -64±6%; carbachol:-28±3%; paclitaxel:-21±6%) ($p<0.001$ carbachol plus paclitaxel vs. carbachol or paclitaxel). All these effects were reverted by the pre-incubation of tumor cells with the non selective muscarinic antagonist atropine (10^{-8}M) (Fig. (**3** panels **A** and **B**)). The combination of carbachol plus paclitaxel or each drug added alone did not exert significant actions on NMuMG cell proliferation (carbachol plus paclitaxel: -7±6%; carbachol: -7±5%; paclitaxel: -14±7%) (Fig. (**3C**)).

Taking into account that atropine not only reverted carbachol action but also paclitaxel effect on the inhibition of tumor cell proliferation, we analyzed the ability of paclitaxel to interact with mAChR expressed in LM2 and LM3 cells. We have previously demonstrated that these cells specifically bind [^{3}H]-QNB [3]. Competition binding assays performed with [^{3}H]-QNB indicate that the muscarinic antagonist atropine as well as paclitaxel inhibited the radioligand binding to tumor cells in a concentration-dependent manner (LM2 pD2 atropine: 6.53, pD2 paclitaxel: 4.00; LM3 pD2 atropine: 6.77, pD2 paclitaxel: 4.95) (Fig. (**4**)).

Considering that the type of cell death may influence cancer patient response to repeated chemotherapy treatment, we investigated the induction of apoptosis/necrosis triggered by: carbachol, paclitaxel or the combination of both drugs, by TUNEL assay. As shown in Fig. (**5**), the combination of 10^{-9}M carbachol with paclitaxel (10^{-11}M) added for 20 h potentiated apoptotic response either in LM2 or in LM3 cells expressed as percent of change (control: LM2: 3.1±3.5; LM3: 4.5±2.7; carbachol plus paclitaxel: LM2: 48.7±6.1; LM3: 47.4±4.9) in comparison to each drug added alone (LM2: carbachol: 15.2±1.8, paclitaxel: 13.1±1.4; LM3: carbachol: 18.3±3.1, paclitaxel: 14.0±3.4) ($p<0.001$ carbachol plus paclitaxel vs. carbachol or paclitaxel). These effects were reverted by the pre-incubation of cells with atropine (10^{-8}M) (Fig. (**5** panels **A** and **B** left)). The combination of paclitaxel plus carbachol produced an additive effect on the stimulation of tumor cell necrosis (LM2: control: 1.9±2.2; carbachol plus paclitaxel: 22.1±3.1; carbachol: 10.1±2.2; paclitaxel: 9.9±1.5. LM3: control: 0.5±0.7; carbachol plus paclitaxel: 18.8±2.9; carbachol: 9.9±2.5; paclitaxel: 7.1±2.6). Atropine also prevented this action (Fig. (**5** panels **A** and **B** right)). The addition of carbachol, paclitaxel or the combination of paclitaxel plus carbachol to NMuMG cells did not modify apoptosis or necrosis in a significant manner (Fig. (**5C**)).

DISCUSSION

GPCR is the largest family of membrane receptors involved in several important biological and pathological functions. mAChR are included in this family. Over the last years, there are several evidences that indicate that the components of the cholinergic system are present in cells without nervous origin, and it was named the non neuronal cholinergic system [1]. Its role in the regulation of important cell functions like mitosis, cell morphology, locomotion, and immune response which are key steps during

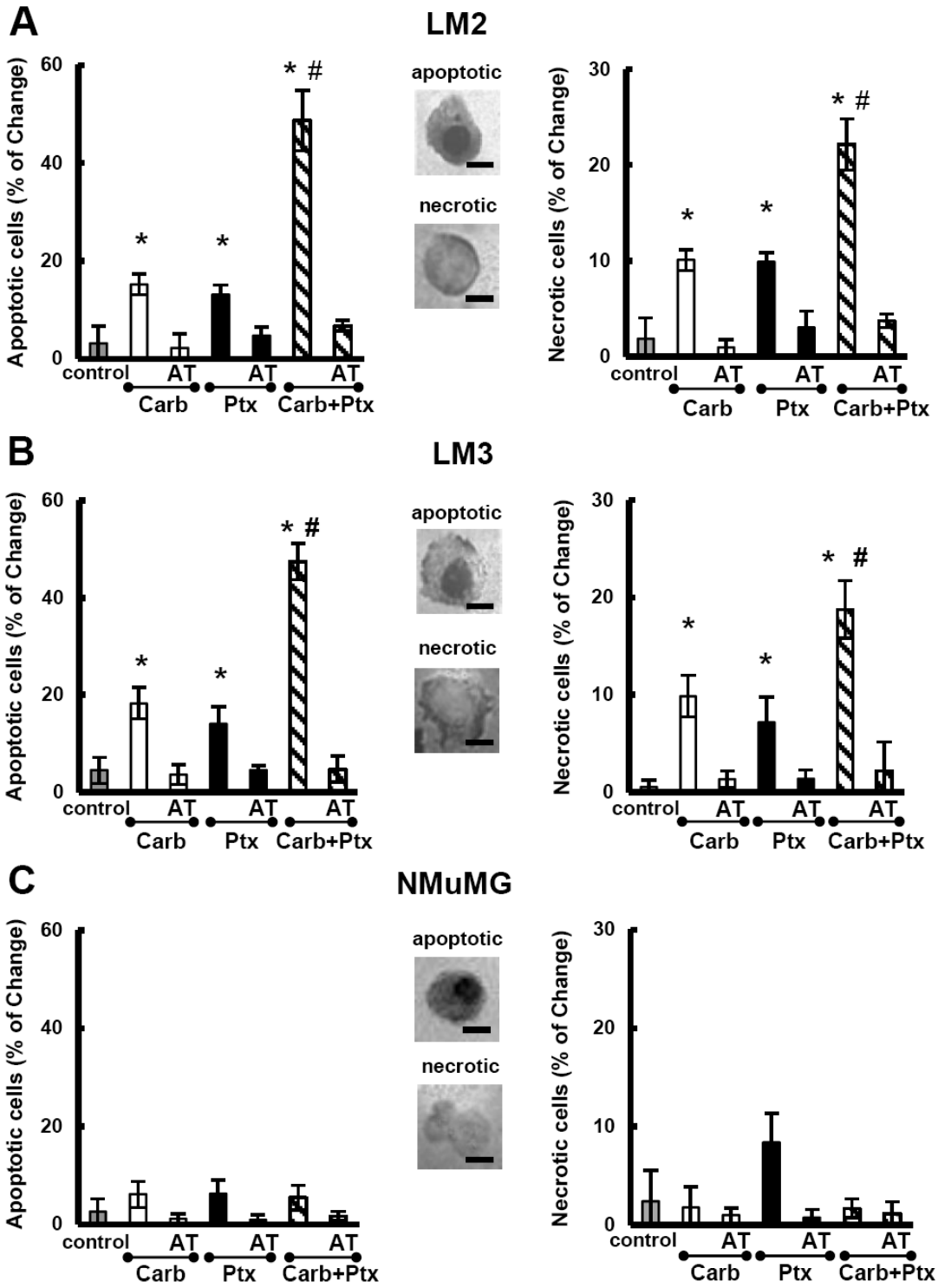

Fig. (5). Effect of carbachol and paclitaxel on tumor cell apoptosis/necrosis by a modified terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay. Carbachol (Carb) (10^{-9}M) or paclitaxel (Ptx) (10^{-11}M) were added separately or together for 20 h in the absence or presence of 10^{-8}M atropine (AT). Values are mean ± S.E.M. of 5 experiments performed in duplicate. * $p<0.001$ vs. control; #$p<0.001$ vs. Carb or Ptx. Microscopic images of apoptotic/necrotic LM2, LM3 and NMuMG cells are shown (Scale bar = 10 μm).

tumor progression, have been documented [16]. We have previously demonstrated that LM2, LM3 and LMM3 cells derived from different murine mammary adenocarcinomas express mAChR [3-5]. The stimulation of these cells with carbachol for short periods of time increased proliferation. However, it is known that the activation of these receptors can up-regulate or down-regulate cell proliferation depending on the context of cellular growth [17]. Here we demonstrate that long term stimulation of LM2 and LM3 tumors with carbachol decreased cell proliferation. Similar results were obtained in different small cell lung carcinomas [18-19]. In these cells, the activation of mAChR with carbachol generated anti-proliferative signals, measured as [^{3}H]-thymidine incorporation after 4 h of incubation, by modulating cadherin-mediated adhesion. These results should add to mAChR therapeutic target properties.

Paclitaxel belongs to taxanes, which are part of a major group of anti-cancer drugs that stabilize microtubules. These structures are composed of tubulin polymers in dynamic equilibrium with tubulin heterodimers consisting of alpha and beta subunits. Their main function is the spindle mitotic formation during cell division, but it is also related to motility, signal transduction and intracellular transport [20]. Unlike other chemotherapeutic drugs that also act on microtubules depolymerization, paclitaxel promotes permanent tubulin polymerization [21]. This effect inhibits cell proliferation in a concentration-dependent manner. Clinical effective concentrations of paclitaxel, that are reported to be near to 10^{-7}M, also produce side effects associated with hypersensitivity reactions, myelosuppression, bradycardia, hypotension, peripheral neuropathy, myalgias, arthralgias, nausea, diarrhea, mucositis and alopecia [22]. Also, subclinical low concentrations (<9 nM) and long period treatment (18 h) with paclitaxel induces apoptosis in A549 cells [23]. These results are in line with ours which evidence pro-apoptotic actions of paclitaxel when suboptimal concentrations were added to LM2 and LM3 cell cultures.

Interestingly, we reported a potentiation of tumor cell death *via* apoptosis induced by the combination of a suboptimal concentration of carbachol plus paclitaxel. The activation of similar signal transduction pathways could be the cause of the potentiation in apoptosis. It has been previously reported that mAChR stimulation with carbachol promotes apoptosis of SH-SY5Y cells by up-regulating the expression of Bcl-2 through the Ras-ERK½ pathway [24]. A similar mechanism which triggers apoptosis by Bcl-2 phosphorylation *via* inactivation of the kinase that phosphorylates and activates MEK/ERK and protein kinase C alpha/beta pathways was described for docetaxel and paclitaxel in H460 human non-small cell lung cancer [25].

Surprisingly, paclitaxel actions on proliferation and apoptosis were reverted by atropine, suggesting an interaction between the cytostatic agent and mAChR. Moreover, paclitaxel shifted in a dose-dependent manner the binding of muscarinic radioligand to LM2 and LM3 cells that express mAChR and not to NMuMG cells that do not express these receptors. McKay *et al*. [26] demonstrated that several microtubule-active drugs block cholinergically mediated catecholamine secretion from adrenal chromaffin cells. They studied interactions of these agents with nicotinic acetylcholine receptor-ion channel complexes using radiolabeled probes for receptor, and described that among others, paclitaxel inhibited radioligand binding to the receptor-gated ion channel. Similar interactions could be occurring between mAChR and paclitaxel.

The combination of cytostatic drugs with coadyuvant agents is common in cancer treatment. Here we reported that the combination of paclitaxel with a muscarinic agonist could be advantageous to decrease cytostatic drug doses and its side effects on normal cells. On the other hand, the ability of chemotherapeutic agents to stimulate apoptosis in tumor bearers exerts immunostimulatory actions as it was previously described, improving the response against malignant neoplasms [27].

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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ABBREVIATIONS

[^{3}H]-QNB	=	quinuclidinyl benzilate tritiated
BSA	=	bovine serum albumine
EDTA	=	ethylenediamine tetraacetic acid
ELISA	=	enzyme-linked immunosorbent assay
FBS	=	fetal bovine serum
GPCR	=	G protein-coupled receptors
mAChR	=	muscarinic acetylcholine receptors
MEK	=	mitogen-activated protein kinase
MTT	=	tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]
PBS	=	phosphate buffered saline
PKC	=	protein kinase C
TUNEL	=	terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling technique

REFERENCES

[1] Wessler, I.; Kirkpatrick, C.J.; Racke, K. The cholinergic 'pitfall': acetylcholine, a universal cell molecule in biological systems, including humans. *Clin. Exp. Pharmacol. Physiol.*, **1999**, *26*(3), 198-205.

[2] Eglen, R.M. Muscarinic receptor subtypes in neuronal and non-neuronal cholinergic function. *Auton. Autacoid. Pharmacol.*, **2006**, *26*(3), 219-233.

[3] Español, A.; Eiján, A.; Mazzoni, E.; Davel, L.; Jasnis, M.; Sacerdote de Lustig, E.; Sales, M. Nitric oxide synthase, arginase and cyclo-oxygenase are involved in muscarinic receptor activation in different murine mammary adenocarcinoma cell lines. *Int. J. Mol. Med.*, **2002**, *9*(6), 651-657.

[4] Español, A.; de la Torre, E.; Fisman, G.; Sales, M. Role of nonneuronal cholinergic system in breast cancer progression. *Life Sci.*, **2007**, *80*(24-25), 2281-2285.

[5] Rimmaudo, L.; de la Torre, E.; Sacerdote de Lustig, E.; Sales, M.E. Muscarinic receptors are involved in LMM3 tumor cells proliferation and angiogenesis. *Biochem. Biophys. Res. Commun.*, **2005**, *334*(4), 1359-1364.

[6] Wang, S.; Huang, X.; Lee, C.K.; Liu, B. Elevated expression of erbB3 confers paclitaxel resistance in erbB2-overexpressing breast cancer cells *via* upregulation of Survivin. *Oncogene*, **2010**, *29*(29), 4225-4236.

[7] Schäfer, H.; Dieckmann, C.; Korniienko, O.; Moldenhauer, G.; Kiefel, H.; Salnikov, A.; Krüger, A.; Altevogt, P.; Sebens, S. Combined treatment of L1CAM antibodies and cytostatic drugs improve the therapeutic response of pancreatic and ovarian carcinoma. *Cancer Lett.*, **2011**, *319*(1), 66-82.

[8] Belfiglio, M.; Fanizza, C.; Tinari, N.; Ficorella, C.; Iacobelli, S.; Natoli, C. Meta-analysis of phase III trials of docetaxel alone or in combination with chemotherapy in metastatic breast cancer. *J. Cancer Res. Clin. Oncol.*, **2012**, *138*(2), 221-229.

[9] Croom, K.; Dhillon, S. Bevacizumab: a review of its use in combination with paclitaxel or capecitabine as first-line therapy for HER2-negative metastatic breast cancer. *Drugs*, **2011**, *71*(16), 2213-2229.

[10] Lanzafame, A.; Christopoulos, A.; Mitchelson, F. Cellular signaling mechanisms for muscarinic acetylcholine receptors. *Receptors Channels*, **2003**, *9*(4), 241-260.

[11] Galli, S.; Colombo, L.; Vanzuli, S.; Daroqui, M.; Vidal, M.; Jasnis, M.; Sacerdote de Lustig, E.; Eiján, A. Characterization of fibroblastoid mammary carcinoma cell line (LM2) originated from a mouse adenocarcinoma. *Int. J. Oncol.*, **2000**, *17*(6), 1259-1265.

[12] Urtreger, A.; Laeda, V.E.; Puricelli, L.; Rivelli, A.; Vidal, M.C.; Sacerdote de Lustig, E.; Bal de Kier Joffé, E.D. Modulation of fibronectin expression and proteolytic activity associated with the invasive and metastasic phenotype in two murine mammary tumor cell lines. *Int. J. Oncol.*, **1997**, *11*(3), 489-496.

[13] Chen, T. In situ detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. *Exp. Cell Res.*, **1997**, *104*(2), 255-262.

[14] Cuello-Carrión, F.; Ciocca, D. Improved detection of apoptotic cells using a modified in situ TUNEL technique. *J. Histochem. Cytochem.*, **1999**, *47*(6), 837-839.

[15] Español, A.; Sales, M. Different muscarinc receptors are involved in the proliferation of murine mammary adenocarcinoma cell lines. *Int. J. Mol. Med*., **2004**, *13*(2), 311-317.

[16] Paleari, L.; Grozio, A.; Cesario, A.; Russo, P. The cholinergic system and cancer. *Semin. Cancer Biol*., **2008**, *18*(3), 211-217.

[17] Nicke, B.; Detjen, K.; Logsdon, C. Muscarinic cholinergic receptors activate both inhibitory and stimulatory growth mechanisms in NIH3T3 cells. *J. Biol. Chem.*, **1999**, *274*(31), 21701-21706.

[18] Williams, C.; Lennon, V. Activation of muscarinic acetylcholine receptors inhibits cell cycle progression in small cell lung carcinoma. *Mol. Biol. Cell*, **1991**, *2*(5), 373-382.

[19] Williams, C. Muscarinic signaling in carcinoma cells. *Life Sci.*, **2003**, *72*(18-19), 2173-2182.

[20] Rowinsky, E.; Cazenave, L.; Donehower, R. Taxol: a novel investigational antimicrotubule agent. *J. Natl. Cancer Inst.*, **1990**, *82*(15), 1247-1259.

[21] Sun, X.; Li, D.; Yang, Y.; Ren, Y.; Li, J.; Wang, Z.; Dong, B.; Liu, M.; Zhou, J. Microtubule-binding protein CLIP-170 is a mediator of paclitaxel sensitivity. *J. Pathol.*, **2012**, *226*(4), 666-673.

[22] Marupudi, N.; Han, J.; Li, K.; Renard, V.; Tyler, B.; Brem, H. Pacliatxel: a review of adverse toxicities and novel delivery strategies. *Expert Opin. Drug Saf.*, **2007**, *6*(5), 609-621.

[23] Torres, K.; Horwitz, S. Mechanisms of taxol-induced cell death are concentration dependent. *Cancer Res.*, **1998**, *58*(16), 3620-3626.

[24] Li, J.; Jing, Z.; Yi, H. Effects of lithium on the activity of ERK-1/2 signal pathway and expression of Bcl-2 family protein in the central nervous system *in vivo*. *Huan. Yi Kr Da Xue Xue Bao*, **2008**, *28*(4), 330-334.

[25] Hu, Y.; Bally, M.; Dragowska, W.H.; Mayer, L. Inhibition of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase enhances chemotherapeutic effects on H460 human non-small cell lung cancer cells through activation of apoptosis. *Mol. Cancer Ther*., **2003**, *2*(7), 641-649.

[26] McKay, D.; Aronstam, R.S.; Schneider, A.S. Interactions of microtubule-active agents with nicotinic acetylcholine receptors. Relationship to their inhibition of catecholamine secretion by adrenal chromaffin cells. *Mol. Pharmacol*., **1985**, *28*(1), 10-16.

[27] Fuentes, D.; Avellanet, J.; Garcia, A.; Iglesias, N.; Gabri, M.R.; Alonso, D.F.; Vazquez, A.M.; Perez, R.; Montero, E. Combined therapeutic effect of a monoclonal anti-idiotype tumor vaccine against NeuGc-containing gangliosides with chemotherapy in a breast carcinoma model. *Breast Cancer Res. Treat.*, **2010**, *120*(2), 379-389.