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Muscarinic Receptors as Targets for Metronomic Therapy in Breast Cancer

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Abstract: ***Background:*** It is actually known that acetylcholine works as a signaling molecule in non-neuronal cells and tissues, in addition to its neuronal function as neurotransmitter. It can act on two types of receptors nicotinic and muscarinic receptors (mAChRs). The latter belong to the G protein coupled receptor family and there are five subtypes genetically cloned. Their activation triggers classical and non-classical intracellular signals that could be linked to the proliferation of normal and/or transformed cells. The M_3 subtype was identified in different types of tumors and its stimulation with agonists triggers cell proliferation, migration, invasion and metastasis. ***Results***: Our laboratory has extensively investigated the expression and function of mAChRs in breast tumors from animal and human origins. We found a profuse expression of mAChRs in breast tumors, but opposite to this, an absence of these receptors in normal breast cells and tissues. The stimulation of mAChRs with the cholinergic agonist carbachol for 20 h increased tumor cell death. Moreover, the combination of subthreshold concentrations of the agonist with paclitaxel potentiates cell death. The usage of low dose chemotherapy with short drug free intervals was named metronomic therapy and it has emerged as a novel regimen for cancer treatment with very low incidence of side effects. ***Conclusion:*** Our work and that of others indicate that mAChRs that are over-expressed in different types of tumor cells could be a useful target for metronomic therapy in cancer treatment.

Keywords: Acetylcholine, muscarinic receptors, signal transduction pathways, tumor progression, breast cancer, metronomic therapy.

EXPRESSION AND FUNCTION OF MUSCARINIC RECEPTORS

It has been proved that the cholinergic system evolved before neurons and, when more complex animals appeared, it was later "utilized" also by neurons [1, 2]. In fact, the cholinergic system had been created by nature about 2.5 billion years before the first appearance of the neuronal tissue. It is probable that acetylcholine (ACh) exists since then, because acetylation of organic molecules like choline, is one of the most common reactions in nature. There is increasing experimental evidence that ACh is widely expressed in pro- and eukaryotic non-neuronal cells. The latter can be proved by the fact that ACh is present in bacteria, blue-green algae, yeast, fungi, protozoa and primitive plants [3]. Thus, ACh works as a signaling molecule in non-neuronal cells and tissues, before its neuronal function spans. For these reasons, Wessler *et al.* have introduced the term "non-neuronal ACh" and "non-neuronal cholinergic system" to underline the presence of ACh in cells independent of neurons or present in organisms free of neuronal tissue during the evolutionary process [3]. In turn, Grando and colleagues applied for the first time the term "universal cytotransmitter", which denotes the involvement of ACh in the regulation of basic and nervous-independent cell functions such as proliferation, differentiation, organization of the cytoskeleton, local release of mediators (*e.g.*, nitric oxide and pro-inflammatory cytokines), locomotion, secretion and ciliary activity [4]. ACh released in the neuronal or in the non-neuronal cholinergic system can activate two types of receptors known as ionotropic and metabotropic receptors. Nicotinic receptors (nAChRs) belong to the family of ionotropic or channel receptors with a molecular mass ~290 kDa. Neuronal nAChRs are integral membrane proteins with a pentameric structure, conformed by different α subunits (α2-10), and β subunits (β2-4) surrounding the cationic channel [5]. Because of the combination among α and/or β subunits several subtypes of homomeric [*e.g.*, $(\alpha7)_5$; $(\alpha9)_5$] or heteromeric [*e.g.*, $(\alpha4)_2(\beta2)_3$; etc.] nAChRs can be detected. Each subtype has different properties regarding the affinities to agonists and antagonists, agonists' potencies, and physiological or pathological functions linked to their activation/inhibition/desensitization [6].

Metabotropic cholinergic receptors were named muscarinic receptors (mAChRs) due to their ability to bind the natural agonist muscarine besides ACh. mAChRs belong to the family of G-protein coupled receptors with seven transmembrane domains. Five different subtypes have been genetically identified: M_1-M_5 [7]. In airways and lung tissue of most mammals including humans, the expression of muscarinic M_1, M_2 and M_3 receptors have been described using different techniques such as binding studies with subtype selective radioligands, molecular biological and immune-biological techniques, and these findings correlate very well with a large number of functional pharmacological studies. M_1 receptors appear to be expressed particularly in peripheral lung tissue and alveolar wall, but could not be detected in larger airways, skin, intestine and glands. M_2 and M_3 receptors represent the major population of mAChRs in smooth muscle fibers, human macrophages from airways and sclera fibroblasts [8, 9]. M_4 and M_5 receptors were described and characterized later than M_1-M_3, and are predominantly expressed in the central nervous system. In urothelium, endothelial and immune cells that mediate inflammatory reactions, the five subtypes of mAChRs have been identified [8]. The coupling of mAChRs to their cellular effector systems is mediated via heterotrimeric G-proteins that are composed of α-, β- and γ-subunits and classified in virtue of their α–subunit subtypes in four families: Gαs, Gαi/o, Gαq and Gα12 [10, 11]. Receptor activation results in dissociation of the heterotrimeric G- protein into its α-and β/γ-subunits. The latter are tightly bound and display one functional unity. Both the α-subunit and the β/γ-subunits are involved in the transduction of muscarinic signals coupling similar or different effectors [12] (Fig. **1**).

It has been extensively documented that, M_1, M_3 and M_5 receptors couple preferentially to Gq proteins, whereas M_2 and M_4 subtypes interact with the Gi/o family of proteins. One important target activated by Gαq represents phospholipase C (PLC) isoenzymes

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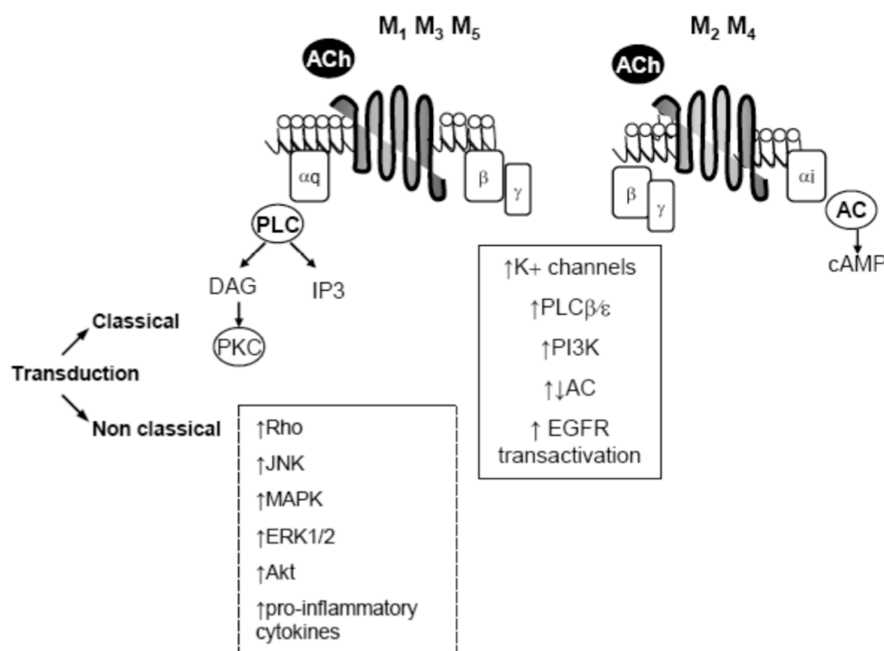

Fig. (1). Mechanism of transduction for different muscarinic acetylcholine receptor (M) subtypes. The binding of acetylcholine (ACh) to different M subtypes leads to the dissociation of G-protein into α and βγ subunits that can activate different effectors. Phospholipase C (PLC); inositol triphosphate (IP3); diacylglycerol (DAG); protein kinase C (PKC); adenylyl cyclase (AC); cyclic adenosine monophosphate (cAMP); phosphoinositide 3-kinase (PI3K); janus kinase (JNK); mitogen activated protein kinase (MAPK); extracellular kinase (ERK); epidermal growth factor receptor (EGFR).

which mediate the hydrolysis of phosphatidylinositol 4, 5-bisphosphate to generate inositol 1, 4, 5-trisphosphate (IP3) and diacylglycerol. IP3 is able to bind to IP3 receptors in the endoplasmic reticulum and triggers the liberation of calcium to the cytosol that can in turn activate several effector enzymes as nitric oxide synthase (NOS). Burstein *et al.* [13] demonstrated that the overexpression of Gαq induces constitutive activation of compatible mAChRs, and that this activity is blocked by muscarinic antagonists. Gαq also increases the potency and efficacy of agonists. These results indicate that regulation of G-protein levels has a profound impact on receptor control of cellular physiology, even in the absence of agonist ligands. Gαi/o proteins inhibit adenylyl cyclase (AC) activity, and reduce calcium channels opening [7]. In turn, Gβγ can activate or inhibit AC, can increase the activity of PLCβ2 or ε isoforms and phosphoinositide-3-kinase (PI3K) as well as the opening of potassium channels. Furthermore, the activation of mAChRs induces transactivation of the epidermal growth factor receptor (EGFR) through βγ -subunits that promote Src-mediated matrix metalloproteinase (MMP)-dependent cleavage and release of EGFR ligands from the cell surface and binding to EGFR and activation of extracellular signal-regulated kinases (ERK)1/2 [14].

mAChRs also regulate a diverse array of signaling intermediates that were not considered as classical either through both Gα or Gβγ members exerting cytoskeleton effects through the activation of small GTPase Rho, and downstream effectors that include non-receptor tyrosine kinases and mitogen-activated protein kinases (MAPK) [11].

MUSCARINIC RECEPTORS IN CANCER

The activation of the Gq-linked receptors M_1, M_3 and M_5 leads to an increment in cell proliferation. The majority of cancers derived from epithelial and endothelial cells express mAChRs. Moreover, many of these cancers also secrete ACh which stimulates cell growth, and for this reason ACh is considered as an autocrine growth factor. It is important to note that, the levels of ACh can be regulated by the enzymes choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) that are also present and active in tumor tissues [15].

For those tumors that do not synthesize ACh, it may be derived from neuronal, endocrine or paracrine sources, and constitutively activates tumor mAChRs and also nAChRs. To a large extent, the expression of mAChRs by malignant tissues follows the expression of the same receptors in the corresponding normal tissue, but patterns of both mAChRs expression and ACh synthesis can change between normal tissues and tumors.

As it is shown in Table **1**, M_3 receptors are expressed in various tumors from different origin. Moreover, these receptors are involved in tumor cell proliferation or invasion during tumor progression, through the activation of distinct signaling pathways. The expression of M_2/M_3 receptors has been detected in head and neck carcinomas, which arise in the mucosal layer of the upper aerodigestive tract. Nearly 90% of head and neck carcinomas are assigned to squamous cell carcinoma. The activation of mAChRs triggers the enzymatic activity of PLC, and as a consequence, IP3 formation [16, 17]. In lung and gastric cancers, the M_3 receptor has been extensively assigned as responsible of tumorigenesis [18, 19]. Muscarinic agonists cause concentration-dependent increases in intracellular calcium and MAPK/Akt phosphorylation in small cell lung carcinoma cell lines [18]. M_3 receptors are also involved in the proliferation of colon cancer cells. Using quantitative real-time PCR, it has been demonstrated that H508, WiDr, and Caco-2 human colon cancer cell lines, express ChAT, that H508 and Caco-2 cells release ACh into cell culture media, and that muscarinic antagonists inhibit proliferation of unstimulated H508 cells by about 40% [20]. In addition, the activation of M_3 receptors in H508 and HT-29 human colon cancer cells triggers a complex mechanism mediated by the stimulation of MMP-7 that cleaves heparin binding epidermal growth factor (HB-EGF) from Pro-HB-EGF, and transactivation of EGFR resulting in intracellular signaling via both the mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK)/ERK and PI3K/Akt signaling pathways. These

Table 1. Subtypes and functions of mAChR in different types of tumors.

Tumor type	mAChR subtype	Function	Signaling pathway	Refs.
Head and neck	M_2, M_3	Cell proliferation	PLC/IP3	[16, 17]
Lung	M_3	Cell proliferation	MAPK/Akt	[18]
Gastric	M_3	Cell proliferation	MAPK	[19]
Colon	M_3	Cell proliferation	Transactivation of EGFR (ERK/PI3K/Akt)	[20]
Skin	M_3	Invasion, migration, metastasis	Calcium mobilization	[21-23]
Brain	M_2 /M_3	Cell proliferation	PLC/PKC/Akt PKC/MAPK	[24-26]
Prostate	M_1/M_3	Cell proliferation	CaM KK/Akt	[27, 28]
Cervix	M_1/M_3/M_4	Cell migration	ERK1/2	[29]

events result in cell proliferation and cell survival (inhibition of apoptosis), both hallmarks of neoplasia [20].

Regarding melanoma, the most serious type of skin cancer, it has been reported that M_1, M_3 and M_5 receptor subtypes are expressed either in tumor tissue or in tumor cell lines from human origin. It was documented that SK-Mel 28 cells express a large number of M_3 receptors and a small amount of the M_5 receptor subtype. Microscopic observation of calcium mobilization after muscarinic stimulation and a standardized chemotaxis assay indicated that cells carried functional mAChRs that induce movement by approximately 30%, an effect abrogated by atropine [21-23].

In brain tumors, mainly astrocytomas, M_2 and M_3 receptors have been identified. Proliferation experiments with subtype-specific mAChR antagonists suggest that carbachol-induced proliferation is due to the activation of M_3 receptors via the PLC/protein kinase C (PKC) pathway also followed by the phosphorylation of Akt [24, 25]. As it is well known, MAPK can be phosphorylated by mitogens binding to G-protein coupled receptors and is considered a major pathway involved in cell proliferation. Yagle *et al*. [26] reported the activation of MAPK coupled to mAChRs in astroglial cells 1321N1 derived from a human astrocytoma. Carbachol caused a rapid and transient phosphorylation of MAPK, particularly ERK1/2, with an increment in its activity, without changing protein levels.

In addition, M_3 receptors are also involved in cell proliferation in prostate and cervix tumors [27, 28]. Their activation conducts to the stimulation of calcium/calmodulin-dependent protein kinase kinase (CaM KK)/Akt and ERK1/2 respectively [29].

MUSCARINIC RECEPTORS IN BREAST CANCER

Our group had previously reported the expression of mAChRs in two different mammary adenocarcinomas named LM2 and LM3 that spontaneously aroused in BALB/c mice. We demonstrated, by competitive binding experiments with the tritiated muscarinic antagonist quinuclidinyl benzilate ([3H]-QNB) that the M_2 subtype predominates in both tumor cell lines. Concordantly, immunoblotting assays indicated that mAChRs exhibit the following order of expression: M_2>M_4>M_3>M_1>>M_5. The activation of mAChRs with carbachol increased proliferation in both cell lines but through different signaling pathways. In LM3 cells, carbachol promoted proliferation via M_3 receptor activation with IP3 and nitric oxide (NO) production. While in LM2 cells, carbachol stimulated proliferation via M_2 and M_1 receptors activation, triggering prostaglandin E_2 liberation and arginase catabolism respectively, both of them involved in tumor cell growth [30, 31]. It is important to note that unlike other tumor types that share mAChRs expression with normal cells of the same strain, cells derived from normal murine mammary gland (NMuMG) did not exhibit mAChRs expression at all. In addition, binding experiments indicated that mAChRs are dramatically over-expressed (40-fold) in LMM3 cells derived from a metastasis of LM3 cells [32]. The order of potency of different muscarinic antagonists to displace [3H]-QNB binding indicates the predominance of M_3 receptor subtype in LMM3 cells. The expression of this receptor was also confirmed by Western blot. The difference in the number of mAChRs between LMM3 and LM3 cells correlates to the difference in their maximal proliferative responses (54% vs. 30% respectively), and in the concentrations of carbachol that produced those responses (10^{-9}M vs. 10^{-7} M) in both cell lines.

In addition, we observed that the stimulatory action of carbachol on LMM3 cell proliferation was mainly due to M_3 receptor activation because the agonist action was totally blunted not only by atropine but also by the M_3 selective antagonist, para-fluoro hexahydro sila-difenidol [32]. These results, together with others obtained by distinct groups pointed to muscarinic antagonists as potential therapeutic tools in cancer [33]. However, the administration of muscarinic antagonists via systemic route frequently exhibits side effects, mainly cardiac complications as it is observed in the treatment of overactive bladder [34].

Breast cancer is the most frequent class of tumor in women and is one of the first causes of death by this illness among them. For this reason, we analyzed the expression of mAChRs in samples surgically obtained from patients with human breast tumors corresponding to different histological grades [35]. The analysis performed by Western blot using specific anti-mAChRs antibodies in homogenates of breast tumors revealed a major expression of M_2 and M_3 subtypes and the growing intensity of the bands correlated with the malignant and invasive breast carcinomas. Patients with benign pathology (*i.e.* fibroadenoma) exhibited low expression of mAChRs. We also confirmed the absence of mAChRs in normal breast tissue [35]. In addition, we provided direct evidence on the constitutive expression of functional mAChRs in MCF-7 cells, derived from a luminal human breast carcinoma estrogen-dependent that represents the most frequent type of tumor in women [36]. We demonstrated for the first time, that MCF-7 cells express mainly M_3 and M_4 receptors. We also confirmed the absence of mAChRs in the non-tumorigenic mammary cell line MCF-10A [36].

The activation of these receptors with carbachol promoted MCF-7 cell proliferation mainly via the M_3 receptor subtype through the stimulation of PLC/PKC/calcium dependent NOS1 and NOS3 expressed in tumor cells. Jiménez and Montiel [37] had previously reported that muscarinic stimulation with carbachol in MCF-7 cells induced an increase in protein synthesis and cell pro-

liferation, and these effects were prevented by PD098059, a specific inhibitor of MAPK kinase (MAPKK). They also demonstrated that mAChRs downstream effectors, PKC-zeta, PI3K, and Src family of tyrosine kinases are key molecules in the signal cascade leading to MAPK/ERK activation.

An additional effect of mAChRs activation in MCF-7 cells is the stimulation of tumor induced angiogenesis and invasion by increasing the number of tumor vessels and the expression of vascular endothelial growth factor-A (VEGF-A) and MMP-9 respectively [38, 39].

TARGETING MUSCARINIC RECEPTORS AS ANTICANCER THERAPY

Since other authors had postulated that mAChR activation can either stimulate or inhibit cellular growth depending on prior levels of cellular activity [12], we investigated the ability of different concentrations of carbachol added during different periods of time to LM2 or LM3 murine mammary tumor cells. As shown in Fig. (**2**), when carbachol was added to cell cultures for ≤10 h, cell proliferation was observed (Figs. **2A** and **B**). On the other hand, the addition of carbachol for longer periods of time exerted an inhibitory effect on tumor cell proliferation (Figs. **2A** and **B**). This cholinergic agonist reduced LM2 and LM3 cell proliferation by 62±6% and 56±8% respectively (Figs. **2A** and **B**). As it was expected, carbachol did not alter NMuMG cell proliferation at any of the concentrations used or tested times, in agreement with the absence of mAChRs in these cells (Fig. **2C**) [40].

In line with these previous results, other authors indicated that the activation of mAChRs can up-regulate or down-regulate cell proliferation depending on the context of cellular growth [41]. They showed that the activation of M_3 receptor subtype ectopically expressed in NIH3T3 cells can cause stimulation and inhibition of growth in the same cell. In quiescent 3T3/M_3 cells, carbachol stimulated DNA synthesis. In contrast, when 3T3/M_3 cells were growing, carbachol inhibited cell duplication. This inhibition was due to both a decrement in cyclin D1 levels, and an increment in p21(cip1) expression. Regarding the later, Pacini *et al*. [42] investigated the expression and function of mAChRs in bladder tumor specimens. All examined samples expressed M_1, M_2 and M_3 receptor subtypes. Moreover, the levels of M_2 transcripts, but not those of M_1 or M_3, significantly increased with the tumor histologic grade. The addition of the M_2 selective agonist arecaidine significantly reduced cell growth and migration of T24 cells, a bladder tumor cell line expressing mAChRs, including the M_2 subtype in a concentration-dependent manner. The silencing of M_2 receptors by siRNA in tumor cells showed the inability of arecaidine to inhibit cell proliferation after 48 h. Whereas, the use of M_1 and M_3 antagonists in T24 cells was not effective, suggesting that the inhibition of cell proliferation was directly dependent on M_2 receptor activation. In addition, Alessandrini *et al*. [43] have demonstrated that the activation of M_2 receptors, also by arecaidine propargyl ester, arrests cell proliferation and induces apoptosis in primary and established glioblastoma cell lines. Considering the high degree of malignancy of this tumor and the inability of conventional drugs to completely block the growth of glioblastoma cancer stem cells (CSCs), they have investigated the effect produced by arecaidine on CSCs growth and survival. Firstly, they detected the expression of M_2 receptors in two cell lines (GB7 and GB8) derived from human biopsies, and confirmed that in both cell lines, the treatment with arecaidine decreased GSC growth. GB7 cells exhibited a time- and dose-dependent decrement of cell proliferation. Moreover, arecaidine caused a reduced cell survival particularly in the GB8 cell line. These effects appeared to be mediated by M_2 receptor activation since experiments performed either in the presence of the M_2/M_4 antagonist methoctramine or by silencing M_2 receptors with a specific siRNA inhibited arecaidine-reduced cell growth.

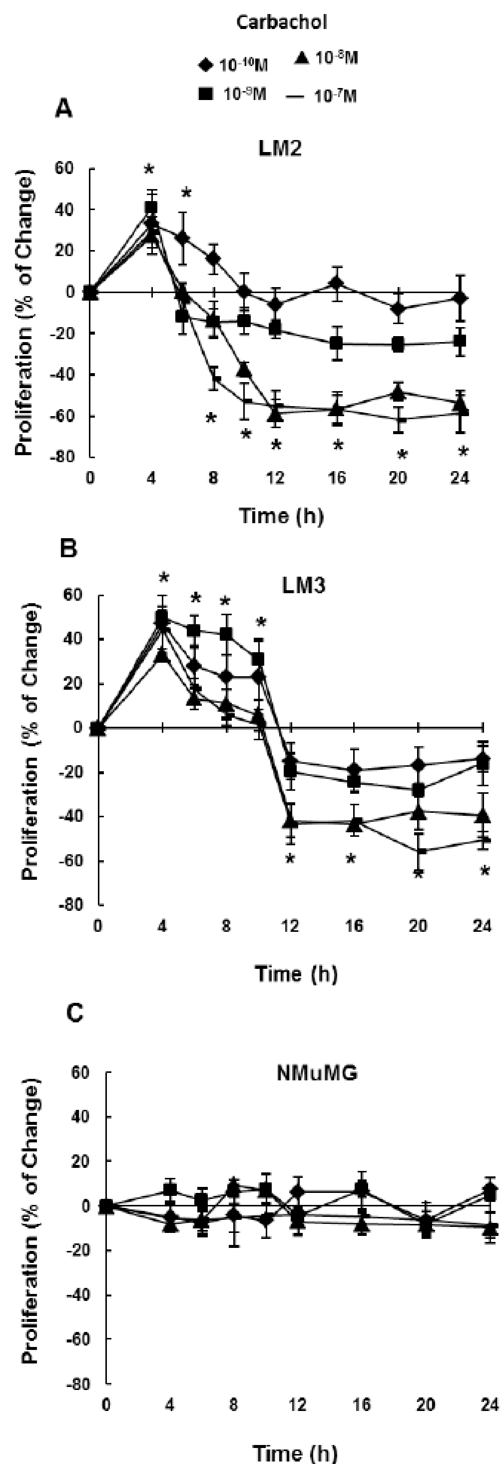

Fig. (2). Effect of carbachol on tumor cell proliferation. Time-response curves of carbachol added at different concentrations to (A) LM2 cells, (B) LM3 cells, and (C) NMuMG cells. Results were expressed as percent of change respect to control (*i.e*., cells without treatment). Values are mean ± S.E.M. of 6 experiments performed in duplicate. *p<0.01 vs. control and it concerns to all values of proliferation higher than 20 % considered as the cut-off value for these experiments [39].

These results opened the spectrum of cholinergic drugs that could inhibit cancer progression by the inclusion of muscarinic agonists.

METRONOMIC THERAPY

Cancer therapy has progressed over the past decade, with the development of targeted treatments [44]. Actually, conventional chemotherapy has also evolved. New drug combinations and/or regimens of administration have been tested to improve efficacy and tolerability of chemotherapy. Conventional administration is generally based on the concept of the maximum tolerated dose (MTD), consisting in the highest effective and survivable dose, which is able to kill as many tumor cells as possible. Although this strategy was supported by very high cure rate in clinical studies requires relative free and long intervals between doses to allow normal host cells, like the hematopoietic precursors and the epithelial cells recover [45]. Because of the latter, low-dose metronomic therapy (MT) emerged as a novel form of chemotherapy utilization, defined as the frequent administration of conventional drugs at low doses with short drug-free breaks. The term metronomic chemotherapy that was originally used in a publication by Hanahan *et al*. [46] appeared in 2000. In the initial study employing MT for the treatment of Lewis lung carcinoma-bearing mice that were resistant to MTD cyclophosphamide, were treated with the same drug in once every six days metronomic schedule. Under this treatment regimen, cyclophosphamide showed an important decrease in tumor growth [47], and authors commented side beneficial effects observed with the employment of MT. This chemotherapy may act inhibiting tumor growth through various mechanisms: (i) increasing tumor cell death; (ii) by eradication and disruption of CSCs; (iii) increasing endothelial cell death through up-regulation of anti-angiogenic factors (*i.e.* thrombospondin-1), and/or down-regulating pro-angiogenic factors such as VEGF, platelet-derived growth factor or hypoxia-inducible factor-1; (iv) blocking cell mobilization and/or decreasing viability of bone marrow-derived circulating endothelial progenitor cells, known to contribute to neo-angiogenesis; and (v) suppressing T regulatory cells, and therefore inducting the activity of T cytotoxic cells and natural killer cells [48] In addition, MT is associated with lower treatment related toxicity than conventional MTD chemotherapy which is an important fact in clinical practice. Furthermore, the cost of a metronomic regimen may be lower than MTD chemotherapy, as a result of fewer side associated effects, and the usage of inexpensive oral drugs such as cyclophosphamide [49].

As many phase II studies have shown, the clinical benefits of MT including tumor growth control and excellent safety profiles, this type of treatment is widely spreading around the world. It is important to know that whereas definitive phase III trial results are still in development, the usage of MT chemotherapy regimens has been shown to improve overall survival (OS) in phase III trials of early lung and breast cancer [50, 51] (Table **2**).

In total, 107 treatment regimens with at least one metronomically used drug were identified to treat tumors from different origins, including breast, prostate, lung, blood, brain, colorectal, skin, liver, adrenal, soft tissue, gastrointestinal, kidney, ovary, neuroendocrine and other. The most frequently used MT drugs were cyclophosphamide, capecitabine, etoposide and vinorelbine [52].

METRONOMIC THERAPY IN BREAST CANCER. ROLE OF MUSCARINIC RECEPTORS

Breast cancer incidence is growing in many developed or developing countries. Actually the treatment of breast cancer involves many therapeutic approaches such as surgery, radiotherapy, hormonal, biological and cytotoxic chemotherapy that attempt to cure patients. The most frequent complications of the disease are recurrence and metastasis although after primary treatments. The progression free survival ranges between 5 and 12 months [53, 54] but these values decreased when patients were treated previously with conventional chemotherapy [55]. When drugs usually used in standard protocols are administered in MT schedules alone or combined with hormones [56] the reported overall response rate (ORR), clinical benefit (CB) and median OS ranged between 19–21, 24–51%, and 12–18 months, respectively improving treatment outcomes. The addition of anti-angiogenic, immunomodulating, anti-inflammatory agents or trastuzumab to this schedule did not allow substantial improvement of the results [57]. Other protocols are focused on achieving CB in metastatic breast cancer patients pre-treated with MTD or refractory to anthracycline or taxanes, by MT with capecitabine and ciclophosphamide and/or bevacizumab [58]. A clinically relevant fraction of the patients (68%) controlled the disease for at least 6 months without significant acute or delayed toxicity. Results indicated that tolerability was generally good and a satisfactory percentage of CB was achieved both in pre-treated patients irrespective to HER2 status [59] and in HER2-negative metastatic breast cancer patients [60].

It is important to note that more data are expected at the end of some ongoing phase III trials, as like the study with NCT01131195, that will provide indeed results about tolerability and efficacy of a combination of bevacizumab plus a metronomic schedule of cyclophosphamide and capecitabine compared to weekly paclitaxel (Px) plus bevacizumab in metastatic breast cancer patients. In addition, the NCT01112826 trial will clarify the impact of MT after standard adjuvant chemotherapy in patients with triple negative breast tumors.

Regarding Px, it belongs to taxanes, which are part of a major group of anti-cancer drugs that stabilize microtubules. Unlike other chemotherapeutic drugs that also act on microtubules depolymerization, Px promotes permanent tubulin polymerization [61]. This effect inhibits cell proliferation in a concentration-dependent man-

Table 2. Different types of chemotherapy in cancer.

	Conventional	Non-conventional	Refs.
Based on	Maximum tolerated dose	Metronomic	[44-46]
Schedule	High doses/relative free long intervals between doses	Frequent administration of low doses/short drug free intervals	[47-49]
Therapeutic effect	Mainly tumor cell death	Tumor cell and cancer stem cell death/tumor dormancy/anti-angiogenic effects/host immunostimulation	[50,51]
Adverse effects	Normal cell death/systemic toxicity/resistance	Nule or low incidence of toxicity/resistance	[45,55]
Chemotherapeutic	Cytotoxic drugs/ hormones/antibodies	Cytotoxic drugs (cyclophosphamide, capecitabine, etoposide and vinorelbine)/ repositioning drugs (propranolol, talidomide)	[52]

ner. Clinical effective concentrations of Px, 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 [62]. 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 combined submaximal concentrations of Px (10^{-11} M) and carbachol (10^{-9} M). We took in mind, that the latter could be a good approach to potentiate both effects, to minimize undesirable effects, and to mimic the action of MT targeting mAChRs in an *in vitro* murine model. As shown in Fig. (**3**), this combination reduced the proliferation of LM2 and LM3 tumor cells in a more potent manner than each drug separately added, without affecting the viability of non-tumorigenic mammary cells NMuMG [40]. Interestingly, we reported a potentiation of tumor cell death via apoptosis induced by the combination of Px with carbachol in these cell lines [40]. The ability of chemotherapeutic agents to stimulate apoptosis in tumor bearers exerts immunostimulatory actions as it was previously described, that improve the response against malignant neoplasms. Also, subclinical low concentrations (<9 nM) and long period treatment (18 h) with Px induce apoptosis in A549 cells [63]. Surprisingly, Px actions on proliferation and apoptosis were reverted by atropine, suggesting an interaction between the cytostatic agent and mAChRs. McKay *et al*. [64] demonstrated that several microtubule-active drugs block cholinergically mediated catecholamine secretion from adrenal chromaffin cells. They studied the interactions of these agents with nAChRs using radiolabeled probes with high selectivity for receptor type, describing that Px, among others inhibited radioligand binding to the receptor-gated ion channel by steric hindrance. Similar interactions could be occurring between mAChRs and Px.

In addition, we demonstrated similar actions of this combination of drugs on human MCF-7 breast tumor cells derived from a luminal adenocarcinoma, that express mAChRs [65]. The addition of carbachol (10^{-11} M) plus Px (10^{-9} M), both at sub-threshold concentrations increased tumor cell death by 46% (Fig. **3A**). This effect was due to both up-regulation of NO production and decrement in arginase II activity [65]. The combination was not effective in the non-tumorigenic cell line MCF-10A that lacks mAChRs. Similarly to that observed in murine mammary tumors, the effect was totally blunted in the presence of atropine [65]. Recently, we observed similar results in the MDA-MB231 cell line derived from a triple negative adenocarcinoma, a type of tumor that lacks estrogen and progesterone receptors as well as HER2 and has a poor prognosis. This type of breast tumor usually requires polychemotherapy (alternating taxanes and/or anthracyclines) with variable rate of success [66]. Since MDA-MB231 cells also express mAChRs, we tested the effect of Px plus carbachol at low doses on these tumor cells. The combination also exerted a significant reduction of cell proliferation through mAChRs activation [67].

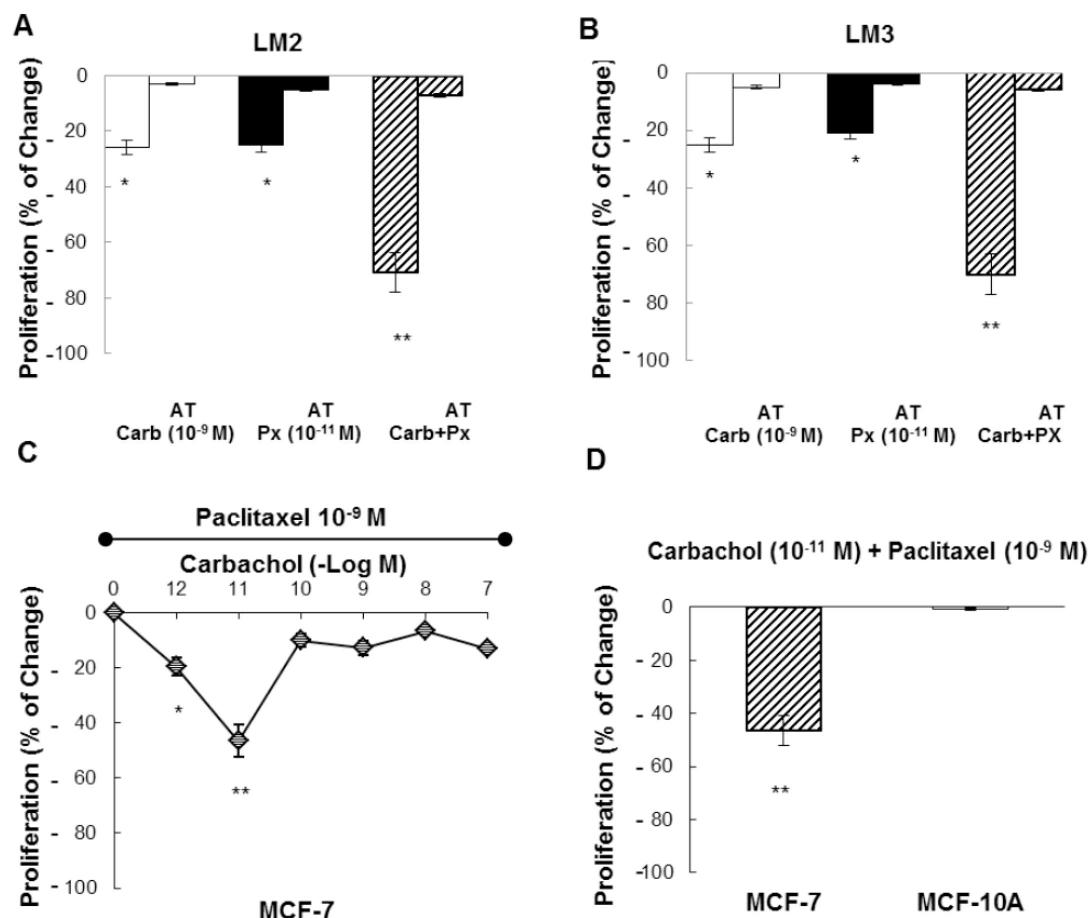

Fig. (3). Effect of carbachol (Carb) and paclitaxel (Px) on tumor cell proliferation. Carb and Px were added separately or together for 20 h to (**A**) LM2 cells, (**B**) LM3 cells, (**C**) MCF-7 and (**D**) MCF-7 and MCF-10A cells in the absence or presence of 10^{-8} M atropine (AT). Results were expressed as percent of change with respect to control (*i.e.*, cells without treatment). Values are mean ± S.E.M. of 4 experiments performed in triplicate. *p<0.01; **p<0.001 vs. control.

CONCLUSION

ACh has been identified as a growth factor for malignant cells from diverse origins, by activating either nAChRs or mAChRs and by triggering cellular signaling pathways that favor tumor progression. For this reason, mAChRs are now considered therapeutic targets in different types of tumors (*i.e.*, brain, lung and breast) in addition to their role in other illness such as chronic obstructive pulmonary disease or gastrointestinal, ocular and cardiac disorders. The use of muscarinic agonists (*e.g.*, arecaidine, carbachol) at pharmacological/suboptimal concentrations alone or in combination with cytotoxic drugs like Px have proved to be a useful strategy to kill tumor cells without affecting normal cells *in vitro*. More *in vivo* experiments using an MT schedule are needed to confirm the effectiveness of this type of therapy in cancer.

LIST OF ABBREVIATIONS

AC	=	Adenylyl cyclase
ACh	=	Acetylcholine
AchE	=	Acetyl cholinesterase
CaM KK	=	calcium/calmodulin-dependent protein kinase kinase
ChAT	=	choline acetyl transferase
EGFR	=	epidermal growth factor receptor
ERK	=	extracellular signal-regulated kinases
HB-EGF	=	heparin binding epidermal growth factor
IP3	=	inositol trisphosphate
mAChRs	=	muscarinic acetylcholine receptors
MAPK	=	mitogen-activated protein kinase
MAPKK	=	mitogen-activated protein kinase kinase
MEK	=	mitogen-activated protein/extracellular signal-regulated kinase kinase
MMP	=	matrix metalloproteinase
MT	=	metronomic therapy
MTD	=	maximum tolerable dose
nAChRs	=	nicotinic acetylcholine receptors
NO	=	nitric oxide
NOS	=	nitric oxide synthase
OS	=	overall survival
PI3K	=	phosphoinositide 3-kinase
PKC	=	protein kinase C
PLC	=	phospholipase C
[3H]-QNB	=	tritiated quinuclidinyl benzilate
VEGF-A	=	vascular endothelial growth factor A

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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