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REVIEW ARTICLE

Role of Muscarinic Acetylcholine Receptors in Breast Cancer: Design of Metronomic Chemotherapy

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Abstract: ***Background*:** muscarinic acetylcholine receptors (mAChRs) have attracted interest as targets for therapeutic interventions in different illnesses like Alzheimer´s disease, viral infections and different tumors. Regarding the latter, many authors have studied each subtype of mAChRs, which seem to be involved in the progression of distinct types of malignancies.

***Methods*:** We carefully revised research literature focused on mAChRs expression and signaling as well as in their involvement in cancer progression and treatment. The characteristics of screened papers were described using the mentioned conceptual framework.

***Results*:** Muscarinic antagonists and agonists have been assayed for the treatment of tumors established in lung, brain and breast with beneficial effects. We described an up-regulation of mAChRs in mammary tumors and the lack of expression in non-tumorigenic breast cells and normal mammary tissues. We and others demonstrated that muscarinic agonists can trigger anti-tumor actions in a dose-dependent manner on tumors originated in different organs like brain or breast. At pharmacological concentrations, they exert similar effects to traditional chemotherapeutic agents. Metronomic chemotherapy refers to the administration of anti-cancer drugs at low doses with short intervals among them, and it is a different regimen applied in cancer treatment reducing malignant growth and angiogenesis, and very low incidence of adverse effects.

***Conclusion*:** The usage of subthreshold concentrations of muscarinic agonists combined with conventional chemotherapeutic agents could be a promising tool for breast cancer therapy.

Keywords: Muscarinic acetylcholine receptors, signal metabolic pathway, breast tumors, metronomic chemotherapy, repurposing drugs, acetylcholine (ACh).

1. INTRODUCTION

Acetylcholine (ACh) was the first neurotransmitter identified and the concept cholinergic system was used to name ACh, its synthesizing and degrading enzymes, transporters and receptors [1]. This system was located in the parasympathetic and the sympathetic nervous system. In 1914, Ewins documented for the first time the presence of ACh in plants [2]. Later, all the components of the cholinergic system including ACh have been detected in blue-green algae, fungi, bacteria, nematodes, sponges and amphibians [3]. Approximately, 400 millions of years ago, the nervous system was originated and included ACh that was already expressed in the cholinergic system as neurotransmitter [1]. Later, evidences about the presence of ACh and/or choline acetyl transferase (ChAT), the enzyme that synthesizes ACh in the immune system [4], placenta [5], urogenital tract [6] and in the skin from animal or human origin [7] were published. ACh is synthesized from acetyl-CoA and choline. The latter is captured from the extracellular media by the specific high-affinity choline transport system (CHT)1 present in many non-nervous cells including epithelial [8] and endothelial ones [9].

These previous results prompted Wessler *et al*. [10] to introduce the term non-neuronal cholinergic system (nNCS) to highlight the idea that ACh is present in cells independent of neurons, and that can act on cells themselves or in neighboring cells. These actions can be exerted by activating nicotinic (nAChRs) and/or muscarinic acetylcholine receptors (mAChRs). In addition, cytosolic ACh can interact with intracellular signaling proteins. In contrast to the cholinergic neurons, there is no storage compartment in non-neuronal cells and ACh appears to be released directly after synthesis

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[11]. It has also been demonstrated in human placenta, a model to study *in vitro* release of non-neuronal ACh, that it is extruded from non-neuronal cells *via* active transport mediated by the OCT (Organic Cation Transporter) family [2, 12]. There are 3 subtypes of OCT (1–3) [13], and using siRNA techniques it was proved that OCT1 and OCT3 mediate the release of ACh in the placenta [11, 12]. The three OCT isoforms were also detected in abraded epithelial cells from rat and human tracheae and human bronchi transcripts [11, 13].

Also acetyl cholinesterase (AChE), the enzyme that degrades ACh is important in nNCS since it is active in non-neuronal cells. For example, the non-innervated parts of skeletal muscle fibers [14], as well as fibroblasts [15] contain AChE activity. Also, erythrocytes possess AChE and its activity together with plasma cholinesterase destroys non-neuronal ACh that escaped into the circulation [12, 15].

2. EXPRESSION AND SIGNALING OF MUSCARINIC ACETYLCHOLINE RECEPTORS. REGULATORY MECHANISMS

ACh can activate nAChRs, which are sodium channels (molecular mass of 290 kDa) [16, 17]. mAChRs belong to the family of G-protein coupled receptors (GPCRs) with seven transmembrane loops, and also bind ACh besides the natural agonist muscarine. The existence of five subtypes of mAChRs was proved by genetically identification: M_1-M_5 [17, 18]. In airways tissues of mammals including human beings, the expression of M_1-M_3 receptors was described [11]. M_1 subtype is mainly expressed in peripheral lung tissue and in the wall of alveoli but is absent in larger airways, skin, intestinal tract and other glands. M_2 and M_3 receptors are the main population of mAChRs in airways human macrophages and sclera fibroblasts [2, 17], and also in smooth muscle fibers [19]. The other subtypes, M_4 and M_5 receptors are predominantly located in the central nervous system [18, 20].

The five subtypes of mAChRs have been detected in urothelium, endothelial and immune cells involved in inflammatory responses [19]. Heterotrimeric G proteins mediate the coupling of mAChRs to their intracellular effector molecules. G proteins were described a long time ago and are composed of α–, β– and γ–subunits. Due to the existence of different subtypes of α–subunit, G proteins are classified into four groups: Gαs, Gαi/o, Gαq and Gα12 [21]. When GPCRs like mAChRs are activated by an agonist, it results in the dissociation of α-and β/γ-subunits. The latter are firmly linked and exert a unique functional activity. Both, α- and β/γ-subunits mediated the signal transduction pathway of mAChRs to similar or different effector molecules [22]. It was extensively described that activated odd receptors (M_1, M_3 and M_5) couple to Gq proteins; its αq subunit stimulates phospholipase C (PLC) yielding the hydrolysis of phosphatidylinositol 4, 5-bisphosphate and the generation of inositol 1, 4, 5-trisphosphate (IP3) and diacylglycerol. IP3 is responsible of the liberation of calcium from the endoplasmic reticulum to the cytosol that produces, in turn, the activation of distinct enzymes like nitric oxide synthase (NOS). On the other hand, M_2 and M_4 receptors bind to Gi/o proteins and inhibit adenylyl cyclase (AC) reducing the synthesis of cAMP; they also reduce the opening of calcium channels [18]. The over-expression of Gαq constitutively activates compatible mAChRs and the latter is prevented by muscarinic antagonists [23]. Changes in Gαq levels also modify the potency and efficacy of agonists exerting a profound impact on cellular physiology, even in the absence of agonists [24].

On the other hand, Gβγ complex can stimulate or inhibit AC, may increase PLCβ2 or ε activity and phosphoinositide-3-kinase (PI3K); it also can trigger the opening of potassium channels.

Another important regulatory event is transactivation. This phenomenon can be produced by the activation of mAChRs and as a consequence, the stimulation of the epidermal growth factor receptor (EGFR) *via* βγ-complex promoting Src-mediated matrix metalloproteinase (MMP)-dependent enzymatic liberation of EGF bound to the cell surface. The interaction EGF-EGFR leads to the activation of extracellular signal-regulated kinases (ERK)1/2 [25].

A complex network involving different intermediates considered as classical/canonical signaling pathways can be also regulated by mAChRs *via* both Gα and Gβγ. In addition, other non-classical/non-canonical metabolic signaling pathways can be triggered exerting cytoskeleton actions *via* the activation of small GTPase Rho, with downstream effector molecules like soluble tyrosine kinases and mitogen-activated protein kinases (MAPK) [17, 26]. It has also been demonstrated that receptors also transduce non-G-protein-mediated signaling *via* arrestins [27] and G-protein receptor kinases (GRKs) [28].

In addition, the regulation of mAChRs activity could be exerted by the activation of different kinases. The binding of an agonist to mAChRs can induce the phosphorylation of these receptors (Fig. **1**). This modification occurs on serine and threonine residues in the third cytoplasmic loop and the C-terminus of the mAChRs. An array of protein kinases is able to phosphorylate mAChRs, including various GRKs, casein kinase 1a, and protein kinase C (PKC) [29, 30]. Free Gβγ subunits at the plasma membrane, which are generated following G protein activation, are required for GRK2/3-mediated receptor phosphorylation. Then, cytosolic β-arrestin interacts with the phosphorylated receptor, leading to uncoupling of the mAChRs from the G proteins. In addition, β-arrestin recruits and activates the tyrosine kinase c-Src. β-arrestin also interacts with clathrin and the clathrin adaptor complex AP-2, leading to immobilization of the receptor-β-arrestin-c-Src complex in the clathrin-coated pit. Then, activated c-Src tyrosine phosphorylates and activates dynamin [30]. Activated dynamin molecules polymerize as a collar around the neck of the endocytic pit and catalyze the fission of the vesicle from the plasma membrane. Following the release of dynamin, clathrin and β-arrestin, the vesicle recycles back to the plasma membrane [30]. The latter mechanism is the most studied and classical for almost all subtype of mAChRs except for M_2 subtype, which is internalized *via* a clathrin-independent mechanism, regulated by Arf6 a member of the ADP-ribosylation factor family of GTP binding protein, and then targeted to lysosomes for degradation [31] (Fig. **1**). Despite M_2 and M_4 receptors are analogous in

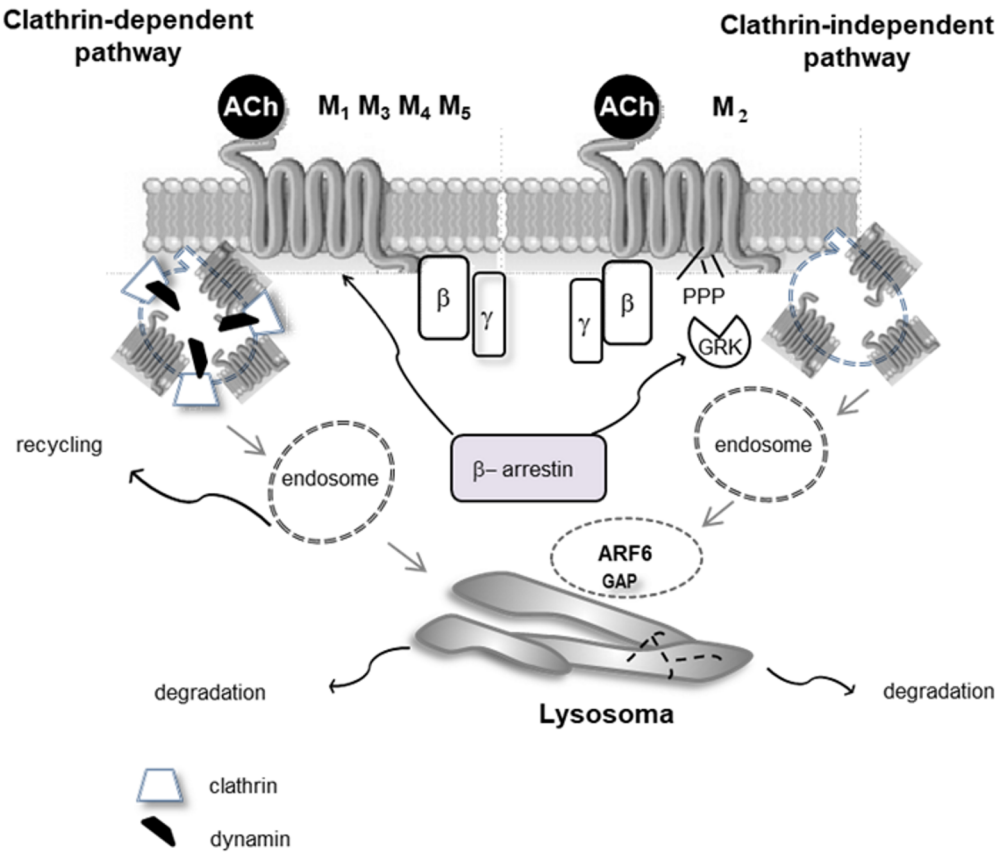

Fig. (1). Mechanisms of internalization and degradation/recycling of muscarinic (M) receptors in a clathrin-dependent or -independent pathway. The binding of acetylcholine (ACh) to different M subtypes besides triggering M receptors signaling leads to the activation of β-arrestins and the recycling of receptors by two different mechanisms. G-protein receptor kinases (GRK); ADP-ribosylation factor 6 (ARF6); GTPase activating protein (GAP).

their coupling to Gi/o protein and in their signal transduction pathway, they differ in the sequence of the i3 loop that is the target of phosphorylation prior to agonist-promoted internalization [31] (Fig. **1**).

3. MUSCARINIC RECEPTORS AND CANCER PROGRESSION

mAChRs are expressed in the majority of tumors derived from epithelial and endothelial cells. In addition, these tumors also liberate ACh which can act as a growth factor promoting tumor cell proliferation. ChAT and AChE that are also expressed and active in malignant tissues are continuously regulating local ACh levels [17, 32]. In the case that tumor lack of this mechanism, ACh may be derived from neuronal, endocrine or paracrine surrounding tissues, and constitutively activates mAChRs and nAChRs present in tumor mass. Mainly, the expression of mAChRs in malignant tissues keeps on the expression of these receptors in the corresponding normal tissue, but also patterns of mAChRs expression can change between tumors and normal tissues [17, 32, 33].

The activation of odd receptors (M_1, M_3 and M_5) couples Gq proteins and increases cell viability. Table **1** recapitulates the expression of M_3 receptors in tumors from different origin and is involved in tumor growth/invasion during malignant progression, *via* distinct metabolic signaling pathways. M_2/M_3 receptor subtypes have been detected in head (glioblastomas) and neck (larynx squamous) carcinomas. Almost 90% of head and neck carcinomas are assigned to squamous cell carcinoma ad it was reported that they express not only mAChRs but also the other components of the nNCS like AChE [34, 35].

In lung [36-38] and gastric [39-41] tumors, it has been extensively assigned that M_3 subtype is responsible of tumor progression. The activation of this receptor with agonists causes proliferation *via* a concentration-dependent increment in cytosolic calcium and the phosphorylation through MAPK/Akt or EGFR/PI3K/Akt in small cell lung carcinoma cell lines [17, 37] and the activation of M1 and M3 receptors induces lung epithelial cells to undergo epithelial–mesenchymal transition proposed as a mechanism in the progression of airway diseases and cancer [38].

It has been reported that M_3 receptor is widely expressed in digestive tract cancer, and may play an important role in the proliferation, differentiation, transformation and carcinogenesis of tumors [39, 42]. mAChR agonists promote the growth of colorectal neoplasia and findings suggest that vagal innervation contributes to gastric tumorigenesis *via* M_3 receptor mediated Wnt signaling [41, 43]. M_3 subtype activation also stimulates colon cancer cell proliferation [44]. The expression of ChAT was detected by real-time PCR in H508, WiDr and Caco-2 human colon cancer cells; moreover, H508

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

Tumor Type	mAChR Subtype	Function	Signaling Pathway	Refs.
Head and neck	M_2/M_3	Proliferation	PLC/IP3	[34, 35]
Lung	M_3	Proliferation/EMT	MAPK/Akt	[36-38]
Gastric	M_3	Proliferation	MAPK	[39, 41]
Colon	M_3	Proliferation/ inhibition of apoptosis	Transactivation of EGFR (ERK/PI3K/Akt) PKC/ERK1/2	[42-44]
Skin	M_3	Invasion, migration, metastasis	Calcium mobilization	[45, 46]
Brain	M_2/M_3	Proliferation	PLC/PKC/Akt MAPK/ERK1/2	[47, 48]
Prostate	M_1/M_3	Proliferation	CaM KK/Akt	[49]
Cervix	$M_1/M_3/M_4$	Migration	ERK1/2	[50]
Breast	$M_3/M_2/M_1$	Proliferation/angiogenesis	PLC/IP3 PGE_2/arginase	[51-53]

PLC=phospholipase C; IP3= inositol trisphosphate; MAPK= mitogen activated protein kinase; EGFR= epidermal growth factor receptor; EMT=epithelial-mesenchymal transition; ERK= extracellular signal–regulated kinase; PI3K= phosphoinositide 3-kinase; PKC= protein kinase C; CaMKK= calcium/ calmodulin-dependent protein kinase kinase; PGE_2= prostaglandin E_2.

and Caco-2 cells liberate ACh into culture media promoting cell proliferation [44]. In H508 and HT-29 human colon cancer cells, the stimulation of M_3 receptors activates MMP-7, which cleaves heparin binding-EGF (HB-EGF) from Pro-HB-EGF. The liberated EGF transactivates its receptor producing intracellular signaling *via* the mitogen-activated protein (MEK)/ERK and PI3K/Akt pathways. EGFR is also involved together with PKC in M_3-mediated activation of ERK1/2 in colon cancer cells [44].

Melanoma is one of the most aggressive classes of skin tumor. In humans, it was documented that M_1, M_3 and M_5 receptors are expressed in tumor tissue and tumor cell lines. One of the most studied cell lines is SK-Mel 28 and it expresses larger amounts of M_3 than M_5 receptor subtype. The activation of receptors implies calcium mobilization and chemotaxis indicating that cells expressed functional mAChRs that induce movement (by near 30 %) an effect blocked by atropine [45, 46].

Similarly to that observed in glioblastomas, astrocytomas express M_2 and M_3 receptors. The addition of carbachol-induced a proliferative effect. The previous treatment with different subtypes-specific antagonists suggests that the effect is due to M_3 receptor activation through PLC/PKC metabolic pathway followed by Akt phosphorylation [47]. As it was mentioned previously, MAPK is considered the major pathway involved in cell proliferation and can be phosphorylated by the binding of mitogens to GPCRs. Yagle *et al.* [48] reported the involvement of the latter mechanism in astroglial cells 1321N1 derived from a human astrocytoma. Carbachol induced the phosphorylation of MAPK, particularly ERK1/2, and increased its activity, without modifying protein levels. M_3 receptors also participate in cell proliferation of tumor cells in prostate [49] and cervix [50]. This effect leads to the stimulation of calcium/calmodulin-dependent protein kinase kinase (CaMKK)/Akt and ERK1/2 respectively [49, 50].

We had previously documented the expression of mAChRs in mammary adenocarcinoma cell lines named LM2 and LM3 that spontaneously appeared in BALB/c mice. By different techniques, we confirmed that M_2 receptor subtype predominates in both tumor cell lines [17]. The activation of mAChRs with carbachol during short periods of time increased proliferation in these tumor cell lines [51] (Fig. **2**). In LM3 tumor, the addition of carbachol-stimulated proliferation through M_3 receptor activation producing IP3 and nitric oxide. In turn, the stimulation of LM2 cells with carbachol activates M_2 and M_1 receptors triggering prostaglandin E_2 liberation and arginase activation respectively [52]. These actions also increased tumor cell proliferation. The analysis of mAChRs expression in normal cells of the same strain, derived from normal murine mammary gland (NMuMG) gave a negative result. Other important results from our laboratory obtained from binding experiments performed in cell lysates indicated that mAChRs are highly up-regulated (40-fold) in LMM3 cells derived from a metastasis of LM3 tumor pointing to an invasive role of mAChRs when they are present at high concentrations [17, 53].

4. MUSCARINIC RECEPTORS AND CANCER TREATMENT

The antagonists that selectively block M_3-mediated responses have been demonstrated to depress the growth of small cell lung cancers (SCLCs) and non-SCLCs [54]. Song *et al.* [36, 55] demonstrated the efficacy of M_3 receptor antagonists to inhibit lung cancer growth *in vitro* including 4-diphenyl-acetoxy-N-methyl-piperidine (4-DAMP), para-fluorohexahydrosila-difenidol (p-F-HHSiD), darifenacin and tiotropium. The last two antagonists also inhibit lung cancer cell growth in nude mice.

Moreover, a very interesting finding is the link of M_3 receptor with EGFR and with human ether-a-go-go-related gene K+ channels to promote tumorigenesis [54, 56]. Carbachol-mediated increment in ether-a-go-go-related gene K+ channels expression was abolished by the selective M_3 antagonist 4-DAMP. In addition, Wang *et al.* [57] demonstrated that M_3 receptor enhanced the proliferation induced by ACh in human gastric cancer cells, whereas the knock-

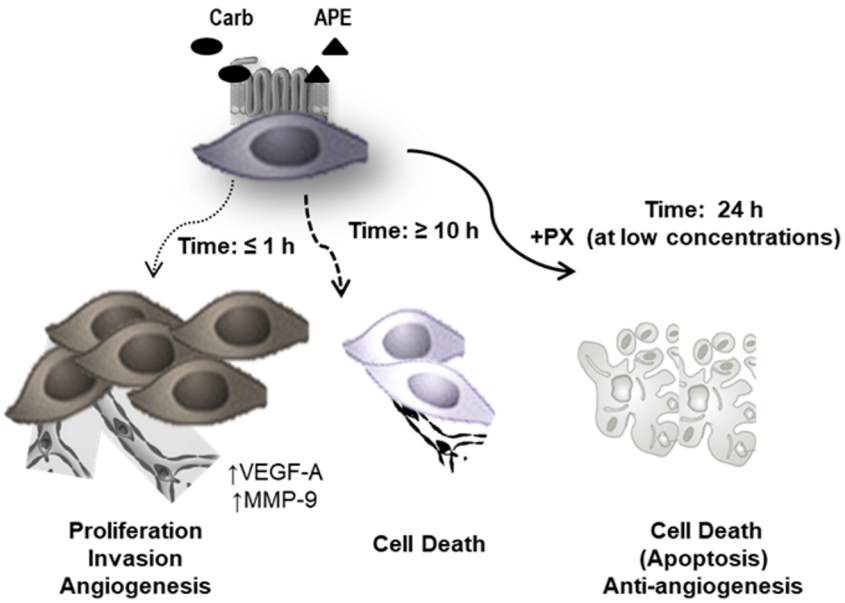

Fig. (2). Action of a muscarinic agonists on breast tumor cells. The addition of the nuscarinic agonist carbachol (Carb) for a short time (1 h or less) exerts stimulatory actions on muscarinic receptors expressed in tumor cells promoting proliferation invasion and angiogenesis. When Carb or arecaidine propargyl ester (APE) is added for longer periods of time to tumor cell cultures it produces cytotoxicity in a concentration dependent-manner increasing either necrosis or apoptosis. Finally if Carb or APE is added together with paclitaxel (PX) both at subthreshold concentrations they increase cell death favoring apoptosis and anti-angiogenesis.

down of this receptor subtype inhibited cell proliferation *in vitro* and suppressed tumorigenesis *in vivo*, suggesting that M_3 antagonists may serve as potential adjuvants to gastric cancer therapies.

Regarding breast cancer, we observed that the selective M_3 antagonist, p-F-HHSiD besides atropine inhibited the effect of carbachol on LMM3 cell growth triggered by the activation of this receptor subtype [53]. The latter observations, together with others obtained by other groups indicated that muscarinic antagonists could be considered as therapeutic tools in cancer. But it should not be ignored that, the systemic administration of these antagonists frequently exhibits adverse effects, mainly on cardiac system as it is published in the treatment of overactive bladder [58].

Breast cancer is one of the first causes of death among women and in spite of the majority of early detected luminal tumor bearers respond to treatment, others (triple negative tumors) lack of specific targets for chemotherapy [17]. For this reason, we analyzed the expression of mAChRs in samples surgically obtained from patients with human breast tumors corresponding to different histological grades and/or TNM classification [33]. By Western blot performed in breast tumors homogenates with specific anti-mAChRs antibodies a major expression of M_2/M_3 subtypes was confirmed. Densitometric analysis indicated that the intensity of the bands correlated with malignancy and invasiveness of breast tumor samples. Patients with fibroadenoma (considered as a benign pathology) or free of illness exhibited low or negative expression of mAChRs respectively [33]. We also analyzed the expression and function of mAChRs in human breast tumor cell lines. MCF-7 cell line derived from a human luminal breast adenocarcinoma (estrogen-dependent) constitutively express mAChRs [59]. By Western blot, we identified M_3 and M_4 receptor subtypes in these cells. Similarly to that observed in normal breast tissue, mAChRs were absent in the non-tumorigenic mammary cell line, MCF-10A [17, 59].

The treatment of MCF-7 cells with carbachol for short periods of time promoted proliferation mainly by M_3 receptor triggering PLC/PKC/calcium-dependent NOS1 and NOS3 activation. It is important to know that muscarinic stimulation of MCF-7 cells also induced malignant angiogenesis with an up-regulation of vascular endothelial growth factor-A (VEGF-A) expression and tumor blood vessels counting. Carbachol was also effective to increase the invasive capacity by increasing the expression and activity of MMP-9 [17, 59-61] (Fig. **2**).

5. IS IT POSSIBLE TO TARGET MUSCARINIC RECEPTORS IN CANCER THERAPY?

Other authors postulated that mAChRs activation could not only stimulate but also inhibit cellular growth depending on cellular metabolic status [24]. Because of the latter, we performed concentration-response and time-response experiments analyzing LM2 and LM 3 cell viability in the presence of carbachol [17]. We observed that carbachol-stimulated cell proliferation when it was added to cell cultures for ≤10 h [60, 62]. But if cells were treated with the agonist for longer periods of time (≥10 h) it exerted an inhibitory action on tumor cell viability (LM2: 62±6% and LM3: 56±8%) [62]. As it was expected, since NMuMG cells do not express mAChRs, carbachol was not able to modify cell proliferation at any of the concentrations added or tested times [17, 62] (Fig. **2**).

Pacini *et al*. [63] investigated mAChRs´ expression and function in tumor specimens from bladder. Comparing the levels of transcripts, M_2 ones but not those of M_1 or M_3 re-

ceptors significantly increased in parallel with tumor histologic grade. The treatment with arecaidine propargyl ester, an M_2 selective agonist for 48 h significantly reduced the growth and migration of T24 cells in a concentration-dependent manner, involving M_2 subtype participation [17]. The transfection of tumor cells with a specific siRNA to silence M_2 receptors inhibited the effect of the agonist. Similar results were obtained by Alessandrini *et al*. in primary and established glioblastoma cell lines [64]. They demonstrated that the activation of M_2 receptors with the same selective agonist arrested cell proliferation and induced apoptosis. Moreover, arecaidine propargyl ester was effective to reduce cancer stem cells growth and survival. The latter effects were prevented in the presence of the M_2/M_4 antagonist methoctramine or by silencing M_2 receptors with a specific siRNA.

All these previous results increased the spectrum of chemotherapeutic agents by introducing cholinergic drugs (agonists) to inhibit cancer progression [17] (Fig. **2**).

One of the most important aims in cancer therapy is the discovery of new specific targets for chemotherapy and the development of new schedules for treatment. Conventional administration of drugs consists in the systemic delivery of the effective highest and tolerable dose in order to kill as much as possible of the tumor cells [17, 65]. This strategy not only kills tumor cells but also normal cells and relative free and extended intervals between doses are needed to allow normal cells (hematopoietic precursors/epithelial cells) to recover [66]. As a consequence of the latter, low-dose metronomic therapy (MT) appeared as a new method of chemotherapy administration, defined as the periodic administration of conventional drugs at low doses with short drug-free intervals [67]. MT may be effective on tumor progression through different mechanisms: (i) by inducing tumor cell death, mainly *via* apoptosis; (ii) by eliminating cancer stem cells; (iii) by stopping angiogenesis through the death of endothelial cells induced by an up-regulation of anti-angiogenic factors (*i.e*. thrombospondin-1), by reducing the expression of VEGF, platelet-derived growth factor or hypoxia-inducible factor-1, and by inhibiting the viability of bone marrow-derived circulating endothelial precursor cells, that contribute to neo-vascularization; and (v) reducing the number and activity of T regulatory cells, and up-regulating T cytotoxic and NK cells [68, 69]. It is important to note that MT exerts lower related toxicity than conventional administration of chemotherapy which is an important fact in patient´s treatment. Moreover, the cost of MT is surely lower than conventional chemotherapy, because associated medication to treat side effects is not needed, and inexpensive oral drugs such as cyclophosphamide can be administered [70, 71]. MT is usually linked to the introduction of repurposing drugs. The latter refers to the assignation of new uses for existing drugs and represents an alternative drug development strategy. In oncology, there is an increasing interest in the prescription of non-cancer drugs for cancer treatments due to the knowledge of pharmacokinetics/dynamics, side effects, and because most of them are available at low cost [72]. This is a main issue for low-income/developing countries, where the incidence of cancer is growing and the availability of drugs for treatment is almost limited to some cytotoxic drugs with high costs and severe adverse effects [73, 74]. Many repurposing drugs have been studied with breast cancer treatment purpose: β2 adrenergic receptor blockers [75], the anti-diabetic drug metformin [76] or the PPRγ ligand, pioglitazone [77].

Nowadays, breast cancer treatment may include many approaches like surgery, radiotherapy, hormonal, immunobiological and cytotoxic drugs with the clinical objective of curing patients. Usually, complications of the disease are recurrence and metastasis even after primary treatments [17]. When drugs usually used in standard protocols are delivered in MT schedules alone or in combination, the overall response rate, clinical benefit and median overall survival ranged between 19–21%, 24–51%, and 1.0–1.5 years, respectively improving beneficial results of treatment [17, 70].

Paclitaxel (PX) is a taxane, a class of diterpenes. They were originally identified from plants of the genus (yews), and as an anti-cancer drug, PX stabilizes microtubules by promoting permanent polymerization of tubulin [78]. This action reduces cell proliferation in a dose-dependent manner [17]. Clinical active concentrations of PX are known to be around 10^{-6} M, but it also produces side non-serious and serious effects associated with hypersensitivity reactions, myelosuppression, peripheral neuropathy, myalgia, arthralgia, mucositis and alopecia [17; 79]. Taking into account that, the best conditions for cancer treatment should be the administration of drugs at doses effective to kill tumor cells with minimal side effects on normal tissues, we associated subthreshold concentrations of PX with carbachol (Table **2**). The latter could be a good approach to produce synergy of both drugs targeting mAChRs and minimal adverse effects in an *in vitro* murine model that could mimic the action of MT [17]. This combination induced tumor cell cytotoxicity on murine LM2 and LM3 cells without affecting the viability of non-tumorigenic mammary cells NMuMG [62]. Moreover, the combination produced a potentiation of tumor cell death *via* apoptosis in these tumor cells [62]. It is important to note that, the stimulation of apoptosis by chemotherapeutic agent in tumor bearers could exert immunostimulation improving the response against tumors. Similar actions for carbachol plus PX on human MCF-7 breast tumor cells derived from a luminal adenocarcinoma, that express mAChRs were observed in our laboratory [80]. The addition of both drugs at

Table 2. Effect of metronomic chemotherapy targeting muscarinic receptors on breast tumors.

Breast Cancer	Metronomic Combination (% of Citotoxicity)
Murine	Carbachol (10^{-9}M) + Paclitaxel (10^{-11}M)
LM2	70.8 ± 5.4
LM3	66.2 ± 5.8
Human	Carbachol (10^{-11}M) + Paclitaxel (10^{-9} M)
MCF-7	46.5 ± 5.8
	Carbachol (10^{-12} M) + Paclitaxel (10^{-8} M)
MDA-MB231	27.4 ± 3.1

subthreshold concentrations stimulated tumor cell cytotoxicity by more than 40% (Table **2**) [17]. The combination did not modify viability in the non-tumorigenic cell line MCF-10A that did not express mAChRs. The effect was totally blunted in the presence of atropine either in murine or in human breast tumor cells [80]. Recently, we analyzed the effect of the same combination of drugs on MDA-MB231 breast tumor cells derived from a triple negative adenocarcinoma, a type of tumor that is negative for the expression of hormone receptors and HER, with a poor prognosis [81]. This type of breast cancer is very aggressive and usually requires chemotherapy with more than one drug (alternating taxanes and/or anthracyclines) with uncertain success [82]. The expression of mAChRs in MDA-MB231 cells make them sensible to low doses of PX plus carbachol exerting a significant reduction in cell viability, but in a less potent manner than in MCF-7 cells [17] (Table **2**).

CONCLUSION

ACh can act as a growth factor for tumor cells in an autocrine or paracrine manner by activating mAChRs. This activation triggers metabolic pathways favoring different steps of tumor progression. Due to the latter, mAChRs should be considered as therapeutic targets in tumors from different origins (*i.e.*, breast, brain and lung) [17]. It has been previously documented that mAChRs have an important role in non–malignant illness (chronic obstructive pulmonary disease or gastrointestinal, ocular and cardiac disorders). The usage of cholinergic agonists (*e.g.*, arecaidine, carbachol) at pharmacological/suboptimal/subthreshold concentrations in combination with traditional drugs like PX is a useful strategy to kill tumor cells without damaging normal cells *in vitro*. More pre-clinical experiments using the mentioned drugs in an MT schedule administered *in vivo* are needed to confirm its effectiveness in breast cancer therapy.

ABBREVIATIONS

4-DAMP	=	4-Diphenyl-Acetoxy-N-Methyl-Piperidine
AC	=	Adenylyl cyclase
ACh	=	Acetylcholine
AChE	=	Acetyl cholinesterase
CaMKK	=	Calcium/calmodulin-dependent protein kinase kinase
ChAT	=	Choline acetyl transferase
CHT	=	Choline transport system
EGFR	=	Epidermal growth factor receptor
ERK	=	Extracellular signal-regulated kinase
GPCRs	=	G-protein coupled receptors
GRKs	=	G-protein receptor kinases
HB-EGF	=	Heparin binding EGF
IP3	=	Inositol 1,4,5-trisphosphate
M	=	Muscarinic
mAChRs	=	Muscarinic Acetylcholine Receptors
MAPK	=	Mitogen-activated protein kinase
MMP	=	Matrix metalloproteinase
MT	=	Metronomic therapy
nAChRs	=	Nicotinic acetylcholine receptors
nNCS	=	Non-neuronal cholinergic system
NOS	=	Nitric oxide synthase
OCT	=	Organic cation transporter
p-F-HHSiD	=	Para-fluorohexahydrosila-difenidol
PI3K	=	Phosphoinositide-3-kinase
PKC	=	Protein kinase C
PLC	=	Phospholipase C
PX	=	Paclitaxel
SCLCs	=	Small cell lung cancers
VEGF	=	Vascular endothelial growth factor

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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