Conference paper

Anna Maria Lucianò, Francesca Mattei, Elisa Damo, Elisa Panzarini, Luciana Dini and Ada Maria Tata*

Effects mediated by M2 muscarinic orthosteric agonist on cell growth in human neuroblastoma cell lines

https://doi.org/10.1515/pac-2018-1224

Abstract: The role of muscarinic receptors has been largely documented over the past few decades. Recently we demonstrated that the activation of M2 muscarinic receptors arrested cell proliferation and induced apoptosis in glioblastoma and in other tumour types. This paper aims to evaluate the expression of the M2 muscarinic receptor subtypes in different neuroblastoma cell lines and its role in the control of cell proliferation and survival. Neuroblastoma is the most common solid extracranial tumour, appearing during childhood and displaying a differentiated clinical behaviour. Considering the high homology between muscarinic receptor subtypes, we have identified Arecaidine Propargyl Ester (APE) as a selective orthosteric agonist for M2 muscarinic receptors. Using this agonist, we demonstrate how a selective activation of the M2 receptor subtype negatively modulates cell growth without affecting cell survival in different human neuroblastoma cell lines. As similarly demonstrated in other cell types, following the M2 receptor silencing by short-interference RNA, the effects of APE are completely abolished. We conclude by confirming the ability of APE to bind selectively M2 muscarinic receptor subtypes. Moreover, for the first time we demonstrate that M2 receptor activation inhibits cell growth also in human neuroblastoma cells, indicating that M2 receptors may be an interesting therapeutic target in several solid tumours.

Keywords: cell proliferation; Eurasia 2018; M2 receptors; neuroblastoma; orthosteric ligand; survival.

Introduction

Muscarinic receptors belong to the G-proteins coupled receptors (GPCRs) family. Five muscarinic receptor subtypes were cloned in different mammalian and non-mammalian species [1]. Variance among subtypes is only limited to the carboxyl- and amino terminals of the proteins and to the third cytoplasmic loop [2]. The identification of selective ligands for muscarinic receptors has encountered difficulties due to the high degree of homology between subtypes and the frequent co-expression of more than one receptor in same tissue. Conversely, the lack of selective agonists has long limited their use in the treatment of different pathologies.

Article note: A collection of invited papers based on presentations at the 15th Eurasia Conference on Chemical Sciences (EuAsC2S-15) held at Sapienza University of Rome, Italy, 5–8 September 2018.

***Corresponding author: Ada Maria Tata,** Department of Biology and Biotechnologies "Charles Darwin", "Sapienza" University of Rome, Rome 00185, Italy; and Research Centre of Neurobiology "Daniel Bovet", "Sapienza" University of Rome, Rome 00185, Italy, e-mail: adamaria.tata@uniroma1.it

Anna Maria Lucianò, Francesca Mattei, Elisa Damo and Luciana Dini: Department of Biology and Biotechnologies "Charles Darwin", "Sapienza" University of Rome, Rome 00185, Italy

Elisa Panzarini: Department of Biological and Environmental Sciences and Technologies (Di.S.Te.B.A.), University of Salento, Lecce, Italy

In fact, the roles of muscarinic receptors in different neurodegenerative and neurological diseases as well as in cancer is well known [3–6]. The identification of muscarinic agonists has proved to be even more complex. Several studies in systems expressing either native or transfected receptor subtypes indicated that the effectiveness of an agonist is not only dependent on the subtype's selective binding affinity, but can be modified also by the availability of the specific receptor subtype [7]. Generally, when we consider molecules with agonist function, we think of several ligands able to interact with the orthosteric site of the receptor. In general, non-selective orthosteric agonists are more common, while it is more difficult to identify agonists able to bind selectively with only one muscarinic receptor subtype (Fig. 1).

Selective binding of a single receptor subtype followed by its functional activation was used to design muscarinic agonists, and potentially in treating specific pathologies (Table 1). In the past, we identified a new M2 receptor subtype agonist named Arecaidine Propargyl Ester (APE). This molecule is a synthetic derivative from Arecoline, an alkaloid extracted by the areca nut, the fruit of the areca palm. Arecoline is a partial agonist of M_1, M_2, M_3 and M_4 muscarinic acetylcholine receptors. On the other hand, its synthetic derivate APE shows high selectivity for M2 subtype, as previously demonstrated by pharmacologically binding experiments [8, 9]. In our previous study, we demonstrated the ability of APE to inhibit cell proliferation and survival in different tumour types via M2 muscarinic receptors [10–12]. Neuroblastoma is the most common type of solid extracranial tumour, appearing during childhood. Among the solid childhood tumours, neuroblastoma is the second most widespread with an incidence of 10 cases per million each year. It originates from the neural crest cell, with prevalence in those cells that should form the sympathetic nervous system and adrenal medulla [13]. The most aggressive form of tumour shows amplification of N-myc, whose overexpression is usually associated with poor survival [14]. Considering the inability of all therapies available to efficiently arrest tumour progression, the identification of new drugs able to impair proliferation and cell survival appears relevant to design new therapeutic protocols. Premised on our previous studies, we have evaluated the ability of M2 orthosteric agonist APE to modulate cell proliferation and survival in human neuroblastoma cell lines and

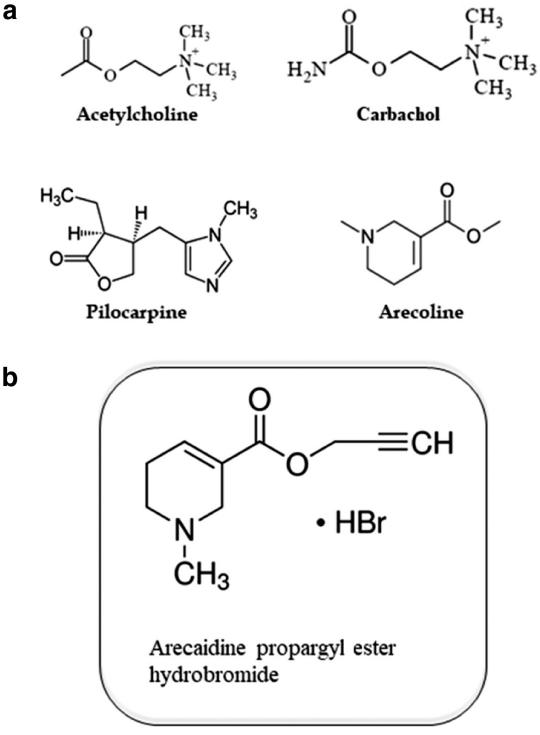
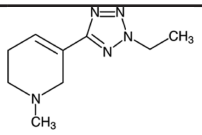
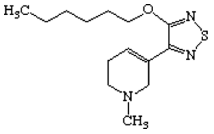
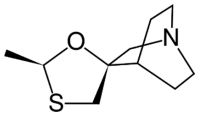
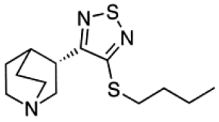


Fig. 1: (a) General structural skeleton of non-selective muscarinic agonists (b) structure of Arecaidine propargyl ester hydrobromide, M2 receptor orthosteric agonist.

Table 1: Muscarinic receptor orthosteric agonists approved for therapeutic protocols.

	Receptor selectivity	Molecular structures	Therapeutic application
Alvameline	M1 agonist		Alzheimer's disease
Xanomeline	M1 > M4 agonist		Alzheimer's disease
Cevimeline	M1 agonist		Sjogren's disease
Vadaclidine (LY297802)	M4 agonist		Antinociceptive effects

neuro-epithelioma cells (SK-N-SH, SK-N-BE, SK-N-MC). Evidence obtained demonstrates the inhibitory effect of an M2 agonist on neuroblastoma cell growth without affecting cell survival.

Materials and methods

Cell cultures

Human Neuroblastoma cell line SK-N-BE was cultured in RPMI 1640 (Roswell Park Memorial Institute, Sigma, St. Louis, MO, USA) supplemented with 10 % fetal bovine serum (FBS) (Immunological Sciences, Rome, Italy), 50 mg/ml streptomycin, 50 IU/ml penicillin, 2 mM glutamine (Sigma), and maintained at 37 °C, in atmosphere of 90 % of air and 5 % of CO_2. Human Neuroblastoma cell line SK-N-SH was cultured in DMEM (Dulbecco's Modified Eagle's Medium, Sigma) supplemented with 10 % FBS, 50 mg/ml streptomycin, 50 IU/ml penicillin, 2 mM glutamine (Sigma), and, 1 % non-essential amino-acids (Sigma) and maintained at 37 °C, in atmosphere of 90 % of air and 10 % of CO_2. Human Neuro-epithelioma cell line SK-N-MC was cultured in MEM (Minimum Essential Medium, Sigma) supplemented with 10 % FBS, 50 mg/ml streptomycin, 50 IU/ml penicillin, 2 mM glutamine (Sigma), and, 1 % non-essential amino-acids (Sigma) and maintained at 37 °C, in atmosphere of 90 % air and 10 % of CO_2.

Cell viability assays

The cells were seeded on 24-well plate at the density of 2×10^4 cells/well. After 24 h cells were treated with cholinergic agonist Arecaidine propargyl ester hydrobromide (***1-Methyl-1,2,5,6-tetrahydro-3-pyridine carboxylic acid propargyl ester hydrobromide;*** **APE**) (Sigma Aldrich, St. Louis, MO, USA) [11, 12] at dif-

ferent time points (ranging from 24 to 72 h). Cell growth was assessed by colorimetric assay based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA) metabolization according to Mossman method [20]. For each well, the OD at 570 nm was measured by GloMax Multi Detection System (Promega, Madison, WI, USA). Cell viability was also determined by trypan blue staining. Trypan blue solution (Sigma-Aldrich, St. Louis, MO, USA) was added to cell suspension (1:10; v/v). After 5 min, the number of blue-stained (non-viable) and unstained cells (viable) was counted by Bürcker chamber. The data were calculated as the percentage of dead on total cells (see Table 2).

Western blot

Cells were lysed with Lysis buffer (Tris 10 mM, NP40 0.5 %, NaCl 150 mM) containing protease inhibitors (50×; Sigma). Protein extracts were loaded on 10 % SDS-polyacrylamide gel (PAGE) and transferred to PVDF membrane (Merck Millipore, KGaA, Darmstadt, Germany). Membranes were blocked for 1 h in 5 % of non-fat milk powder (Sigma-Aldrich, St. Louis, MO, USA) in PBS containing 0.1 % Tween-20 (Sigma), and then incubated with monoclonal anti-M2 antibody (1:800 Abcam, Cambridge, UK) overnight at 4 °C. Blots were washed with PBS + Tween and then incubated for 1 h with anti-mouse secondary antibody horseradish-peroxidase-conjugated (Promega Italia, MI, Italy). Signal was revealed by ECL reagent (Immunological Science, MI, Italy). Immunoreactive bands were visualized in a Chemidoc system (Molecular Imager ChemiDoc XRS + System with Image Lab Software, Biorad, CA, USA) and the optical density of the bands quantified by ImageJ software (National Institutes of Health). Beta-actin was used as protein reference for loading control.

RNA extraction and RT-PCR analysis

Total RNA was extracted using Total RNA extraction Mini kit (FMB, PA, USA) following the manufacturer' instructions, and then digested with DNAse I (Ambion-Life Technologies Italia, Monza, Italy). For each sample,

Table 2: Percentage of dead cells in untreated and 100 μM APE treated cells.

	% Dead cells	±SEM	*p*-Value
SK-N-BE			
Ctrl 24 h	10.77	±0.92	
APE 24 h	12.17	±0.50	0.229
Ctrl 48 h	10.07	±2.43	
APE 48 h	13.87	±3.20	0.398
Ctrl 72 h	10.52	±1.91	
APE 72 h	11.79	±1.56	0.624
SK-N-SH			
Ctrl 24 h	4.98	±1.08	
APE 24 h	6.40	±0.72	0.334
Ctrl 48 h	8.73	±0.89	
APE 48 h	12.30	±0.53	0.025
Ctrl 72 h	12.73	±0.69	
APE 72 h	22.23	±0.64	**0.0003**
SK-N-MC			
Ctrl 24 h	8.33	±1.25	
APE 24 h	10.56	±0.70	0.169
Ctrl 48 h	8.73	±0.17	
APE 48 h	13.92	±1.12	**0.003**
Ctrl 72 h	12.85	±3.15	
APE 72 h	17.55	±1.07	0.183

Bold values are significant.

2 µg of total RNA was reverse transcribed for 60 min at 37 °C with Random Primers (Promega, Madison, WI, USA) and M-MLV reverse transcriptase (Promega, Madison, WI, USA). Primers and GoTaq Green Master Mix (Promega, Madison, WI, USA) were added to 100 ng of cDNA. The expression of all the five muscarinic receptors transcripts was evaluated by semi-quantitative RT-PCR analysis using the following primers:

- M1: up 5′-agagagaccctgccaacttt-3′;
  low 5′-ctcctgactttcctgcctaaa-3′
- M2: up 5′-ccaagaccccgtttctccaag-3′;
  low 5′-ccttctcctctcccctgaacac-3′
- M3: up 5′-cgctccaacaggaggaagta-3′;
  low 5′-ggagttgaggatggtgctgt-3′
- M4: up 5′-aatgaagcaagagcgtcaagaa-3′;
  low 5′-tcattggaagtgtccttatca-3′
- M5: up 5′-cctggctgatctccttcatc-3′;
  low 5′-gtccttggttcgcttctctg-3′
- c-myc up 5′-cagcagcgactctgaggagg-3′
  low 5′-gactctgacctttgccagga-3′
- N-myc up 5′-cctccatgacagcgctaaac-3′
  low 5′-aggcggatgtgtcaatggta-3
- 18 s; up-5′-ccagtaagtgcgggtcataagc-3′
  low, 5′-aacgatccaatcggtagtagcg-3′

Knockdown of M2 receptor

SK-N-BE cells were plated in 6-well plates at the density of 3×10^5 cells/well or in 24-well plate at the density of 5×10^3 cell/well for western blot analysis and MTT assay, respectively. After 24 h were transfected with a pool of siRNAs selective for human M2 receptor (CHRM2) as previously described [11, 12]. The transfection was carried out for 60 h with 60 nM M2-siRNA pool (Riboxx Life Sciences, Radebeul, Germany) using the transfection reagent HappyFect (Tecrea, London, UK) according to the manufacturer's instructions. Cells were harvested 60 h following transfection and western blot analysis was performed to determine the level of M2 receptor. For the analysis of cell growth by MTT assay, 100 mM APE was added for additional 48 h in fresh medium (except for the control) after transfection. Sequences of the M2-siRNA pool used were previously described [12].

Statistical analysis

Student's t test and one-way ANOVA test followed by Bonferroni's post test were used to evaluate statistical significance within the different samples. Results were considered statistically significant at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

Results and discussion

Expression of muscarinic receptors in neuroblastoma cell lines

We have investigated the effects of M2 muscarinic receptors in several neuroblastoma cell lines. The semi-quantitative RT-PCR analysis showed that transcript for all five muscarinic receptors subtypes were expressed in SK-N-BE. Only the M4 transcript appeared absent or faintly expressed in SK-N-SH and SH-N-MC (Fig. 2a). Interestingly, M2 mRNA appeared expressed in all cell lines, although at a different level.

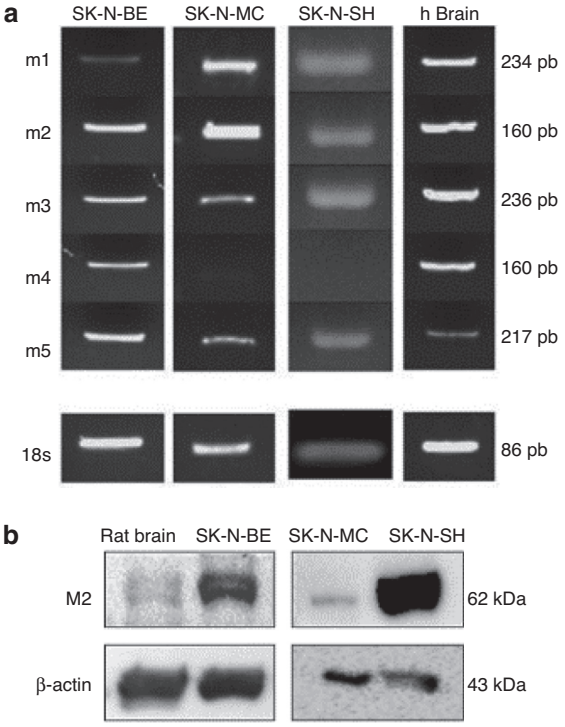


Fig. 2: (a) Representative RT-PCR analysis for different muscarinic receptor subtypes in neuroblastoma cell lines (SK-N-BE; SK-N-SH) and in neuroepithelioma cell line SK-N-MC. Human brain RNA was used as positive control. 18s was used as housekeeping gene. (b) Representative western blot analysis for M2 receptor expression in neuroblastoma cell lines. Rat brain was used as positive control. β-Actin was used as reference protein.

Western blot analysis revealed the expression of M2 receptor proteins in different neuroblastoma cell lines (Fig. 2b). As results, the level of the expression of the M2 protein appeared inversely correlated with the mRNA levels. In fact where the M2 transcript level was apparently lower (SK-N-SH), the protein level results higher (Fig. 2a and b).

Analysis of cell growth and survival

Albeit several muscarinic receptor subtypes were expressed in neuroblastoma cell lines, the activation of all muscarinic receptors by no selective orthosteric agonist muscarine (100 μM) did not induce any modifications in neuroblastoma cell proliferation (see Supplementary Fig. 1). This effect is probably dependent on the simultaneous activation of all muscarinic receptor subtypes that activating several signal transduction pathways, may induce a balance between the second messengers downstream different muscarinic receptor subtype activation. On the contrary, previous research demonstrated how the M2 agonist APE is able to significantly inhibit cell growth in different tumour types (glioblastoma and urothelial bladder cancer cells) [10–12]. Considering the expression of the M2 subtype also in neuroblastoma cell lines, we investigated the ability of APE to modulate cell growth in three different of them. The MTT assay revealed the ability of APE to downregulate cell growth, although in unequal manners in the three cell lines (Fig. 3). In particular, the low concentration of APE (3 and 12.5 μM) did not appear to affect neuroblastoma cell growth. In accordance with previous studies [10–12], only 50 and 100 μM concentrations appeared to significantly inhibit cell growth. A 25 μM APE concentration inhibited cell growth at a lower level only in the SK-N-SH cell line. It is interesting to observe how the sensitivity of neuroblastoma cell line is directly correlated to the M2 protein levels. In fact, the cell line that resulted more responsive to M2 agonist was the SK-N-SH, which showed higher levels of the M2 receptor protein. Contrarily, the SK-N-MC cell line resulted

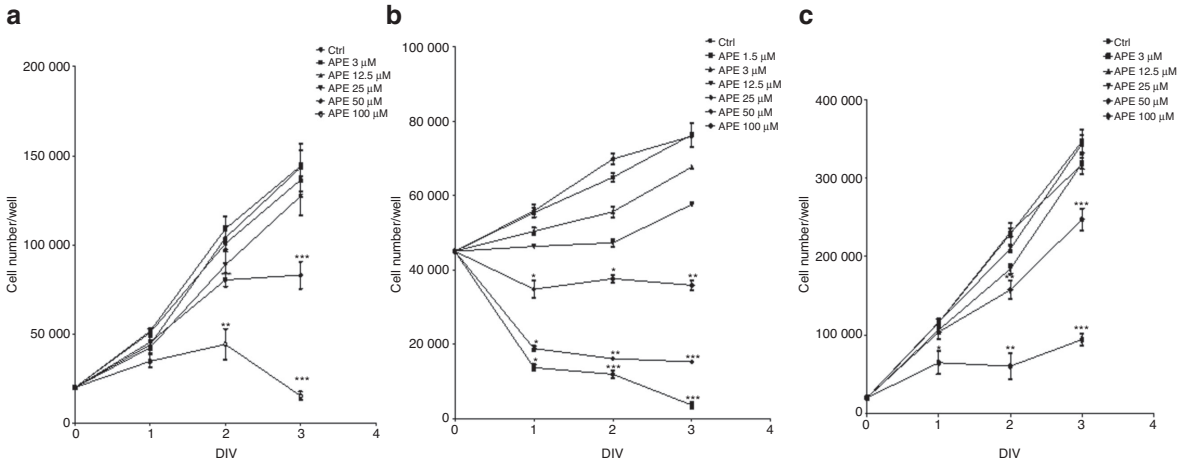


Fig. 3: Analysis of cell growth by MTT assay at different APE concentrations at time points of treatment (Day *in vitro*, DIV). (a) SK-N-BE; (b) SK-N-SH; (c) SK-N-MC. Data are the mean ± S.E.M. of three independent experiments performed in triplicate (*p* value: APE treated cells vs. ctrl) (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$).

more resistant to the treatment; apparently, 3, 12.5, 25, and 50 μM APE concentrations did not cause any significant reduction of cell growth, but only a mere slowdown.

In order to explain whether a reduction in cell growth had been the result of decreased cell proliferation or survival, we have assessed the number of dead cells using the exclusion test by trypan blue staining. The micrographs reported in Fig. 4 showed that the cells treated with 100 μM APE, presented a decreased cell density compared with untreated cells (control condition). However, there were no cells clearly detached from the substrate thereby signalling absence of dead cells.

This observation was confirmed by the data obtained by trypan blue staining, which showed a percentage of dead cells in APE-treated samples not significantly different from the respective control in all experimental conditions and in all three cell lines (Table 2). Seemingly, an increase in dead cells resulted after 48 h of APE treatment only in SK-N-MC. This result proves how APE has no toxic effect on neuroblastoma cells in general, as reported in the case of other tumours [15]. Altogether these evidence suggests that APE may negatively modulate cell proliferation in neuroblastoma and hence cause a slowdown of cell division at low doses (12.5, 25 μM), paired with an arrest of cell proliferation when used at high doses (50 and 100 μM). This notwithstanding, the three cell lines respond differently depending on APE concentration. This may depend upon levels of expression of the M2 receptors as well as the possibly different binding affinity for APE.

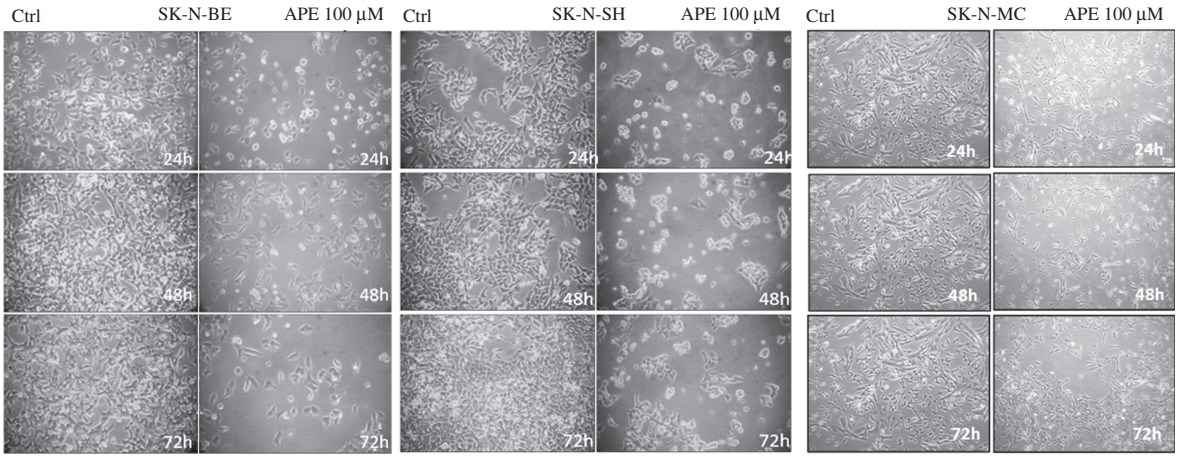


Fig. 4: Neuroblastoma cell lines in control conditions and after APE (100 μM) treatment at 24 h. 48 h and 72 h of treatment (100×).

In order to confirm that APE selectively binds only M2 receptor subtypes and to exclude that the high dose of APE may activate other muscarinic receptor subtypes, the SK-N-BE cells were transfected with a pool of siRNA, able to knock down the expression of human M2 muscarinic cholinergic receptors (CHRM2). In this instance, our western blot analysis demonstrated how after siRNA transfection (60 nM each/well) the expression of M2 receptors was significantly reduced (Fig. 5a). MTT assay performed in un-transfected cells and in M2 siRNA transfected cells, in the absence or in the presence of 100 μM APE, showed that the decreased cell number observed with APE in un-transfected cells disappeared after siRNA transfection (Fig. 5b).

Negative modulation of protoncogenes by M2 receptor activation

In order to explain the possible mechanism causing the decreased neuroblastoma cell proliferation after APE treatment, we investigate the ability of APE to modulate the expression of protoncogenes implicated in the neuroblastoma tumorigenesis. Upregulation or genomic amplification of oncogenes c-myc and N-myc are associated to high tumor malignancy and poor prognosis for the patients [16, 17]. Considering the role played by these oncogenes in neuroblastoma cell proliferation, we investigated the ability of APE to modulate the expression of c-myc and n-myc expression in all neuroblastoma cells lines considered in the present study. The analysis by RT-PCR analysis has clearly indicated the ability of M2 agonist APE to negatively modulate the expression of both oncogenes expression in SK-N-BE and SH-N-MC (Fig. 6a, b). SK-N-SH instead does not express N-myc as previously demonstrated [18], but only c-myc. However the expression of c-myc appeared down-regulated after APE treatment also in SK-N-SH (Fig. 6c).

Conclusions

The results obtained in this paper, following suit from our previous data [11, 12] confirm the APE as preferred M2 orthosteric muscarinic agonist. Considering that M2 receptors in general have inhibitory effects, we have dem-

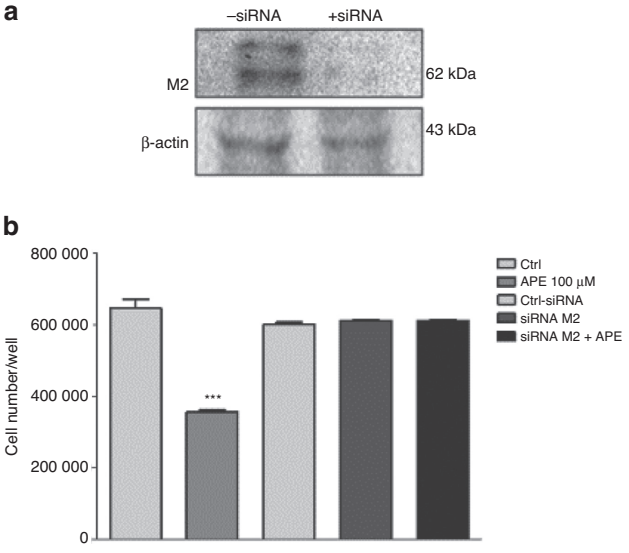


Fig. 5: (a) Representative Western blot analysis for M2 receptor expression in SK-N-BE cells in the absence and in the presence of 60 nM M2-siRNA pool. β-Actin was used as a reference protein. (b) MTT assay performed in SK-N-BE cells after 60 h of siRNA transfection and 48 h of APE (100 μM) treatment. Data are the mean ± S.E.M. of three independent experiments performed in triplicate. (APE vs. ctrl: ***$p < 0.001$) (APE siRNA M2 vs. ctrl siRNA M2: $p = 0.32$ *n.s.*).

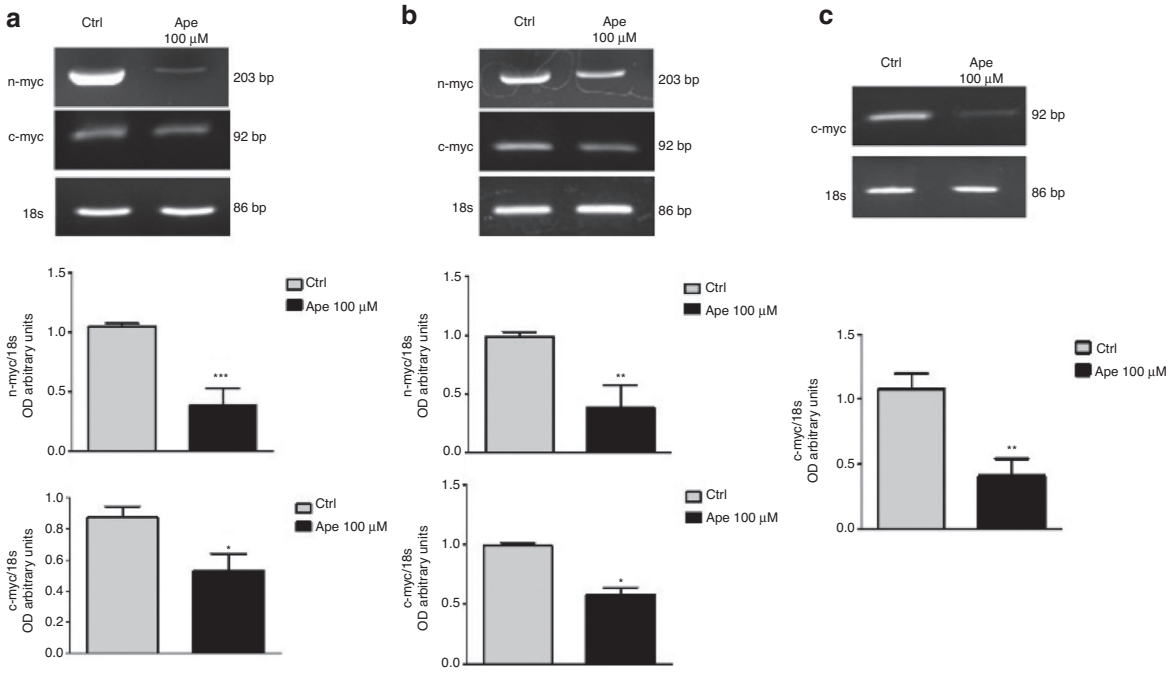


Fig. 6: Analysis by RT-PCR of c-myc and n-myc expression after 24 h of treatment with 100 μM APE. (a) SK-N-BE; (b) SK-N-MC; (c) SK-N-SH. The graphs below indicate the densitometric analysis of the bands normalized with 18 s, used as housekeeping gene. The results are the mean ± S.E.M. of three independent experiments (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

onstrated that its selective activation in neuroblastoma as well as in other tumour types (glioblastoma, breast cancer and urothelial bladder cancer cells) can cause a significant inhibition of tumour cell proliferation.

In particular in neuroblastoma, the anti-proliferative effects mediated by M2 receptor activation may be mediated by the downregulated expression of c-myc and N-myc oncogenes, largely involved in neuroblastoma cell proliferation and malignancy.

Altogether, these data confirm the M2 receptors as a new promising therapeutic target in cancer therapy. However, further research should aim at identifying a more selective agonist, able to produce similar effects but at a lower concentration in order to reduce potential side effects of high dosage of APE. Recently we have tested a new M2 dualsteric muscarinic agonist that may come out as an excellent therapeutic device for treatment of several solid tumours [7, 19].

Acknowledgments: This work was supported by Ateneo Sapienza, University of Rome, and FABBR 2018 funds (MIUR) to A.M.T. The authors are grateful for G. Lucianò's English revision.

Conflict of Interest: The authors declare there are no conflicts of interest.

References

[1] E. C. Hulme, N. J. M. Birsall, N. J. Buckley. *Annu. Rev. Pharmacol. Toxicol*. **30**, 33 (1990).
[2] E. G. Peralta, A. Ashkenazi, J. W. Winslow, D. H. Smith, J. Ramachandran, D. J. Capon. *EMBO J.* **6**, 3923 (1987).
[3] C. C. Felder, F. P. Bymaster, J. Ward, N. Delapp. *J. Med. Chem.* **43**, 4333 (2000).
[4] A. Confaloni, G. Tosto, A. M. Tata. *Curr. Pharm. Des*. **22**, 20150 (2016).
[5] F. De Angelis, A. M. Tata. *Cent. Nerv. Syst. Ag. Med. Chem*. **16**, 218 (2016).
[6] M. Di Bari, G. Di Pinto, M. Reale, G. Mengod, A. M. Tata. *CNS Agents Med. Chem*. **17**, 109 (2017).
[7] C. Matera, A. M. Tata. *Rec. Pat. CNS Drug Discov*. **9**, 85 (2014).
[8] R. Piovesana, S. Melfi, M. Fiore, V. Magnaghi, A. M. Tata. *J. Cell Physiol*. **233**, 5348 (2018).

[9] S. Loreti, R. Ricordy, M. E. De Stefano, G. Augusti-Tocco, A. M. Tata. *Neuron Glia Biol.* **3**, 269 (2007).
[10] L. Pacini, E. De Falco, P. Ruggieri, C. Fabbiano A. L. Pastore, G. Palleschi, M. Di Bari, V. Petrozza, A. Carbone, A. M. Tata, A. Calogero. *Canc. Biol. Ther.* **15**, 1489 (2014).
[11] M. Ferretti, C. Fabbiano, M. Di Bari, C. Conte, E. Castigli, M. Sciaccaluga, D. Ponti, P. Ruggieri, A. Raco, R. Ricordy, A. Calogero, A. M. Tata. *J Cell Mol. Med*. **17**, 552 (2013).
[12] F. Alessandrini, I. Cristofaro, M. Di Bari, J. Zasso, L. Conti, A. M. Tata. *Int. Immunopharmacol*. **29**, 105 (2015).
[13] C. U. Louis, J. M. Sholet. *Ann. Rev. Med.* **66**, 49 (2015).
[14] N. Tanaka, M. Fukuzawa. *Int. J. Oncol*. **33**, 815 (2008).
[15] M. Di Bari, V. Tombolillo, C. Conte, E. Castigli, M. Scaccaluga, E. Iorio, G. Carpinelli, R. Ricordy, M. Fiore, F. Degrassi, A. M. Tata. *Neurochem. Int*. **90**, 261 (2015).
[16] M. W. Zimmerman, Y. Liu, S. He, A. D. Durbin, B. J. Abraham, J. Easton, Y. Shao, B. Xu, S. Zhu, X. Zhang, Z. Li, N. Weichert-Leahey, R. A. Young, J. Zhang, A. Thomas Look. *Cancer Discov*. **8**, 320 (2018).
[17] D. S. Rickman, J. H. Schulte, M. Eilers. *Cancer Discov.* **8**, 150 (2018).
[18] E. Piccinni, A. Chelstowska, J. Hanus, P. Widlak, S. Loreti, A. M. Tata, G. Augusti-Tocco, M. M. Bianchi, R. Negri. *Acta Biochim. Pol.* **58**, 529 (2011).
[19] I. Cristofaro, Z. Spinello, C. Matera, M. Fiore, L. Conti, M. De Amici, C. Dallanoce, A. M. Tata. *Neurochem. Int.* **118**, 52 (2018).
[20] T. Mosmann. *J. Immunol. Methods* **65**, 55 (1983).

Supplementary Material: The online version of this article offers supplementary material (https://doi.org/10.1515/pac-2018-1224).

Graphical abstract

Anna Maria Lucianò, Francesca Mattei, Elisa Damo, Elisa Panzarini, Luciana Dini and Ada Maria Tata

Effects mediated by M2 muscarinic orthosteric agonist on cell growth in human neuroblastoma cell lines

https://doi.org/10.1515/pac-2018-1224
Pure Appl. Chem. 2019; x(x): xxx–xxx

Conference paper:

Keywords: cell proliferation; Eurasia 2018; M2 receptors; neuroblastoma; orthosteric ligand; survival.