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PII: S0014-2999(16)30665-3  
DOI: <http://dx.doi.org/10.1016/j.ejphar.2016.10.027>  
Reference: EJP70893

To appear in: *European Journal of Pharmacology*

Received date: 2 August 2016  
Revised date: 17 October 2016  
Accepted date: 20 October 2016

Cite this article as: Annunziatina Laurino, Rosanna Matucci, Giulio Vistoli and Laura Raimondi, 3-iodothyronamine (T1AM), a novel antagonist of muscarinic receptors, *European Journal of Pharmacology* <http://dx.doi.org/10.1016/j.ejphar.2016.10.027>

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**3-iodothyronamine (T1AM), a novel antagonist of muscarinic receptors**

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**Abstract**

3-iodothyronamine (T1AM) is a trace amine suspected to derive from thyroid hormone metabolism. T1AM was described as a ligand of G-protein coupled monoaminergic receptors, including trace amine associated receptors, suggesting the amine may exert a modulatory role on the monoaminergic transmission. Nothing is known on the possibility that T1AM could also modulate the cholinergic transmission interacting with muscarinic receptors.

We evaluated whether T1AM (10 nM- 100  $\mu$ M) was able to i) displace [ $^3$ H]-NMS (0.20 nM) binding to membrane preparations from CHO cells stably transfected with human muscarinic receptor subtypes (M1-M5); ii) modify basal or acetylcholine induced pERK<sub>1/2</sub> levels in CHO expressing the human muscarinic type 3 receptor subtype by Western blot iii) modify basal and carbachol-induced contraction of isolated rat urinary bladder. T1AM fitting within rat muscarinic type 3 receptor was simulated by Docking studies.

T1AM recognized all muscarinic receptor subtypes (pK<sub>i</sub> values in the micromolar range). Interacting at type 3, T1AM reduced acetylcholine-increased pERK<sub>1/2</sub> levels. T1AM reduced carbachol-induced contraction of the rat urinary bladder. The fenoxyl residue and the iodide ion were found essential for establishing contacts with the active site of the rat muscarinic type 3 receptor subtype.

Our results indicate that T1AM binds at muscarinic receptors behaving as a weak, not selective, antagonist. This finding adds knowledge on the pharmacodynamics features of T1AM and it may prompt investigation on novel pharmacological effects of T1AM at

conditions of hyper-activation of the muscarinic tone including the overactive urinary bladder.

**Keywords:** 3-iodothyronamine; 3-iodothyroacetic acid; thyroid; muscarinic receptors.

Accepted manuscript

## 1. Introduction

3-iodothyronamine (T1AM) is an iodinated primary amine circulating in trace in human and rodents (Galli et al., 2012) (Saba et al., 2010). T1AM is produced in the thyroid as well as in extra-thyroid tissues (Hoefig et al., 2012) (Hoefig et al., 2015) and is rapidly metabolized by monoamine oxidases (MAO) and deiodinase activities to 3-iodothyroacetic acid (TA1) and iodothyronamine, respectively (Hackenmueller et al., 2012). The physiological meaning of T1AM tissue levels is currently unknown. Much is instead known on the pharmacological effects of T1AM. In this respect, we described that T1AM acutely modifies mice behavior and metabolism (Manni et al., 2012) (Manni et al., 2013) (Laurino et al., 2015) with a mechanism which remains to be defined but that depends, at least in part, on the activation of the histaminergic system and on T1AM biotransformation into TA1 (Manni et al., 2012)(Manni et al., 2013) (Laurino et al., 2015). In 2004, Scanlan et al. described the hibernating effect of T1AM while recently James et al (2013) reported a pro-awaking effect of T1AM in rats.

Notwithstanding the increasing numbers of hypotheses, the mechanisms responsible for such effects remain elusive yet.

However, T1AM has the potential to interact at several targets as documented by different experimental approaches. Scanlan et al. (2004) described T1AM as a high affinity ligand (in the nanomolar range of affinity) for the trace amine-associated receptor type 1 (TAAR1). Later, by virtue of its  $\beta$ -phenylethyl skeleton, *in-vitro* studies indicated T1AM recognized, in the micromolar range of concentrations, monoaminergic targets including beta 2 and alpha 2 receptors (Regards et al., 2007) (Dinter et al., 2015), working as an agonist and as an inverse agonist respectively (Dinter et al., 2015a) (Dinter et al., 2015b). More recently, Dinter et al. (2015) reported T1AM as an

inverse agonist of TAAR type 5 (TAAR5) (Dinter et al., 2015c). Further, recent electrophysiological evidence indicated T1AM (in the range of 0.1-10  $\mu$ M) as an activator of the TRPM8 cold channel (Lucius et al., 2016). Except for alpha2 adrenoceptors, whether any of these targets are involved in pharmacological effects of T1AM are currently unknown. This uncertainty also includes the involvement of TAAR1. In fact, Chiellini et al. (2005), reported findings which argued against the involvement of TAAR1 in T1AM-induced reduction of body temperature. However, the relationship between TAAR1 and T1AM would merit to be further explored in light of the findings from Szumska et al (2015). These authors reported TAAR1 expression on thyroid follicles, a localization supporting their role, and of T1AM, its endogenous ligand, in thyroid regulation. Taken together all these evidence indicate that T1AM, as other trace amines, may be considered a modulator of the monoaminergic transmission (Borowsky et al., 2001). Nothing is known instead about the possibility that T1AM could also modulate the cholinergic transmission interacting with muscarinic receptors.

Muscarinic receptors are a family of five different G-coupled receptors (M1-M5) linked to two main intracellular cascades governed by Gi or Gq proteins. Muscarinic receptors are expressed at the central nervous system, where they have a main role in memory and pain circuits, and in the periphery where they are involved in smooth muscle contraction, in exocrine and endocrine gland secretion, including the thyroid (Szumska et al., 2015) and the pancreas (Nakajima et al., 2013). The recognition of muscarinic receptors by T1AM might rationally sustain some pharmacological effects of the amine and open new perspectives for investigating unexplored T1AM functions governed by muscarinic signaling, including the control of visceral smooth muscle tone.

To explore this possibility we performed binding experiments with T1AM and with TA1 on human muscarinic receptor subtypes (M1-M5) stably expressed in CHO cells. Then, to investigate on the agonistic/antagonistic nature of T1AM towards muscarinic receptors, we evaluated whether T1AM modified i) basal or acetylcholine-induced pERK1/2 levels in CHO stably transfected with human muscarinic type 3 receptor and ii) basal or carbachol-induced contraction of the rat urinary bladder.

## 2. Materials and methods

### 2.1 Animals

Male Sprague Dawley rats (275–300 g) from ENVIGO (San Pietro al Natisone, Udine, Italy) were used. Animals were kept at  $23 \pm 1^\circ\text{C}$  with a 12 h light–dark cycle (light on at 07:00 h) and were fed a standard laboratory diet with water *ad libitum*.

Experiments and animal use procedures were in compliance with ARRIVE guidelines (McGrath et al., 2010). All the experimental procedures were in accordance with EU Directive 2010/63/EU for animal experiments.

### 2.2 Membrane preparation

CHO cells stably expressing cDNA encoding the human muscarinic receptors (M1-M5) were generously provided by Prof. R. Maggio (Department of Experimental Medicine, University of L'Aquila, Italy).

Confluent CHO cell lines were trypsinized, washed with buffer (25 mM sodium phosphate-containing 5 mM  $\text{MgCl}_2$  at pH 7.4) and homogenized for 30 s using an Ultra-Turrax (16,000 rpm). The pellet was centrifuged ( $17,000 \times g$  for 15 min at  $4^\circ\text{C}$ ) and the

membranes were resuspended in the same buffer, re-homogenized with Ultra-Turrax and stored at  $-80^{\circ}\text{C}$  until use.

### 2.2.1 Binding assay in CHO cells

The radioligand binding assay was run in polypropylene 96-well plates (Sarstedt, Verona, Italy) and performed for 120 min at room temperature in a final volume of 0.25 mL of 25 mM sodium phosphate buffer, pH 7.4, containing 5 mM  $\text{MgCl}_2$ . Final membrane protein concentrations were: 30  $\mu\text{g}/\text{mL}$  for human muscarinic type 1, 70  $\mu\text{g}/\text{mL}$  for human muscarinic type 2, 25  $\mu\text{g}/\text{mL}$  for human muscarinic type 3, 50  $\mu\text{g}/\text{mL}$  for human muscarinic type 4 and 25  $\mu\text{g}/\text{mL}$  for human muscarinic type 5. In heterologous competition curves, fixed concentrations of the tracer (0.2 nM [ $^3\text{H}$ ]-NMS, ca.  $K_D$ ) were displaced by increasing concentrations of T1AM and TA1 (Dei et al., 2005). All measurements were performed in duplicate. At the end of the binding reaction, free radioligand was separated from bound ligand by rapid filtration through UniFilter GF/C plates (Perkin–Elmer Life and Analytical Science, Boston, MA, USA) using a FilterMate Cell Harvester (Perkin–Elmer Life and Analytical Science, Boston, MA); after filtration, the filters were washed several times with ice cold water, allowed to dry overnight at room temperature under air flow. 25  $\mu\text{l}$  of scintillation liquid (Microscint-20, Perkin–Elmer Life and Analytical Science, Boston, MA) were then added and the filter-bound radioactivity counted by TopCount NXT Microplate Scintillation Counter (Perkin–Elmer Life and Analytical Science, Boston, MA).

Off-Rate assays were performed to estimate the affinity of T1AM for the [ $^3\text{H}$ ]NMS occupied receptor. In these experiments, designed to investigate the effects of a range of



modulator concentrations on the [ $^3\text{H}$ ]NMS dissociation rate, a “one-point kinetic assay” approach was used, where the effect on radioligand dissociation of the test ligand was determined at 0 min and at one time point, which was chosen to be ca 2.5 dissociation half-lives of [ $^3\text{H}$ ]NMS alone (Lazareno et al., 1995). A high concentration of human M1-M5 CHO membranes (50-75  $\mu\text{g}/\text{ml}$ ) was incubated with a high concentration of [ $^3\text{H}$ ]NMS (2 nM) for about 60 min at room temperature. Then, 100  $\mu\text{l}$  aliquots were distributed into tubes which contained 10  $\mu\text{l}$  of 100  $\mu\text{M}$  atropine alone, or in the presence of a different concentrations of test compounds, and diluted in 1 ml total volume of buffer. The time zero data point was obtained using only 100  $\mu\text{l}$  of the mixture containing membranes plus [ $^3\text{H}$ ]NMS. Incubation (100 min for hM<sub>3</sub>) was terminated by rapid filtration through Whatman GF/C filters (Brandel, USA), that had been presoaked in a 0.05 % polyethylenimine (PEI) solution for at least 1 h, using a Brandell cell harvester (Biomedical Research and Development Laboratory, Inc Atlas Drive, Gaithersburg, MD, USA). Filters were washed three times with 3 ml aliquots of ice-cold milliQ water and dried before the addition of 4.5 ml of scintillation cocktail (Filter Count, Perkin-Elmer Life and Analytical Science, Monza, Italy). The radioactivity was determined using scintillation counting (TRI-CARB 1100, Perkin-Elmer Life and Analytical Science, Monza, Italy).

### 2.3 Docking studies

All derivatives were simulated in their protonated state, that one involved in ligand recognition by rat muscarinic type 3 receptor. The ligand's conformational profile was

analyzed by MonteCarlo simulations which produced 1000 conformers by randomly rotating the rotatable bonds. In detail, the study was focused on the X-ray structure of the rM3R in complex with tiotropium (PDB Id: 4DAJ). The protein structure was prepared by removing the water molecules and all crystallization additives and then underwent energy minimation by keeping the backbone atoms fixed to preserve the resolved folding. After deleting the bound ligand, the so optimized protein structure was utilized by docking simulations as performed by using Plants which generates reliable ligand poses by ant colony optimization (ACO) algorithms (Korbe et al., 2009).

In detail, the search was focused on a 10 Å radius sphere around the key aspartate residue (Asp147 in rat muscarinic type 3 receptor) thus including the entire binding site; 10 poses were generated and scored by the ChemPlp function and the speed was equal to 1. The so obtained lowest score complex was minimized by keeping fixed all atoms outside a 10 Å radius sphere around the bound ligand and finally underwent a targeted 1 ns simulations with constant temperature at 300 K and the above mentioned constraints to assess the relative stability of the computed complex. All cited calculations were performed by using the conjugate gradient algorithm until a rms equal to 0.001. Namd2.9 was used with the CHARMM force field and the Gasteiger's atomic charges (Phillips et al., 2005).

#### 2.4. Western blot analysis

For Western-blot analysis CHO cells transfected with human muscarinic type 3 receptor were cultured up to a sub-confluence density and then starved for 12 h in serum-free

medium. Cells were then washed 3 times with phosphate saline buffer (PBS), pH 7.4 and exposed Ach as follows:

To assess the effect of T1AM on basal pERK cell levels, cells were exposed to vehicle (Veh; i.e. DMSO 0.001-0.01% in H<sub>2</sub>O) or T1AM (1, 10  $\mu$ M prepared in Veh) for 5 min.

To assess the stimulatory effect of acetylcholine, cells were pre-treated with atropine (1 mM) and after 30 min, acetylcholine (3, 30, 100 and 1000 nM) was added and incubated further for 5 min with cells;

To evaluate the effect of T1AM on acetylcholine-induced pERK1/2, cells were exposed to T1AM (1, 10  $\mu$ M) for 10 min and then to acetylcholine (3, 30, 100 and 1000 nM) for additional 5 min.

After the last drug addition, cells were washed with PBS and scraped with 1% sodium dodecyl sulfate (SDS) and 1 mM EDTA to collect proteins. To note, CHO cell membranes do not possess cholinesterase activity (Gnagey et al., 1996) and acetylcholine could therefore be used in the absence of a cholinesterase inhibitor.

Furthermore, CHO cells do not express muscarinic receptors (Popper and Batra 1994). Because of this, experiments were only run on transfected CHO cells. Cell viability following exposure at each treatment was evaluated by the (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide test (MTT; Mosmann, 1983).

Cell proteins (20  $\mu$ g) were separated on 10% SDS-PAGE and transferred into PVDF membranes (60 min at 398 mA) using standard procedures. Blots were incubated overnight at 4°C with specific antibody against ERK<sub>1/2</sub>, phosphorylated on Thr202/Tyr204 (Cell Signaling Technology, Denver, CO, USA) or GAPDH (Santa Cruz Biotechnology, Inc. Dallas, TX, USA) as reference protein. The antigen-antibody

complexes were visualized using appropriate secondary antibodies. Blots were immunorecognized for the proteins of interest using an enhanced chemiluminescence detection system (Pierce, Rodano, Italy). Exposition and developing time were standardized for all blots. Densitometric analysis of scanned images was performed on an iMac computer using the public domain NIH Image program. Results are expressed as arbitrary units (AU; representing the ratio between the expression levels of the protein of interest normalized to that of GAPDH and considering this ratio as 1 in basal conditions) and they represent the mean  $\pm$  S.E.M. of three different gels.

### 2.5 Isolation of urinary bladder

The urinary bladders were exposed, removed, and cleaned of connective tissue and adherent fat. The lowest half of the bladder was longitudinally bisected and used for *in vitro* studies. The urothelium–suburothelium was kept intact. The rat bladder was vertically mounted under 1.0 g resting tension in organ baths. Preparations were aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> and bathed in a physiological salt solution of the following composition (mM): NaCl 119, KCl 4.7, NaHCO<sub>3</sub> 15, KH<sub>2</sub>PO<sub>4</sub> 1.2, MgCl<sub>2</sub> 1.2, CaCl<sub>2</sub> 1.5, and glucose 11.1. Changes in isometric tension were recorded on a polygraph chart (Battaglia Rangoni, San Giorgio di Piano, Bologna, Italy). The KCl (80 mM)-induced increase in tension value was taken as 100% and the increase recorded in the presence of different concentrations of various drugs refer to this value. As contracting agent we used carbachol, the muscarinic agonist, at concentrations of 0.5, 5, 10  $\mu$ M and its responsiveness was not affected by previous exposure to high potassium solution (data not shown). T1AM cumulative concentrations (from 10 nM to 100  $\mu$ M) were added to the carbachol concentration used.

## 2.6 Statistical analysis

For radioligand inhibition binding experiments, a one-site binding equation was fitted to the specific binding of acetylcholine ligand to evaluate the  $IC_{50}$  which is the concentration of inhibitor that reduces half the maximal radioligand binding for acetylcholine binding site.  $IC_{50}$  values were converted to  $K_i$  values (inhibitor equilibrium dissociation constant) using the Cheng and Prusoff equation (Lazareno et al., 1993).

Data are expressed as mean  $\pm$  S.E.M. of independent experiments. Statistical analysis was performed by one-way ANOVA. When the experimental setting included only two groups, the unpaired t-test was used. The threshold of statistical significance was set at  $P < 0.05$ . All data analysis was performed by GraphPad Prism 5.02 statistical program (GraphPad Software, San Diego, CA, USA).

### 3. Results

#### 3.1 T1AM but not TA1 binds to human muscarinic receptors

Binding experiments on human muscarinic receptor subtypes expressed in CHO cells were performed as described in the “Methods” using [<sup>3</sup>H]NMS as radioligand.

Our results showed that T1AM displaced [<sup>3</sup>H]NMS from all the 5 different human muscarinic receptor subtypes and a pKi value in the micromolar range was calculated (Table 1). In particular, T1AM showed similar pKi values for muscarinic type 1, type 3, type 4 and type 5 (Table 1; Fig. 1). Instead, the T1AM value of affinity towards muscarinic type 2 receptor was one order of magnitude lower than for other subtypes. These pKi values indicated a higher affinity of T1AM than that measured by carbachol, an agonist at muscarinic receptors (Table 1) which, as T1AM, did not show any preferential binding towards one specific muscarinic receptor subtype (Table 1).

TA1, the acid derivative of T1AM, showed displacement of [<sup>3</sup>H]NMS from all the muscarinic receptor subtypes only at 1 mM concentration. Because of this, we considered the TA1 interaction at muscarinic receptors irrelevant and not worth exploring further (data not shown).

To determine the time course of [<sup>3</sup>H]NMS dissociation, preliminary assays in full time mode were performed as described in the Methods section. The mean rate of dissociation of [<sup>3</sup>H]NMS ( $k_{off}$ ) and the mean half-life of dissociation ( $t_{1/2}$ ) was calculated for M3 receptor subtype (Table 2). Similar results were obtained for M1, M2, M4 and M5 receptor subtypes (data not showed).

Since [ $^3\text{H}$ ]NMS dissociation was found to follow a monoexponential time course, a one-point kinetic protocol was applied. The residual binding of [ $^3\text{H}$ ]NMS at only one time point was measured in the absence and presence of different concentrations (0.01-100  $\mu\text{M}$ ) of the putative allosteric ligand (T1AM) and a single-point estimate of [ $^3\text{H}$ ]NMS ( $k_{\text{off}}$ ) was determined, to assess its potency in inhibiting the radioligand dissociation from the human  $\text{M}_3$  receptor subtype.

Accordingly, T1AM did not produce any effect on the dissociation of [ $^3\text{H}$ ]NMS, revealing its feature of only competitive ligand.

### *3.2 T1AM interaction at human muscarinic type 3 receptor reduces acetylcholine-induced pERK<sub>1/2</sub> levels*

Activation of ERK<sub>1/2</sub> is recognized as a fingerprint of all muscarinic receptor subtype activation independently of the type of G protein coupled (Giovannini et al., 2015). In order to evaluate whether T1AM worked as an agonist or an antagonist at muscarinic receptors we chose to investigate the effect of T1AM on pERK<sub>1/2</sub> levels in cell expressing human muscarinic type 3 receptor, the subtype for which T1AM showed the lowest pKi value (Table 1). Experiments were run in the absence and in the presence of acetylcholine.

As shown in Fig. 2, when CHO cells transfected with human muscarinic type 3 receptor were exposed to T1AM (1, 10  $\mu\text{M}$ ; *Setting 1*), pERK<sub>1/2</sub> cell levels were similar to those

measured in control cells, not exposed to T1AM (Fig. 2, panels A), thus excluding that the amine can activate the intracellular cascade and thus work as an agonist.

Instead, in the same cells, acetylcholine produced a concentration-dependent increase of pERK<sub>1/2</sub> levels (pEC50M  $-7.81 \pm 0.072$ ). This increase was completely prevented when cells were pre-incubated with atropine (1  $\mu$ M) for 25 min before exposure to acetylcholine (Fig. 3, panels a-d). Acetylcholine-induced increase of pERK<sub>1/2</sub> levels was reduced by pre-incubating cells with T1AM. In this latter condition, the pEC50M values of Acetylcholine shifted to  $-6.96 \pm 0.11$  and  $-6.67 \pm 0.07$  in the presence of 1 and 10  $\mu$ M T1AM respectively. Higher concentrations of T1AM could not be tested because percentage of DMSO higher than 0.01% reduced cell viability.

These results were indicative of an antagonistic behavior of T1AM at human muscarinic type 3 receptor

### *3.3 Docking studies indicate T1AM interactions at the active site of the rat muscarinic type 3 receptor*

The putative complex of T1AM with the rat muscarinic type 3 receptor subtype was studied. Figure 3 shows that T1AM stabilizes a rich set of polar contacts involving the ligand ammonium head with Asp147 plus the surrounding tyrosine residues. In addition, the phenolic function elicited H-bonds with the conserved Thr231 and Thr234. When considering that the mentioned residues are highly conserved in all muscarinic subtypes,



such a common network of interactions could explain the affinity of the T1AM towards rat muscarinic type 3 receptor and, more generally, towards all muscarinic subtypes.

Importantly, Fig. 3 emphasizes the stabilizing role of the iodine atom which is seen to elicit clear halogen bonds with Asn587 and Cys532 even though the steric clashes due to its marked atomic size tend to counterbalance the relevance of its contacts.

The performed MD run confirms the notable stability of the computed complex as documented by the very low rmsd mean values of the bound ligand ( $2.21 \pm 0.22 \text{ \AA}$ ) and the surrounding residues ( $1.80 \pm 0.17 \text{ \AA}$ ). Notably, the ion-pair with Asp147 and the halogen bond with Asn587 are stably retained throughout the simulation as evidenced by their distance averages ( $\text{N}^+ - \text{Asp147} = 2.90 \pm 0.08 \text{ \AA}$  and  $\text{I-Asn587} = 3.76 \pm 0.29 \text{ \AA}$ ). In contrast, the H-bonds involving the phenolic group with Thr231 and Thr234 shows a more dynamic behavior reasonably due to flexibility of the bound T1AM which assumes progressively more extended conformations in order to maximize the p-p stacking contacts elicited by its aromatic rings.

#### *3.4 T1AM reduces carbachol-induced contraction of rat urinary bladder*

The rat urinary bladder expresses all subtypes of muscarinic receptors producing contractions of the detrusor muscle with the M3 subtype recognized as among the main involved in urinary bladder micturition. Accordingly, carbachol produced a concentration dependent increase of muscle contraction.

T1AM (10 nM-100  $\mu\text{M}$ ) was not able to modify rat bladder spontaneous contraction (data not shown) but instead it reduced carbachol-induced muscle contraction (Fig. 4)

and a  $pIC_{50M}$  value of  $-3.99 \pm 0.29$ ,  $-4.77 \pm 0.155$  and  $-4.48 \pm 0.068$  on 10, 5 and 0.5  $\mu M$  carbachol respectively was calculated. T1AM (10 nM-100  $\mu M$ ) did not modify KCl-induced (80 mM) contraction (data not shown).

#### 4. Discussion

Our report on the capacity of T1AM, but not of TA1, to bind to all muscarinic receptor subtypes at the orthosteric site is a novel finding. In the range of concentrations consistent with  $pK_i$  values calculated by binding experiments, T1AM reduces acetylcholine-induced  $pERK_{1/2}$  in CHO cells stably expressing the human type 3 muscarinic receptor, the subtype chosen as prototype for studying T1AM pharmacodynamics features. Furthermore, in rat urinary bladder, a tissue expressing all the muscarinic receptors subtypes, T1AM reduces carbachol-induced contraction. Taken together our results indicate that T1AM behaves as a weak antagonist at muscarinic receptors. To the best of our knowledge this is the first evidence indicating a pharmacodynamics feature of T1AM translated into a functional effect.

Our results recognize T1AM as a not selective ligand for muscarinic receptors. In fact, even if T1AM shows the lowest affinity for the type 2 than for the other subtypes, the differences among  $pK_i$  values calculated from binding studies are not so high to consider the ligand as “selective” for one specific muscarinic subtype. This is not surprising since T1AM is an endogenous compound and because the amino acids lining the orthosteric binding site for muscarinic ligands are highly conserved among the five muscarinic subtypes (Wess et al., 2007)(Kruse et al., 2014). This finding does not exclude that T1AM skeleton might serve to develop novel small-molecule orthosteric

ligands endowed with a high degree of selectivity for individual muscarinic subtypes, a goal which remains a major challenge for medicinal chemists.

Since T1AM endogenous tissue levels are several orders of magnitude lower than those recognized to interact at muscarinic receptors (Laurino et al., 2015)(Manni et al., 2013) it is unlikely that the antagonistic features on muscarinic receptors are part of the physiological role of T1AM. Instead, T1AM could have pharmacological effectiveness in reducing functional consequences of an hyper-activated muscarinic signaling. Among the functions controlled by the muscarinic pathway are the control of exocrine and endocrine gland secretion, including the thyroid and the pancreas, and of the smooth muscle tone (Maayan et al., 1983)(Montiel et al., 2007).

However, we evaluated the anti-muscarinic features of T1AM in rat urinary bladder, an *ex-vivo* model suitable for studying the effect of muscarinic and anti-muscarinic drug activities. In this preparation, all muscarinic subtypes are expressed and their activation evokes micturition. Consensus evidence indicates that the pharmacological effects mediated by the different subtypes are hard to distinguish (Hegde et al., 1997) and that, in preparations from normal bladders with an intact urothelium, studies indicate that carbachol contraction is mediated by a homogenous muscarinic type 3 population (Hegde et al., 1997). In this tissue exposed to carbachol, mimicking increased cholinergic tone, we demonstrated that the addition of T1AM reduced contraction calculating an IC<sub>50</sub>M perfectly overlapping with pK<sub>i</sub> values measured from binding experiments. To note, T1AM does not modify basal rat bladder smooth muscle tone and it does not reduce KCl contraction. Importantly, the reduction of carbachol contraction is a typical effect of the amine and it does not involve the production of TA1.)

The present data contribute to the knowledge of the pharmacodynamics features of T1AM, confirming the amine is a low affinity ligand for several targets, among which the muscarinic receptors. These features potentially makes T1AM as an endogenous modulator of the aminergic and of the cholinergic transmission. From that it is known, T1AM has agonistic activity at alfa2 and behaves as an inverse agonist at beta2 adrenoceptors, both features positively controlling the sympathergic transmission. To note, at very low doses, T1AM behaves as a neuromodulator of the histaminergic system.

To note, different portions of T1AM moiety are involved in the recognition of adrenergic and of muscarinic receptors. In fact, while the  $\beta$ -phenylethyl moiety was recognized essential for interacting at adrenergic receptors, the diphenoxyl and iodide ion residues are here identified as crucial for establishing chemical connections with strategic amino acid residues in the active site of the muscarinic type 3 receptor which is however conserved among receptor subtypes and among species (Kruse et al., 2014). Furthermore, T1AM structure may represent a new fruitful chemotype to design novel anti-muscarinic drugs with higher affinity for the receptors than T1AM. In respect of the thyroid, anti-muscarinic drugs derived from T1AM might have some potential usefulness within the therapies for hyperthyroidism offering synergism with drugs currently used to reduce hormone secretion.

**7. Funding:** This paper was supported by a local grant to LR from the University of Florence

## 5. Acknowledgements

We thanks prof. Zucchi from the University of Pisa, Italy for supplying us T1AM and TA1

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**Fig. 1 T1AM binds all human muscarinic receptor subtypes stably expressed in CHO cells.** Plasma membranes were obtained from CHO cell expressing the 5 different human muscarinic receptor subtypes as described in the “Methods”. Membranes were probed with 0.2 nM [<sup>3</sup>H]-*N*-methyl-scopolamine and displacement was attempted by T1AM or TA1 (from 10 nM to 100 μM). Results are the means±S.E.M. of three experiments run in triplicate.

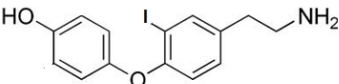
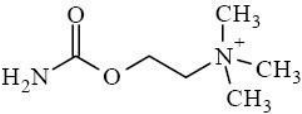
**Fig. 2 Acetylcholine activates ERK<sub>1/2</sub> in CHO cells stably transfected with human muscarinic type 3 receptor subtype: the effect of atropine and of T1AM** CHO Cells expressing human muscarinic type 3 receptor subtype were exposed for 5 min to Panel

a: 3 nM acetylcholine in the absence or in the presence of atropine (Atrop; 1  $\mu$ M) or to T1AM (1, 10 and 100  $\mu$ M), Panel b: 10 nM acetylcholine in the presence or in the presence of atropine (Atrop; 1  $\mu$ M) Panel c: to acetylcholine (30, 100, 1000 nM) in the presence or in the presence of atropine (Atrop; 1  $\mu$ M)) or to T1AM (1, 10  $\mu$ M); Following exposure, cells were collected, lysed and analyzed for pERK1/2 cell levels as described in “Methods”. A densitometry analysis is reported (AU; representing the ratio between the expression levels of the protein of interest normalized to that of GAPDH and considering this ratio as 1 in basal conditions) and they represent the mean  $\pm$  S.E.M. of three different gels. A representative experiment for each acetylcholine concentration is shown. Samples run on the gels derive from the same experiment and the blots were processed in parallel. Panel d-: Concentration-effect curves of acetylcholine on pERK<sub>1/2</sub> levels were built from results from densitometry. \*\*P<0.01 and \*\*\*P< 0.001 vs. Veh.; #P<0.05 , ##P<0.01, ###P<0.001 and #### P<0.0001 vs. acetylcholine.

**Fig. 3 Computed complex of T1AM with rat muscarinic type 3 receptor subtype**  
T1AM computed complex with rat muscarinic type 3 receptor subtype is showed

**Fig. 4 T1AM reduces carbachol-induced contraction of rat urinary bladder** Rat urinary bladder was isolated as described in the “Methods” and put in an oxygenated organ bath. The rat urinary bladder was contracted by adding carbachol 0.5, 5 and 10  $\mu$ M. Cumulative concentration-response curves of T1AM (from 10 nM to 100  $\mu$ M) on each carbachol concentration were obtained. Carbachol-induced contracture was measured (cm) and then compared to the contraction (cm) elicited by 80 mM KCl, taken as 100%. Results are expressed as the means  $\pm$ S.E.M. of at least three different experiments for each carbachol concentration.

**Table 1 Affinity values of 3-iodothyronamine and carbachol binding to human muscarinic receptors (M1-M5)**

Compounds	Name	hM1R	hM2R	hM3R	hM4R	hM5R
	<b>T1AM</b>	6.018 ±0.069	5.119 ±0.098	6.594 ±0.052	6.184 ±0.071	6.553 ±0.057
	<b>Carbachol</b> <b>I</b>	5.71 ±0.09	5.49 ±0.06	5.62 ±0.10	5.04 ±0.07	4.65 ±0.07

Binding experiments were performed as described in “Methods” in plasma membrane of CHO cells stable transfected with human muscarinic receptors (M1-M5). Each determination was run in triplicate and calculation of pKi was made by Graph-Pad fitting

**Table 2. Dissociation rate constants of [<sup>3</sup>H]NMS in the absence and in the presence of T1AM obtained by single time point assays.**

	± T1AM (μM)			
$k_{-1}$ (min <sup>-1</sup> )	0.018±0.002	0.010±0.002	0.016±0.001	0.017±0.001

Values are shown as mean ± standard error of at least three experiments

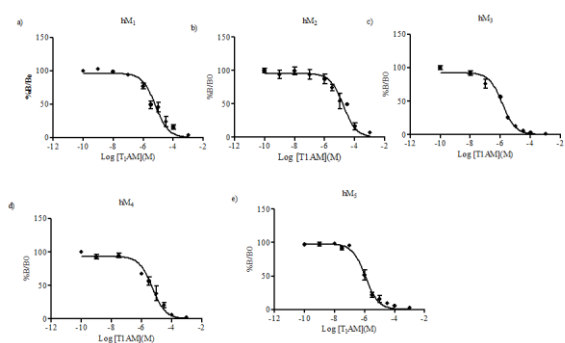


Fig. 2

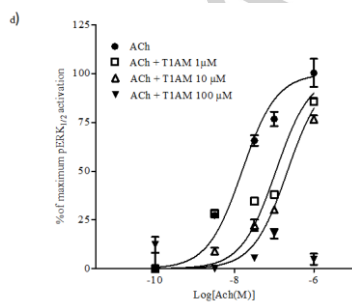
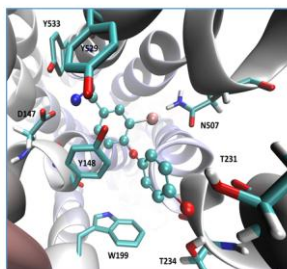


Fig. 3

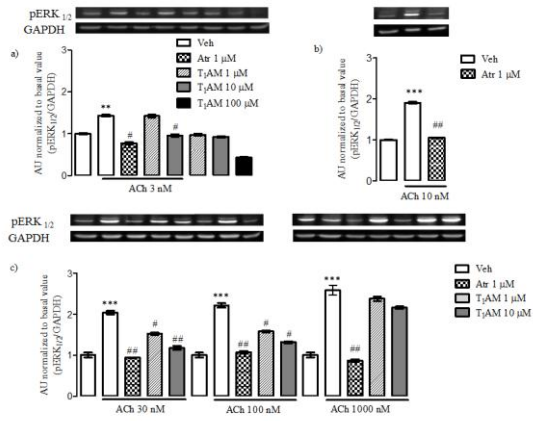


Fig. 4

