Arch. Pharm. Res. (2013) 36:731–738
DOI 10.1007/s12272-013-0084-8

Archives of Pharmacal Research
www.springer.com/12272

RESEARCH ARTICLE

In situ screening of 3-arylcoumarin derivatives reveals new inhibitors of mast cell degranulation

Marcela de Souza Santos · Maria Perpétua Freire de Morais Del Lama · Laila Aparecida Deliberto · Flávio da Silva Emery · Mônica Tallarico Pupo · Rose Mary Zumstein Georgetto Naal

Published online: 22 March 2013
© The Pharmaceutical Society of Korea 2013

Abstract Due to the severity and high prevalence of allergic diseases, there is growing interest in the development of inhibitors of such conditions. 3-Arylcoumarin derivatives emerge as promising compounds for the treatment of allergic disorders, in particular due to their close structural similarity to flavonoids, whose anti-allergic activity has been extensively reported. The aim of this work was to perform a screening of a set of 3-arylcoumarins as potential inhibitors of mast cell degranulation, a key event for the development of allergic reactions. For that purpose, it was utilized a biosensor model based on mast cells, whose in vitro assay allows for such screening, in a high throughput fashion, and also permits bringing to attention some coumarin structural features that are important for their biological activity. The mast cell-based biosensor was shown to discriminate, with high sensitivity and reproducibility, between coumarins that did not affect or caused different degrees of inhibition of degranulation. Among active coumarins, some substituents could be accounted for their inhibitory activity, such as the hydroxylation of positions 6 and 2′ of 3-phenylcoumarins, in addition to catechol, amino and thiophene moieties. In summary, 3-arylcoumarins could be suggested as potential candidates for the development of new anti-allergic drugs.

Keywords 3-Arylcoumarins · Allergy · RBL-2H3 cells · Mast cell degranulation · β-Hexosaminidase · Cell-based biosensor

M. de Souza Santos · M. P. Freire de Morais Del Lama · L. A. Deliberto · R. M. Zumstein Georgetto Naal (✉)
Departamento de Física e Química, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Avenida do Café, s/n-Campus Universitário, Ribeirão Preto, SP 14040-903, Brazil
e-mail: rmzgnaal@fcfrp.usp.br

M. de Souza Santos · M. P. Freire de Morais Del Lama · L. A. Deliberto · R. M. Zumstein Georgetto Naal
Instituto de Ciência e Tecnologia de Bioanalítica, Campinas, Brazil

F. da Silva Emery · M. Tallarico Pupo
Departamento de Ciências Farmacêuticas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, Brazil

Introduction

Coumarins (1,2-benzopyrone or *o*-hydroxycinnamic acid-8-lactone) comprise a large class of oxygen heterocycles that occur naturally as plant and microorganism secondary metabolites (Hoult and Payá 1996; Wu et al. 2009; Smyth et al. 2009). Examination of the coumarin structure reveals that multiple substitutions can occur at any of the six available sites of the coumarin core, during the biosynthetic pathway, which readily contributes to explain how at least 1,300 coumarin derivatives have been isolated from natural sources (Hoult and Payá 1996; Wu et al. 2009). As a result of such expressive structural diversity, the coumarin family has been reported to exhibit a wide spectrum of pharmacological actions, which include anti-coagulant (Hoult and Payá 1996), anti-inflammatory (Pedersen et al. 2007; Bissonnette et al. 2009; Kang et al. 2009; Riveiro et al. 2010), anti-oxidant (Kontogiorgis and Hadjipavlou-Litina 2003; Melagraki et al. 2009), antimicrobial (Waffo et al. 2000; Smyth et al. 2009), anticancer (Riveiro et al. 2010), and anti-HIV activities (Kostova et al. 2006). In fact, it can be established that this family of compounds, in general, obeys Lipinski's rule of

five and exhibits cell membrane permeability, which are commonly found characteristics in most available drugs today (Riveiro et al. 2010).

The capacities of coumarin compounds to inhibit allergic reactions have been reported as well. For instance, Choi and Yan (2009) demonstrated that scoparone (6,7-dimethoxycoumarin) suppressed local allergic reactions induced in rats. Joo et al. (2010) reported that the ethanolic extract of *Angelica gigas*, enriched in coumarin derivatives, strongly alleviated the symptoms of allergic contact dermatitis in animal models. Allergic inflammation is mediated by the expansion of the T helper 2-subset of T cells and isotype switching of B cells to generate IgE antibodies towards specific allergens (Kim et al. 2009). IgE, in turn, binds tightly to its immune receptor, FcεRI, localized at the surface of mast cells (Masuda and Schmitz 2008). Antigen-mediated cross-linking of IgE-FcεRI complexes activates divergent signaling cascades that, ultimately, lead to mast cell degranulation, i.e., rapid release of preformed granule contents (e.g., histamine and serotonin), followed by secretion of newly synthesized lipid mediators (e.g., leukotrienes and eicosanoids), and, hours later, chemokines and cytokines (Kambayashi et al. 2007; Masuda and Schmitz 2008; Huber et al. 2012). On that account, mast cells are widely regarded as the central effectors during the acute and chronic phases of allergic reactions (Moriya et al. 1997; Rossi et al. 2006; Kambayashi et al. 2007; Moon et al. 2007; Nakano et al. 2008; Tanifuji et al. 2010).

Most of the current available anti-allergic medications antagonize chemical mediators released by activated mast cells, such as anti-histamine and anti-leukotriene drugs, which provide partial relief of allergy symptoms. Rather than antagonizing single mediators, an alternative therapeutic strategy consists of preventing mast cells from releasing several of those allergic mediators (Matsuda et al. 2003; Rossi et al. 2006). In this context, the RBL-2H3 cell line has been largely used as a convenient model system to study regulated mast cell secretion (Metzger 1992). We previously reported on the use of RBL-2H3 cells to develop a mast cell-based biosensor for monitoring, in situ, the capacity of potential new drugs to block mast cell activation and degranulation (Naal et al. 2004; Curtis et al. 2008; Andrioli et al. 2012). Conventional assays to quantify mast cell degranulation are widely based on measurement of released β-hexosaminidase, which is located in secretory granules and is secreted along with histamine upon mast cell activation. However, those assays are carried out in a large number of steps, including the transfer of cell supernatant, making them unwieldy for high throughput analysis. The RBL-2H3 cell-based biosensor, on the other hand, allows facile, more rapid, and less labor intense quantification of released β-hexosaminidase with high reproducibility and sensibility (Naal et al. 2004).

The mast cell-based biosensor was applied in the present study for the screening of a collection of 17 3-arylcoumarin derivatives (Table 1) as potential inhibitors of RBL-2H3 mast cell degranulation. The synthesis of those coumarin compounds was described earlier (de Marchi et al. 2004; Kabeya et al. 2007) and their design was primarily based on their analogy to flavonoids, previously identified as biologically active compounds. As depicted in Table 1, this set of coumarin derivatives comprises molecules sharing a common 3-phenyl- or 3-piperonylcoumarin skeleton, to which hydroxyl and acetoxy groups were introduced at different positions, in addition to other substituents such as amino and heterocyclic substituted enamine groups. Such a pattern of substitution allowed us to explore the relationships of structure to the inhibitory effect of the studied molecules on RBL-2H3 mast cell degranulation. Considering that mast cell activation is a key event for the development of allergic reactions, the search for inhibitors of mast cell degranulation offers great therapeutic promise towards the development of new anti-allergic drugs.

Materials and methods

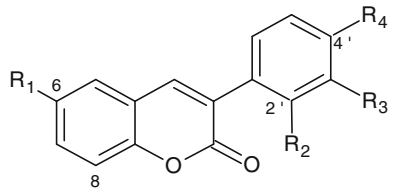
Materials

Potassium chloride, sodium chloride, magnesium chloride 6-hydrate, calcium chloride dihydrate and HEPES were purchased from Mallinckrodt Baker (Phillipsburg, USA). 4-Methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (MUG), ketotifen fumarate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were supplied from Sigma-Aldrich (St. Louis, USA); and bovine serum albumin (BSA) from Gibco (Grand Island, USA). BSA conjugated with an average of 15 dinitrophenyl groups (DNP-BSA) was prepared as previously described (Hardy 1986). Mouse monoclonal anti-DNP IgE from concentrated cell culture was purified as earlier described (Posner et al. 1992). 3-Arylcoumarin derivatives were synthesized as previously described (de Marchi et al. 2004; Kabeya et al. 2007).

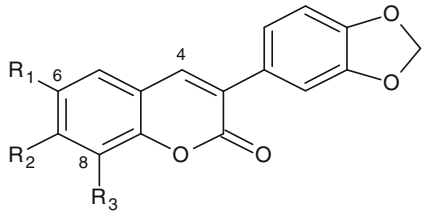
Cell preparation

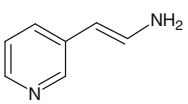
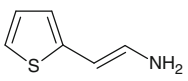
RBL-2H3 cells (Barsumian et al. 1981) were maintained in a monolayer culture in minimum essential medium with L-glutamine and Eagle's salt, supplemented with 20 % fetal bovine serum and 50 μg/mL gentamicin sulfate. The cells were used from 3 to 5 days after passage, and released from culture flasks by treatment with 0.5 % trypsin–EDTA for 5 min at 37 °C, centrifuged for 3 min at 1,000 rpm

Table 1 Inhibitory activity of 3-arylcoumarins against antigen-induced RBL-2H3 mast cell degranulation



Coumarin	R_1	R_2	R_3	R_4	IC_{50} (μM)
1	H	H	H	H	–
2	OH	H	H	H	28.0 ± 2.6**
3	H	H	H	OH	–
4	OH	H	H	OH	28.3 ± 1.7**
5	OH	OH	H	H	13.4 ± 1.5**
6	OH	H	OH	OH	15.9 ± 1.7**
7	$OCOCH_3$	H	H	H	~100**
8	$OCOCH_3$	H	H	$OCOCH_3$	–
9	$OCOCH_3$	$OCOCH_3$	H	H	20.5 ± 1.8**



Coumarin	R_1	R_2	R_3	IC_{50} (μM)
10	OH	H	H	–
11	H	OH	H	–
12	H	H	OH	–
13	OH	OH	H	–
14	H	OH	OH	–
15	NH_2	H	H	28.9 ± 4.2**
16		H	H	18.2 ± 1.6**
17		H	H	10.5 ± 1.8**
Ketotifen	–	–	–	154.7 ± 7.6**

Asterisks indicate statistical significance at 100 μM in comparison to control

** $p < 0.01$

(centrifuge Sorvall Legend, model Mach 1.6R, Thermo Fisher Scientific, Walthman, USA) and resuspended at 1×10^6 cell/mL. Cells were sensitized with a fivefold molar excess of anti-DNP IgE over FcεRI. Then, 250 μL of sensitized cells were seeded onto 96-well plates at 1.5×10^5 cell/mL and incubated overnight at 37 °C in an atmosphere of 5 % CO_2. All cell culture reagents were obtained from Gibco unless otherwise noted.

β-Hexosaminidase release assay

The in situ assay for quantification of released β-hexosaminidase from mast cells was performed as described by (Naal et al. 2004). RBL-2H3 cells, prepared as described above, were washed twice with 200 μL of Tyrode's buffer (135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.6 mM glucose, 20 mM HEPES and 1 mg/mL BSA, pH 7.4). In sequence, 100 μL of each concentration (0.01–100 μM) of tested 3-arylcoumarins, prepared in Tyrode's buffer (from a 1.0 mg/mL DMSO stock solution, volume of DMSO minimized to avoid cell toxicity), was added to each well, followed by addition of 100 μL of 0.2 μg/mL antigen (final concentration 0.1 μg/mL) and 100 μL of 3.6 mM β-hexosaminidase substrate, MUG (final concentration 1.2 mM), both in Tyrode's buffer. Samples stimulated with antigen in the absence of arylcoumarins were taken as samples where degranulation was at a maximum. The samples were incubated at 37 °C for 90 min, after which cells were placed on ice to stop degranulation. The product of the β-hexosaminidase-mediated cleavage of MUG, methylumbelliferone, had its fluorescence measured in a microplate reader (BioTEK, Winooski, USA) with 360 nm excitation and 450 nm emission filters. Spontaneous release of β-hexosaminidase was determined from cells untreated with either coumarin derivatives or antigen. Total β-hexosaminidase cell content was obtained by lysing cells with 0.1 % Triton X-100. The percentage of stimulated β-hexosaminidase release was calculated according to Eq 1. Values of IC_{50} (concentration of tested arylcoumarin necessary to inhibit the release of β-hexosaminidase from cells stimulated with antigen by 50 %) were determined graphically. The inhibitory effect of arylcoumarins on β-hexosaminidase secretion was compared with that of ketotifen fumarate (see Table 1).

$$\%\beta\text{-hexosaminidase} = \left(\frac{S - N}{T - N}\right) \times 100 \quad (1)$$

where N is normal, fluorescence from vehicle (Tyrode's buffer), (−) cells. S is sample, fluorescence from (+) DNP-BSA, (+) or (−) arylcoumarin samples. T is total, fluorescence from Triton X-100-lysed cell samples.

β-Hexosaminidase activity assay

RBL-2H3 cell suspension (1.5×10^5 cell/mL) was sonicated and centrifuged. Next, 45 μL of supernatant, containing the enzyme β-hexosaminidase, was incubated with

5 μL of 3-arylcoumarin sample solutions (final concentration range 0.01–100 μM) and 50 μL of β-hexosaminidase substrate (MUG) solution (final concentration 1.2 mM), for 60 min. The enzyme-substrate reaction was stopped by the addition of 200 μL of stop solution (0.1 M Na_2CO_3/$NaHCO_3$ buffer, pH 10.0). Fluorescence was measured in a microplate reader, as described above.

Cytotoxic assay

RBL-2H3 cells were cultured at 8×10^4 cell/mL overnight. After the washing step with PBS buffer, cells were treated with different concentrations of tested 3-arylcoumarins solutions (0.01–100 μM), prepared in culture medium, for 24 h. Next, the culture medium was removed and replaced with new medium supplemented with 10 % MTT (5 mg/mL stock in PBS). After incubation for additional 4 h, followed by removal of the supernatant, formazan crystals were dissolved in DMSO. Cell viability was determined using a microplate reader at 570 nm. Optical density measured from formazan formed in non-treated control samples was taken as 100 % of cell viability.

Statistical analysis

Results presented in Table 1 were expressed as mean ± SD for three independent experiments. Statistical significance was assessed by one-way ANOVA followed by Dunnett's *posthoc* pairwise comparisons. Differences were considered statistically significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Results

As shown in Table 1, 3-phenylcoumarin (compound 1) did not inhibit antigen-induced degranulation of RBL-2H3 mast cells. The substitution of 3-phenylcoumarin with a hydroxyl group at position 6 (6-hydroxy-3-phenylcoumarin, compound 2) led to an active derivative, capable of inhibiting stimulated degranulation with an IC_{50} equal to 28 μM. On the other hand, the compound 3, hydroxylated at C-4′ (4′-hydroxy-3-phenylcoumarin) did not influence stimulated-mast cell degranulation. Interestingly, cell treatment with compound 4, which is hydroxylated at both positions 6 and 4′ (6,4′-dihydroxy-3-phenylcoumarin), inhibited antigen-induced degranulation with equivalent potency to compound 2, bearing a single hydroxylation at C-6 (compound 2—$IC_{50} = 28$ μM, compound 4—$IC_{50} = 28.3$ μM). Those results suggest that the inhibitory effect of compound 4 did not derive from a combined effect of the two hydroxyl groups, but rather, from the single positive influence of 6-hydroxylation on the activity of 3-phenylcoumarin. The introduction of a second hydroxyl group at C-2′ improved the potency of 6-hydroxy-3-phenylcoumarin. The resulting 6,2′-dihydroxy-3-phenylocoumarin, compound 5, inhibited RBL-2H3 degranulation with an IC_{50} of 13.4 μM, which is about twice smaller than that for the monohydroxylated compound 4. Those results suggest that the presence of both hydroxyl groups could have contributed to the increase in the inhibitory potency of the coumarin derivative. Likewise, the addition of a third hydroxylation to the C-3′ of 6,4′-dihydroxy-3-phenylcoumarin enhanced its inhibitory potency by a factor of almost two (6,4′-dihydroxy-3-phenylcoumarin, compound 4—$IC_{50} = 28.3$ μM > 6,3′,4′-trihydroxy-3-phenylcoumarin, compound 6—$IC_{50} = 15.9$ μM).

While the 6-hydroxylation of 3-phenylcoumarin enabled this derivative to strongly inhibit mast cell degranulation, the esterification of the same position resulted in a very weak inhibitor (6-acetoxy-3-phenylcoumarin, compound 7—$IC_{50} \sim 100$ μM > 6-hydroxy-3-phenylcoumarin, compound 2—$IC_{50} = 28$ μM). Similarly, the esterification of both 6 and 4′ positions of 3-phenylcoumarin led to an inactive compound (6,4′-diacetoxy-3-phenylcoumarin, compound 8), in contrast to the strong inhibition of RBL-degranulation exhibited by its hydroxylated counterpart (6,4′-dihydroxy-3-phenylcoumarin, compound 4—$IC_{50} = 28.3$ μM). Although not very significant, the 6,2′-diacetoxy-3-phenylcoumarin (compound 9) was slightly less potent than the 6,2′-dihydroxy-3-phenylcoumarin (compound 5), with IC_{50} values equal to 13.4 and 20.5 μM, respectively.

When 3′- and 4′-hydroxyl of 6,3′,4′-trihydroxy-3-phenylcoumarin (compound 6) became protected as a methylenedioxy group, the resulting compound, 6-hydroxy-3-piperonylcoumarin (compound 10), lacked inhibitory activity against mast cell degranulation. The 3-piperonylcoumarin with different degrees of hydroxylation groups at different positions and in number, did not improve the inhibitory potential. The compounds 11, 12, 13, and 14, bearing hydroxylations at positions 7-; 8-; 6,7-; and 7,8-, respectively, were all incapable of decreasing the secretion of β-hexosaminidase. Despite the poor application of the 3-piperonylcoumarin derivatives tested so far as inhibitors of mast cell degranulation, the substitution of 3-piperonylcoumarin with an amino group at C-6 (6-amino-3-piperonylcoumarin, compound 15) represented a considerable gain of activity ($IC_{50} = 28.9$ μM). The extension of this substitution by a pyridine ring (6-{[(*E*)-2-(pyridin-2-yl)ethenyl]amino}-3-piperonylcoumarin, compound 16) led to an increase in the inhibitory potency (6-amino-3-piperonylcoumarin, compound 15—$IC_{50} = 28.9$ μM > 6-{[(*E*)-2-(pyridin-2-yl)ethenyl]amino}-3-piperonylcoumarin, compound 16—$IC_{50} = 18.2$ μM). When the extension to the 6-amino group was provided by a thiophene ring, the inhibitory potency was improved by a factor of almost

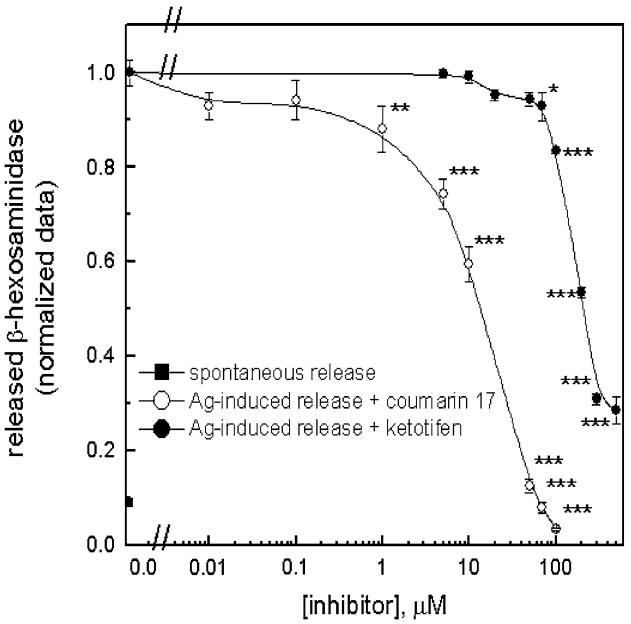


Fig. 1 Coumarin 17 is a more potent inhibitor of mast cell degranulation than ketotifen. Anti-DNP IgE-sensitized RBL cells were treated with either coumarin 17 or ketotifen fumarate at the indicated concentrations, followed by antigen (Ag) stimulation and addition of MUG for 90 min. Released β-hexosaminidase was calculated as indicated at materials and methods section. All data represent arithmetic mean of triplicate determinations. One-way ANOVA, followed by Dunnett's test, indicate statistical significance of $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, in comparison to antigen-control samples

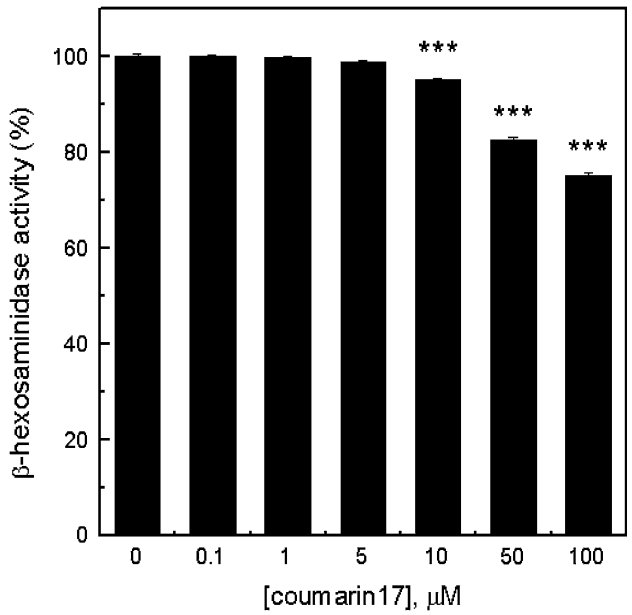


Fig. 2 Coumarin 17 causes a weak inhibiton of β-hexosaminidase activity. RBL cell supernatant was treated with the indicated concentrations of coumarin 17, in the presence of MUG, for 60 min. β-hexosaminidase activity was calculated as indicated at materials and methods section. All data represent arithmetic mean of triplicate determinations. One-way ANOVA, followed by Dunnett's test, indicate statistical significance of $***p < 0.001$, in comparison to untreated-control samples

three, and the compound was the most active (6-amino-3-piperonylcoumarin, compound 15—$IC_{50} = 28.9$ μM > 6-{[(*E*)-2-(thiophen-2-yl)ethenyl]amino}-3-piperonylcoumarin, compound 17—$IC_{50} = 10.5$ μM). The respective π-deficient and π-excessive characters of pyridine and thiophene rings might have accounted for the significant difference in degranulation inhibition exhibited by the 3-piperonylcoumarin derivatives bearing such moieties.

It is noteworthy that, for all active 3-arylcoumarin derivatives, their inhibitory potency against antigen-stimulated mast cell degranulation was greatly higher than that exhibited by ketotifen ($IC_{50} = 154.7$ μM, Fig. 1). Ketotifen, a commonly used positive control for degranulation assays (Matsuda et al. 2002; Serna et al. 2006), has been recently classified as a multiple action drug, suggested to combine anti-histaminic, anti-inflammatory, and, mast cell stabilization properties (Lambiase et al. 2009).

Next, we certified that the inhibitory activity of the active compounds could be mostly attributed to their capacity to decrease the antigen-induced release of β-hexosaminidase, i.e. the inhibition of mast cell degranulation. In this way, we investigated the possibility that such compounds could inhibit the catalytic activity of β-hexosaminidase (conversion of MUG into methylumbelliferone) and/or that they could be toxic to RBL-2H3 cells, both of which possibilities could have provided a false positive result. The coumarins were shown to similarly cause any or a slight inhibition of both the activity of β-hexosaminidase and cell viability, consistent with a primary inhibitory effect of the active coumarins on antigen-stimulated mast cell degranulation. Figures 2 and 3 depict the effects of coumarin 17 on β-hexosaminidase catalytic activity and cell toxicity, respectively, as a representative example for all active coumarins.

Discussion

The coumarin derivative artekeiskeanol A, isolated from *Artemisia keikeana* Miq., was shown to inhibit, in a concentration-dependent manner, antigen-stimulated degranulation of RBL-2H3 cells (Hong et al. 2009). Likewise, Ryu et al. (2001) reported on the anti-degranulation activity of cnidicin, a coumarin from the root extract of *Angelika koreana*. In the present study, we have presented 3-arylcoumarin derivatives as potent inhibitors of antigen-induced mast cell degranulation. Our results strengthen the application of the coumarin family as anti-allergic drug candidates.

As mentioned earlier, the rationale for the synthesis of the studied group of 3-arylcoumarins was primarily based

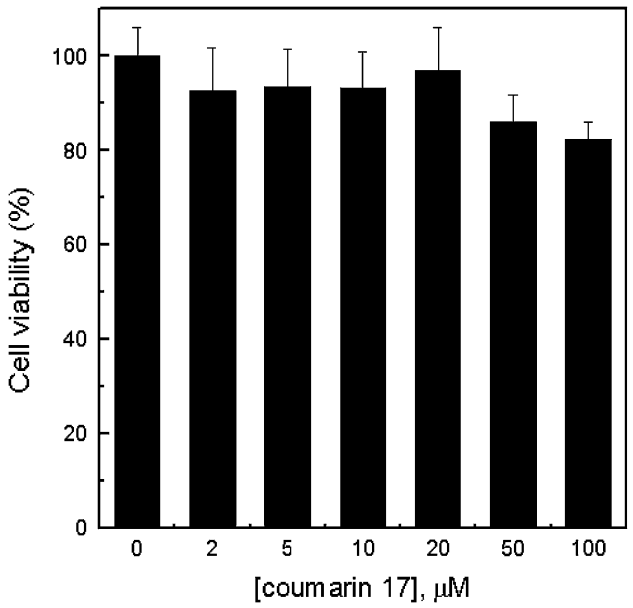


Fig. 3 Coumarin 17 does not affect RBL-2H3 cell viability. RBL cells were incubated with coumarin 17 at the indicated concentrations for 24 h. Cell viability was assessed as described at materials and methods section. All data represent arithmetic mean of triplicate determinations. One-way ANOVA, followed by Dunnett's test, indicate no statistical significance among triplicates

on their structural resemblance to flavonoids (de Marchi et al. 2004). Matsuda et al. (2002) demonstrated that the 2,3-double bond within the structure of flavones and flavonols is essential for their anti-degranulation activity. 3-Phenylcoumarins exhibit a 3,4-double bond that mimics the double-bond moiety of flavonoids. Wang et al. (2007) demonstrated that 3-phenylisocoumarins lacking the 3,4-double bond exhibited weaker inhibitory activity against RBL-2H3 cell degranulation than their analogs bearing the double bond. However, the close structural similarity of the 3-phenylcoumarin skeleton to flavonoids did not, by itself, make those coumarins inhibitors of mast cell degranulation. In agreement with our findings, Kabeya et al. (2007, 2008) demonstrated that non-substituted 3-arylcoumarins did not inhibit either horseradish catalytic activity or neutrophil oxidative metabolism.

Considering that non-substituted 3-arylcoumarin derivatives do not modulate the stimulated release of β-hexosaminidase, we evaluated the contributions of different substituents within the 3-arylcoumarin skeleton according to its anti-degranulation activity. It was found that hydroxylation at position 6 greatly improved the inhibitory activity of 3-phenylcoumarins. Matsuda and others found that the hydroxylation of flavonoids at the same position enhanced their capacity to inhibit degranulation. Similarly, Wang et al. (2007) reported that the anti-degranulation activity of 3-phenylisocoumarin derivatives bearing 6-hydroxylation was stronger than that of compounds lacking the 6-hydroxyl group. While 4′-hydroxylation was previously reported to be important for the inhibition of degranulation by flavonoids and 3-phenylisocoumarins (Cheong et al. 1998; Matsuda et al. 2002; Wang et al. 2007), here, we clearly demonstrated that the substitution of 3-phenylcoumarins with a 4′-hydroxyl group did not contribute to reducing the release of β-hexosaminidase.

Although 4′-hydroxylation did not change the activity of the substituted coumarin, 3′,4′-dihydroxylations represented an increase in the inhibitory potency. The presence of a catechol moiety within the B ring of flavonoids or at the phenyl ring of 3-phenylcoumarins was previously suggested to positively influence their biological activities (Matsuda et al. 2002; Kabeya et al. 2007, 2008). Those findings are consistent with the fact that the protection of the catechol group, as in 3-phenyl-piperonylcoumarins, turns the latter derivatives into biologically inactive compounds. In agreement with our results, Kabeya et al. (2007), (2008) reported that 6,2′-dihydroxy-3-phenylcoumarin was more effective than its 6,4′-dihydroxylated analog at inhibiting both horseradish catalytic activity and neutrophil-mediated generation of reactive oxygen species. It was suggested that the spatial proximity between the 2′-hydroxyl group and the coumarin lactone could mimic the interaction of catechol moiety with the active site (Kabeya et al. 2007). Like the methylenedioxy moiety, which reduced the inhibitory activity of 3-phenylcoumarins, the protection of hydroxyl groups by esterification of the accessed coumarins, decreased the anti-degranulation action of their hydroxylated counterparts, in agreement with previous studies (Kabeya et al. 2007, 2008).

It was observed that, among the studied set of 3-piperonylcoumarins, the derivative bearing a thiophene moiety was the most potent compound. The thiophene ring is one of the most interesting heterocycles from a pharmacological point-of-view and is usually used as a bioisostere of benzene, because of the similarity in electronic and physico-chemical properties. Thiophene-carrying compounds exhibit various therapeutic applications such as anti-inflammatory, serotonin antagonists, anti-cancer, and anti-atherosclerotic agents (Mishra et al. 2011).

In summary, the application of the mast cell-based biosensor for the screening of inhibitors of degranulation enabled us to discriminate, with high sensitivity and reproducibility, between coumarins that did not affect or caused different degrees of inhibition of stimulated degranulation. Ours results point out 3-arylcoumarin derivatives as strong inhibitors of antigen-induced RBL-2H3 mast cell degranulation and highlight that some substitutions, such as 6- and 2′-hydroxylations, catechol, amino, and thiophene moieties, are important for the anti-degranulation activity, the first step during the investigation new prototypes with anti-allergic applications.

Acknowledgments The authors thank Drs. Barbara Baird and David Holowka (Cornell University) for the kind gifts: DNP-BSA and anti-DNP IgE. This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), grant # 1277/10-3, Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), grant # 2008/01712-6 and Instituto Nacional de Ciência e Tecnologia de Bioanalítica. MTP also thanks Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for grant fellowship.

Conflict of interest The authors declare they have no conflict of interest.

References

Andrioli, W.J., M.S. Santos, V.B. Silva, R.B. Oliveira, A. Chagas-Paula, J.A. Jorge, N.A. Furtado, M.T. Pupo, C.H. Silva, R.M. Naal, and J.K. Bastos. 2012. δ-Lactam derivative from thermophilic soil fungus exhibits in vitro anti-allergic activity. *Natural Product Research* 26: 2168–2175.

Barsumian, E.L., C. Isersky, M.G. Petrino, and R.P. Siraganian. 1981. IgE-induced histamine release from rat basophilic leukemia cell lines: isolation of releasing and nonreleasing clones. *European Journal of Immunology* 11: 317–323.

Bissonnette, E.Y., G.M. Tremblay, V. Turmel, B. Pirotte, and M. Reboud-Rabaux. 2009. Coumarinic derivatives show anti-inflammatory effects on alveolar macrophages, but their anti-elastase activity is essential to reduce lung inflammation in vivo. *International Immunopharmacology* 9: 49–54.

Cheong, H., S.Y. Ryu, M.H. Oak, S.H. Cheon, G.S. Yoo, and K.M. Kim. 1998. Studies of structure activity relationship of flavonoids for the anti-allergic actions. *Archives of Pharmacal Research* 4: 478–480.

Choi, Y.H., and G.H. Yan. 2009. Anti-allergic effects of scoparone on mast cell-mediated allergy model. *Phytomedicine* 16: 1089–1094.

Curtis, T., R.M.Z.G. Naal, C. Batt, J. Tabb, and D. Holowka. 2008. Development of a mast cell-based biosensor. *Biosensors & Bioelectronics* 23: 1024–1031.

Hardy, R. 1986. *Handbook of Experimental Immunology*. Oxford: London.

Hong, J., H. Sasaki, N. Hirasawa, K. Ishihara, J.H. Kwak, O. Zee, F.J. Schmitz, T. Seyama, and K. Ohuchi. 2009. Suppresion of the antigen-stimulated RBL-2H3 mast cell activation by Artekeiskeanol A. *Planta Medica* 14: 1494–1498.

Hoult, J.R., and M. Payá. 1996. Pharmacological and biochemical actions of simple coumarins: natural products with therapeutic potential. *General Pharmacology* 27: 713–722.

Huber, R., F.C. Stintzing, D. Briemle, C. Beckmann, U. Meyer, and C. Gründemann. 2012. In vitro antiallergic effects of aqueous fermented preparations from Citrus and Cydonia fruits. *Planta Medica* 78: 334–340.

Kabeya, L.M., C.H. da Silva, A. Kanashiro, J.M. Campos, A.E. Azzolini, A.C. Polizello, M.T. Pupo, and Y.M. Lucisano-Valim. 2008. Inhibition of immune complex-mediated neutrophil oxidative metabolism: a pharmacophore model for 3-phenylcoumarin derivatives using GRIND-based 3D-QSAR and 2D-QSAR procedures. *European Journal of Medicinal Chemistry* 43: 996–1007.

Kabeya, L.M., A.A. de Marchi, A. Kanashiro, N.P. Lopes, C.H. da Silva, M.T. Pupo, and Y.M. Lucisano-Valim. 2007. Inhibition of horseradish peroxidase catalytic activity by new 3-phenylcoumarin derivatives: synthesis and structure-activity relationships. *Bioorganic & Medicinal Chemistry* 15: 1516–1524.

Kambayashi, T., and G.A. Koretzky. 2007. Proximal signaling events in Fc epsilon RI-mediated mast cell activation. *The Journal of Allergy and Clinical Immunology* 119: 544–552.

Kang, K.H., C.S. Kong, Y. Seo, M.M. Kim, and S.K. Kim. 2009. Anti-inflammatory effect of coumarins isolated from *Corydalis heterocarpa* in HT-29 human colon carcinoma cells. *Food and Chemical Toxicology* 47: 2129–2134.

Kim, J.W., J.H. Lee, B.Y. Hwang, S.H. Mun, N.Y. Ko, K. Kim, B. Kim, H.S. Kim, Y.M. Kim, and W.S. Choi. 2009. Morin inhibits Fyn kinase in mast cell and IgE-mediated type I hypersensitivity response in vivo. *Biochemical Pharmacology* 77: 1506–1512.

Kontogiorgis, C., and D. Hadjipavlou-Litina. 2003. Biological evaluation of several coumarin derivatives designed as possible anti-inflammatory/antioxidant agents. *Journal of Enzyme Inhibition and Medicinal Chemistry* 18: 63–69.

Kostova, I., Raleva, S., Genova, P., Argirova, R., (2006). Structure-activity relationships of synthetic coumarins as HIV inhibitors. *Bioinorganic Chemistry and Applications* 2006: Article ID 68274.

Joo, S.S., D. Park, S. Shin, J.H. Jeon, T.K. Kim, Y.J. Choi, S.H. Lee, J.S. Kim, S.K. Park, B.Y. Hwang, B.Y. Lee, and Y.B. Kim. 2010. Anti-allergic effects and mechanisms of action of the ethanolic extract of *Angelica gigas* in dinitrofluorobenzene-induced inflammation models. *Environmental Toxicology and Pharmacology* 30: 127–133.

Lambiase, A., A. Micera, and S. Bonini. 2009. Multiple action agents and the eye: do they really stabilize mast cells? *Current opinion in Allergy and Clinical Immunology* 9: 454–465.

de Marchi, A.A., M.S. Castilho, P.G. Nascimento, F.C. Archanjo, G. del Ponte, G. Oliva, and M.T. Pupo. 2004. New 3-piperonylcoumarins as inhibitors of glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) from *Trypanosoma cruzi*. *Bioorganic & Medicinal Chemistry* 12: 4823–4833.

Masuda, E.S., and J. Schmitz. 2008. Syk inhibitors as treatment for allergic rhinitis. *Pulmonary Pharmacology & Therapeutics* 21: 461–467.

Matsuda, H., T. Morikawa, H. Managi, and M. Yoshikawa. 2003. Antiallergic principles from *Alpinia galanga*: structural requirements of phenylpropanoids forinhibition of degranulation and release of TNF-alpha and IL-4 in RBL-2H3 cells. *Bioorganic & Medicinal Chemistry Letters* 13: 3197–3202.

Mastuda, H., T. Morikawa, K. Ueda, H. Managi, and M. Yoshikawa. 2002. Structural requirements of flavonoids for inhibition of antigen-Induced degranulation, TNF-alpha and IL-4 production from RBL-2H3 cells. *Bioorganic & Medicinal Chemistry* 10: 3123–3128.

Melagraki, G., A. Afantitis, O. Igglessi-Markopoulou, A. Detsi, M. Koufaki, C. Kontogiorgis, and D.J. Hadjipavlou-Litina. 2009. Synthesis and evaluation of the antioxidant and anti-inflammatory activity of novel coumarin-3-aminoamides and their alpha-lipoic acid adducts. *European Journal of Medicinal Chemistry* 44: 3020–3026.

Metzger, H. 1992. The receptor with high affinity for IgE. *Immunological Reviews* 125: 37–48.

Mishra, R., K.K. Jha, S. Kumar, and I. Tomer. 2011. Synthesis, properties and biological activity of thiophene: a review. *Der Pharma Chemica* 3: 38–54.

Moon, P.D., B.H. Lee, H.J. Jeong, H.J. An, S.J. Park, H.R. Kim, S.G. Ko, J.Y. Um, S.H. Hong, and H.M. Kim. 2007. Use of scopoletin to inhibit the production of inflammatory cytokines through inhibition the IkappaB/NF-kappaB signal cascade in the human mast cell line HMC-1. *European Journal of Pharmacology* 555: 218–225.

Moriya, K., J. Rivera, S. Odom, Y. Sakuma, K. Muramato, T. Yoshiuchi, M. Miyamoto, and K. Yamada. 1997. ER-27319, an acridone-related compound, inhibits release of antigen-induced

allergic mediators from mast cells by selective inhibition of Fcε receptor I-mediated activation of Syk. *Proceedings of the National Academy of Sciences of the United States of America* 94: 12539–12544.

Naal, R.M.Z.G., J. Tabb, D. Holowka, and B. Baird. 2004. In situ measurement of degranulation as a biosensor based on RBL-2H3 mast cells. *Biosensors & Bioelectronics* 20: 791–796.

Nakano, N., C. Nishiyama, T. Tokura, Y. Nagasako-Akazome, Y. Ohtake, K. Okumura, and H. Ogawa. 2008. Procyanidin C1 from apple extracts inhibits Fc epsilon RI-mediated mast cell activation. *International Archives of Allergy and Immunology* 147: 213–221.

Pedersen, J.Z., C. Oliveira, S. Incerpi, V. Kumar, A.M. Fiore, P. De Vito, A.K. Prasad, S.V. Malhotra, V.S. Parmar, and L. Saso. 2007. Antioxidant activity of 4-methylcoumarins. *Journal of Pharmacy and Pharmacology* 59: 1721–1728.

Posner, R.G., B. Lee, D.H. Conrad, D. Holowka, B. Baird, and B. Goldstein. 1992. Aggregation of IgE-receptor complexes on rat basophilic leukemia cells does not change the intrinsic affinity but can alter the kinetics of the ligand-IgE interaction. *Biochemistry* 31: 5350–5356.

Riveiro, M.E., N. De Kimpe, A. Moglioni, R. Vázquez, F. Monczor, C. Shayo, and C. Davio. 2010. Coumarins: old compounds with novel promising therapeutic perspectives. *Current Medicinal Chemistry* 17: 1325–1338.

Rossi, A.B., E. Herlaar, S. Braselmann, S. Huynh, V. Taylor, R. Frances, S.D. Issakani, A. Argade, R. Singh, D.G. Payan, and E. Masuda. 2006. Identification of the Syk kinase inhibitor R112 by a human mast cell screen. *Journal of Allergy and Clinical Immunology* 118: 749–755.

Ryu, S.Y., N.Y. Kou, H.S. Choi, H. Ryu, T.S. Kim, and K.M. Kim. 2001. Cnidicin, a coumarin, from the root of *Angelica koreana*, inhibits the degranulation of mast cell and the NO generation in RAW 264.7 cells. *Planta Medica* 67: 172–174.

Serna, H., M. Porras, and P. Vergara. 2006. Mast cell stabilizer ketotifen [4-(1-methyl-4-piperidylidene)-4 h-benzo[4,5]cyclohepta[1,2-b]thiophen-10(9H)-one fumarate] prevents mucosal mast cell hyperplasia and intestinal dysmotility in experimental *Trichinella spiralis* inflammation in the rat. *Journal of Pharmacology and Experimental Therapeutics* 319: 1104–1111.

Smyth, T., V.N. Ramachandran, and W.F. Smyth. 2009. A study of the antimicrobial activity of selected naturally occurring and synthetic coumarins. *International Journal of Antimicrobial Agents* 33: 421–426.

Tanifuji, S., E. Aizu-Yokota, M. Funakoshi-Tago, Y. Sonoda, H. Inoue, and T. Kasahara. 2010. Licochalcones suppress degranulation by decreasing the intracellular Ca^{2+} level and tyrosine phosphorylation of ERK in RBL-2H3 cells. *International Immunopharmacology* 10: 769–776.

Waffo, A.K., G.A. Azebaze, A.E. Nkengfack, Z.T. Fomum, M. Meyer, B. Bodo, and F.R. van Heerden. 2000. Indicanines B and C, two isoflavonoid derivatives from the root bark of *Erythrina indica*. *Phytochemistry* 53: 981–985.

Wang, Q., H. Matsuda, K. Matsuhira, S. Nakamura, D. Yuan, and M. Yoshikawa. 2007. Inhibitory effects of thunberginols A, B, and F on degranulations and releases of TNF-alpha and IL-4 in RBL-2H3 cells. *Biological & Pharmaceutical Bulletin* 30: 388–392.

Wu, L., X. Wang, W. Xu, F. Farzaneh, and R. Xu. 2009. The structure and pharmacological functions of coumarins and their derivatives. *Current Medicinal Chemistry* 16: 4236–4260.