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COMMUNICATION

Imaging histamine in live basophils and macrophages with a fluorescent mesoionic acid fluoride†

Nicola Kielland,‡[a] Marc Vendrell,‡[b] Rodolfo Lavilla*[cd] and Young-Tae Chang*[be]

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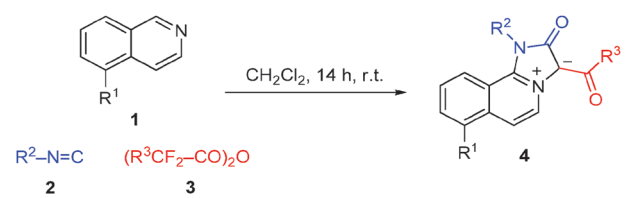
Histamine is a biogenic amine with fundamental roles in circulatory and immune systems. We report a fluorescent small molecule (Histamine Blue) for imaging intracellular histamine in live basophils and macrophages. Histamine Blue is a fluorescent mesoionic acid fluoride that turns on upon reaction with histamine. The selective response of Histamine Blue enabled the visualization of intracellular histamine under different physiological conditions.

Histamine is a ubiquitous biogenic amine, synthesized by histidine decarboxylation and with multiple functions in the physiology of the body.[1] Histamine is especially abundant in mast cells and basophils, where it is stored and released under chemical stimulation,[2] and plays essential roles in gastric acid secretion,[3] allergic and inflammatory responses,[4] neurotransmission,[5] thermoregulation[6] and cognition and behavioural events.[7] A major challenge in elucidating the function and dynamics of histamine in all these processes is represented by the lack of tools for tracking histamine in live cells. Conventional methods to determine histamine levels[8–12] (*e.g.* chromatography, capillary electrophoresis, fluorimetry, immunoassays) rely on multistep protocols that employ cell extracts, which are limited in spatial and temporal resolution. While the localization of histamine in the sympathetic nervous system has been demonstrated by immunohistochemistry in permeabilized samples[13] and Imato's group described fluorescent nickel-based complexes to monitor histamine levels in macrophages,[14,15] there is a demand for new fluorescent probes to image intracellular histamine in real-time and in a variety of live cells under different conditions.

We have developed a simple and rapid method to image histamine in live basophils and macrophages using a fluorescent mesoionic acid fluoride. Our discovery has been achieved by combining the synthetic methodology of multicomponent reactions (MCR),[16] which can provide a straightforward access to complex heterocycles in a single step,[17] and the systematic screening of fluorescent molecules.[18] We recently described a new MCR involving azines **1**, isocyanides **2** and fluorinated anhydrides **3** to yield unprecedented mesoionic adducts **4** in good yields[19] (Scheme 1) and prepared a set of 12 compounds (**4a–4h** and **5a–5d**, Scheme S1 in Electronic Supporting Information (ESI†)) based on the isoquinoline scaffold. These derivatives exhibited absorption bands compatible with commercially available excitation sources (*i.e.*, xenon and mercury lamps as well as violet lasers), blue fluorescence emission wavelengths (from 402 to 450 nm) and moderate-to-low quantum yields (0.24 as average) with potential to behave as fluorescent turn-on probes (Table S1 in ESI†). We examined their spectral properties in the presence of a broad panel of biomolecules (*e.g.* proteins, saccharides, nucleic acids, peptides and small molecule metabolites) and observed that acid fluorides **4a–4d** (Scheme S1 in ESI†) displayed a distinct fluorescence increase after incubation with histamine (Fig. S1 in ESI†). In particular, the reaction of histamine with **4a** resulted in the bathochromic shift of its emission spectrum (*i.e.*, from 406 to 432 nm) and a significant fluorescence increase that was distinguishable to the naked eye under a 365 nm lamp (Fig. 1).

As mesoionic acid fluorides were found to be remarkably stable to hydrolysis in neutral and basic solutions, we monitored the reaction between **4a** and histamine in phosphate buffer saline (PBS) by HPLC-MS (Fig. S2 in ESI†). After 20 min, **4a** was fully converted to the highly fluorescent conjugate **5i** (Fig. 1). We hypothesize that the formation of the amide **5i** may block the quenching electron transfer from the mesoionic core to the electron-deficient fluoride and lead to the observed fluorescence turn-on effect. In order to confirm the exact structure of the fluorescent species formed, we synthesized different amide



Scheme 1 General MCR scheme for mesoionic isoquinoline adducts **4**.

[a] *Institute for Biomedical Research, Barcelona Science Park, Spain.*
[b] *Laboratory of Bioimaging Probe Development, Singapore Bioimaging Consortium, A*STAR, Singapore.*
[c] *Barcelona Science Park, University of Barcelona, Spain.*
[d] *Laboratory of Organic Chemistry, Faculty of Pharmacy, University of Barcelona, Spain. E-mail: rlavilla@pcb.ub.es; Fax: +34 4021896; Tel: +34 4037126*
[e] *Department of Chemistry & MedChem Program of Life Sciences Institute, National University of Singapore, Singapore. E-mail: chmcyt@nus.edu.sg; Fax: +65 6779 1691; Tel: +65 6516 6774*

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‡ These authors contributed equally to this work.

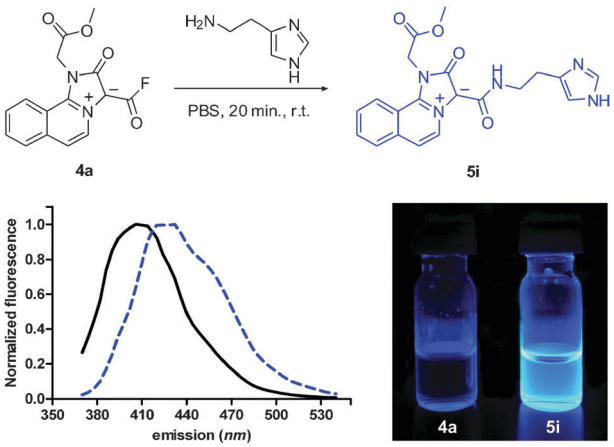

Fig. 1 Reaction of **4a** with histamine in PBS. Fluorescence spectra of **4a** before (black) and after (blue) reaction with histamine; exc: 340 nm. Fluorescent images of PBS solutions of **4a** and **5i** under a 365 nm lamp.

conjugates by the reaction of mesoionic acid fluorides with histamine, imidazole and *N*-Boc-histamine (Scheme S2 in ESI†). Mesoionic amides from histamine were highly fluorescent, while amides from imidazole and *N*-Boc-histamine were non-fluorescent due to the quenching PeT (photoinduced electron transfer) of the imidazole group (Fig. S3 in ESI†). This observation indicates that the linkage between **4a** and histamine is through the primary amine and not through the nitrogen of the imidazole.

Next we evaluated the selectivity of **4a** against histamine and different signaling molecules, mainly neurotransmitters and hormones. **4a** displayed a very good selectivity towards histamine with a maximum 14-fold fluorescence increase (Fig. 2). The combination of both imidazole and amine groups of histamine appeared to be critical for this response, as only minor enhancements were observed with other primary amines. The kinetic analysis of these reactions indicated that **4a** exhibited an optimum selectivity toward histamine at incubation times between 15 and 30 min (Fig. 2 inset). Furthermore, **4a** showed a very weak fluorescent response against other intracellular metabolites (*e.g.* glutathione, glucose, ATP, ADP or cAMP), which asserted its suitability for imaging intracellular histamine (Fig. S4 in ESI†). In view of this selectivity and the fluorescent colour of the resulting histamine adduct, we named **4a** as **Histamine Blue**.

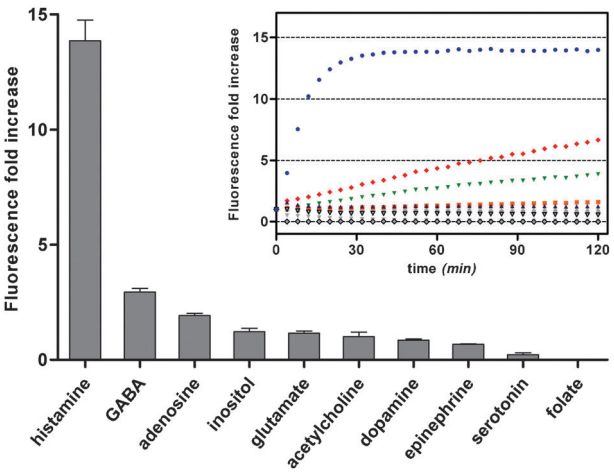

Fig. 2 Fluorescent response of **4a** (**Histamine Blue**) (10 μM) after incubation with different signaling molecules (5 mM) for 30 min in PBS (pH: 7.3). Inset: Kinetic analysis of the reactions of **Histamine Blue** with histamine (blue), GABA (red), adenosine (green), glutamate (orange), inositol (purple) and others (grey); exc: 370 nm. Values are represented as means (n = 4) and errors bars as standard deviations.

The fast kinetics of **Histamine Blue** prompted us to investigate the mechanisms of its preferential reactivity. Hence we examined the role of the sp^2 nitrogen of histamine by comparing the condensation rates of **Histamine Blue** with two similar primary amines including or not an sp^2 heterocyclic nitrogen (*i.e.*, 2-(pyridin-2-yl)ethylamine and 2-phenylethylamine respectively). The reactions of **Histamine Blue** with both amines proceeded at a similar rate, around 20-fold slower than histamine (Fig. S5 in ESI†). These results suggested that the role of the sp^2 nitrogen of the heterocycle was not the main factor in controlling the rate determining step of the reaction. Moreover, the unique catalytic effect observed with histamine might be more related to the imidazole group acting as a proton transfer moiety than as a nucleophilic catalyst (Scheme S3 in ESI†).[20,21]

In view of the characteristics of **Histamine Blue**, we evaluated its application for imaging histamine in live RBL-2H3 basophils and RAW 264.7 macrophages. **Histamine Blue** stained the cytoplasm of RBL-2H3 basophils, where histamine-storing granules are located, after incubating the cells with the dye (20 μM) for 15 min at 37 °C (Fig. 3). The fluorescence of **Histamine Blue** did not co-localize with DRAQ5 nuclear staining and matched the high levels of intracellular histamine in basophils, in the range of nmol histamine per 10^6 cells (Fig. 4d).[22,23] Moreover, the treatment of basophils with **Histamine Blue** did not affect their morphology or induced any cytotoxicity (Fig. S6, S7 in ESI†).

We also examined the ability of **Histamine Blue** to image the uptake and *de novo* synthesis of histamine in RAW 264.7 macrophages, which do not contain high levels of intracellular

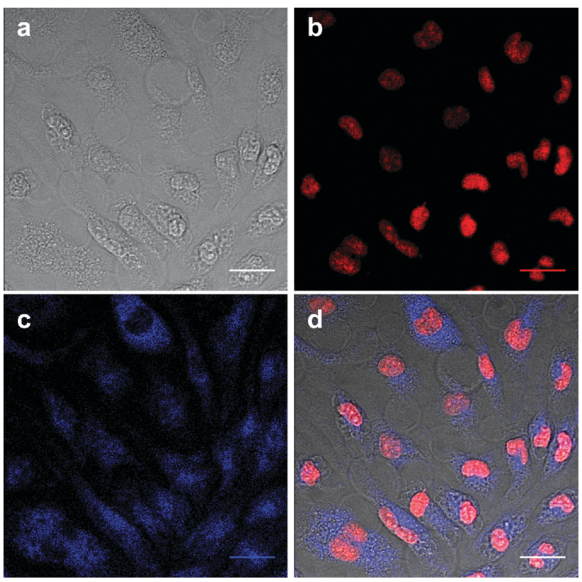

Fig. 3 RBL-2H3 basophils after incubation with **Histamine Blue**. (a) Bright field image, (b) DRAQ5 nuclear counterstaining (TXR), (c) **Histamine Blue** staining (DAPI), (d) merged a, b and c. Scale bar: 20 μm. Pearson's co-localization coefficient for TXR and DAPI channels: 0.16.

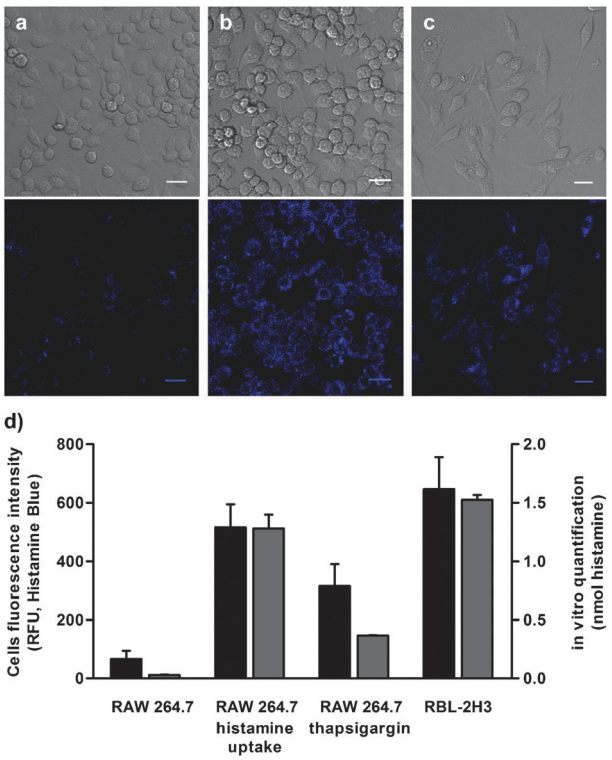

Fig. 4 RAW 264.7 macrophages after treatment with **Histamine Blue**. Bright field (upper) and fluorescent (lower) images after incubation with **Histamine Blue** of: (a) RAW 264.7, (b) RAW 264.7 after histamine uptake and (c) RAW 264.7 after treatment with thapsigargin. Scale bar: 20 μm. (d) Average fluorescence intensities of cells stained with **Histamine Blue** (black) and quantification of histamine levels per 10^6 cells (grey) in RAW 264.7 before and after chemical treatment and in RBL-2H3 basophils.

histamine under normal conditions. **Histamine Blue** did not stain non-treated macrophages (Fig. 4a), while brightly stained them after the uptake of histamine (Fig. 4b). Titration experiments showed that **Histamine Blue** stained RAW 264.7 cells in a histamine uptake-dependent manner (Fig. S8 in ESI†). These results are consistent with previous reports indicating that RAW 264.7 macrophages can reach histamine levels similar to basophils after incubation with 10 mM histamine for 2 h.[24] Furthermore, we determined the amount of histamine in extracts of RBL-2H3 basophils and RAW 264.7 macrophages by the conventional method using *o*-phthaldialdehyde.[25] *In vitro* quantification of the histamine levels correlated well with the fluorescence intensity of **Histamine Blue** in live cell imaging (Fig. 4d). Finally we induced *de novo* synthesis of histamine in RAW 264.7 by treatment with thapsigargin, an endomembrane Ca^{2+}-ATPase inhibitor.[26] **Histamine Blue** stained thapsigargin-treated macrophages (Fig. 4c) more brightly than non-treated macrophages (Fig. 4a). Altogether we proved that **Histamine Blue** can be used for imaging histamine in live basophils and macrophages under different physiological conditions.

In summary, we developed a new fluorescent probe (**Histamine Blue**) for imaging histamine in live cells. **Histamine Blue** is a mesoionic acid fluoride showing a 14-fold fluorescence increase upon condensation with histamine. **Histamine Blue** displays very good selectivity over a broad range of signalling molecules and metabolites due to the catalytic effect of the imidazole group, and is the first fluorescent small molecule for imaging histamine in live RBL-2H3 basophils. **Histamine Blue** can also monitor the uptake and *de novo* synthesis of histamine in live RAW 264.7 macrophages and its fluorescence intensity correlates well with the histamine levels in cell extracts. This discovery may assist the investigation of the roles that histamine plays in mammalian physiology and a number of pathologies.

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