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ORIGINAL ARTICLE

Histone deacetylase inhibitors restore IL-10 expression in lipopolysaccharide-induced cell inflammation and reduce IL-1β and IL-6 production in breast silicone implant in C57BL/6J wild-type murine model

Rosa Di Liddo[1]*, Sergio Valente[2]*, Samanta Taurone[3], Clemens Zwergel[2], Biagina Marrocco[2], Rosaria Turchetta[3], Maria Teresa Conconi[1], Carlotta Scarpa[4], Thomas Bertalot[1], Sandra Schrenk[1], Antonello Mai[2,5], and Marco Artico[3]

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Abstract

Among epigenetic enzymes, histone deacetylases (HDACs) are responsible for regulating the expression of an extensive array of genes by reversible deacetylation of nuclear histones as well as a large number of non-histone proteins. Initially proposed for cancer therapy, recently the interest for HDAC inhibitors (HDACi) as orally active, safe, and anti-inflammatory agents is rising due to their ability in reducing the severity of inflammatory and autoimmune diseases. In particular, selective HDAC3, HDAC6, and HDAC8 inhibitors have been described to downregulate the expression of pro-inflammatory cytokines (TNF-α, TGF-β, IL-1β, and IL-6). Herein, using KB31, C2C12, and 3T3-J2 cell lines, we demonstrated that, under lipopolysaccharide-induced *in vitro* inflammation, HDAC3/6/8 inhibitor MC2625 and HDAC6-selective inhibitor MC2780 were effective at a concentration of 30 ng/mL to downregulate mRNA expression of pro-inflammatory cytokines (IL-1β and IL-6) and to promote the transcription of *IL-10* gene, without affecting the cell viability. Afterwards, we investigated by immunohistochemistry the activity of MC2625 and MC2780 at a concentration of 60 ng/kg animal weight to regulate silicone-triggered immune response in C57BL/6J female mice. Our findings evidenced the ability of such inhibitors to reduce host inflammation in silicone implants promoting a thickness reduction of peri-implant fibrous capsule, upregulating IL-10 expression, and reducing the production of both IL-1β and IL-6. These results underline the potential application of MC2625 and MC2780 in inflammation-related diseases.

Keywords

Breast silicone implants, epigenetics, HDAC inhibitors, inflammation, pro-inflammatory cytokines

History

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Introduction

Epigenetic regulation of gene expression is now recognized as a novel approach to treat several diseases. Histone deacetylases (HDACs) are responsible for regulating the expression of an extensive array of genes by catalyzing the reversible deacetylation of ε-amino groups located on the N-terminal lysine residues of nuclear histones as well as a large number of non-histone proteins [1,2]. Although initially used mainly for cancer therapy, research now suggests that inhibitors of HDAC (HDACi) could also be utilized for treating diseases ranging from neurodegenerative [3–5] to inflammatory ones, such as asthma and rheumatoid arthritis [6,7]. Anti-inflammatory properties of HDACi are interestingly observed in the low nanomolar range, compared with the micromolar range needed to treat cancer. The recent expanding interest in HDAC inhibitors as orally active, safe, and anti-inflammatory agents has been increased by the ability of these inhibitors to reduce disease severity in several *in vitro* and *in vivo* models of inflammatory and autoimmune diseases [6,8,9]. Histone hyperacetylation results in up-regulation of cell-cycle inhibitors ($p21^{Cip1}$, $p27^{Kip1}$, and $p16^{INK4}$), repression of inflammatory cytokines (IL-1 and IL-8), tumor necrosis factor-α (TNF-α), and downregulation of immune stimulators (IL-6, IL-10, and CD154) [10]. The role of HDACs in the modulation of inflammatory mediators has been well reviewed: these enzymes are mainly involved in the activation of T-cells (HDAC1, HDAC6, HDAC7, and HDAC9), macrophages (HDAC2-5, HDAC6, and HDAC7), differentiation and proliferation processes (HDAC5), adhesion mechanisms of monocytes to endothelium (HDAC3-5), and osteoclast activity (HDAC3, HDAC5, HDAC7, and HDAC8) [11]. Among HDAC isoforms, HDAC3 is reported to play a pivotal role in monocyte recruitment to sites of inflammation and in macrophage cytokine production [12], while HDAC6 regulates the activity of $Foxp3^+$ T-regulatory cells [13].

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Interestingly, HDAC3-deficient macrophages stimulated with lipopolysaccharide (LPS) demonstrated a reduced expression of inflammatory cytokines [12]. Further studies [14] reported that histone deacetylase isoforms regulate innate immune response by deacetylating mitogen-activated protein kinase phosphatase-1 and thus inhibiting Toll-like receptor (TLR) signaling. Since their first use in aesthetic and reconstructive mammoplasty, breast prostheses demonstrated to cause a natural foreign body reaction (FBR) characterized by the infiltration of inflammatory cells, including neutrophils, monocytes/macrophages, T-cells (helper and cytotoxic subtypes) and B-lymphocytes [15], and non-inflammatory cells, including fibroblasts and myofibroblasts [16,17]. It is well documented that FBR response leads to fibrotic encapsulation around the expander in the first 20 d after surgery. In susceptible individuals, the progression of inflammatory reaction could promote the formation of capsule contracture, a local complication of unknown causes [18]. The periprosthetic capsule represents a dynamic inflammatory reaction that involves in early-phase monocytes/macrophages and, later on, $CD4^+$ T helper cells, recruited by inflammatory microenviroment. Among cytokines released by macrophages, TNF-α and TGF-β stimulate neo-angiogenesis, proliferation of fibroblasts, and deposition of collagenous matrix [19]. The current knowledge suggests that the local application of oligonucleosides targeting Connective Tissue Growth Factor (CTGF) and TGF-β could be effective in reducing CTGF synthesis and capsular formation. Zimman et al. [20] detected a reduction of TGF-β1, autoantibodies against collagen III and periprosthetic fibrosis in rats treated with enalapril, an inhibitor of angiotensin-converting enzyme and angiotensin II receptor antagonist. Moreover, leukotriene receptor antagonists, such as zafirlukast and montelukast, showed an antifibrotic activity [21,22]. In patients with rheumatoid arthritis (RA), the co-treatment with methotrexate and etanercept revealed to be useful to modulate T helper-1 and -2 cells and to regulate the secretion of the main cytokines involved in the formation of fibrous capsule (IL-1, IL-6, IL-17, TNF-α, and TGF-β) [23]. Several studies have demonstrated that HDAC inhibitors act as modulators of the inflammatory response: the HDAC pan-inhibitors SAHA (Zolinza®, Merck, Darmstadt, Germany) and ITF2357 (givinostat, Merck, Darmstadt, Germany) were demonstrated to reduce the production of TNF-α, IL-1α, IL-1β, and IFN-γ from peripheral blood mononuclear cells (PBMCs) stimulated with LPS [9,24]. The expression of inflammatory cytokines is regulated by reversible acetylation and deacetylation of histones and transcription factors. HDACi were demonstrated to be effective therapeutics in animal models of rheumatoid arthritis (RA) and have anti-inflammatory effects in RA synovial macrophages and tissue via modulation of mRNA stability. In particular, HDACi trichostatin A (TSA) suppressed IL-6 production by RA fibroblast-like synoviocytes stimulated with IL-1β [25]. Similarly, the treatment of LPS-stimulated macrophages with TSA reduced IL-6, TNF-α, and IL-1β expression [26]. In an experimental colitis model, SAHA treatment resulted in a dose-dependent suppression of cytokine synthesis and apoptosis induction of lamina propria lymphocytes [27] and promoted the reduction of LPS-induced septic shock in rodents downregulating the expression of TNF-α and IL-1β [28]. In addition, TSA suppressed the ability of IL-1β and TNF-α to up-regulate matrix metalloproteinase (MMP)-3, consistently with a cartilage protective effect [29]. Moreover, ITF2357 reduced joint inflammation in rat and mouse models, and, in contrast to SAHA, was able to prevent joint destruction as well as inflammation [30]. Recently, the HDAC3-selective inhibitor MI192 was found to reduce TNF-α production and to dose dependently suppress IL-6 production in PBMCs from RA patients [31]. Noteworthy, the HDAC6-selective inhibitor tubastatin A [32,13] was shown to enhance the suppressive effects of $Foxp3^+$ regulatory T cells (Tregs), suggesting to be a potential therapeutic approach to slow or reverse the pathogenesis of autoimmune disorders and prevent allograft rejection, inflammatory bowel disease, and RA [33–35]. Recently, the specific inhibition of HDAC8 by ITF3056, an analogue of ITF2357, was reported to downregulate both gene expression and production of pro-inflammatory cytokines [36]. Therefore, based on the findings correlating HDACs with innate immune response, herein we investigated *in vitro* and *in vivo* the anti-inflammatory activity of two different HDAC inhibitors (MC2625 and MC2780) selected from our own library. MC2625 (Figure 1) is an isosteric pyridine analog of a series of aroylamminocinnamyl hydroxamates [37–40], that carries a branched bulky group at the cap moiety, a chemical modification previously demonstrated to furnish isoform-selective HDAC inhibition [41]. MC2780 (Figure 1) bears a *tert*-butoxycarbonylamino group bound to the phenyl cap moiety, a structural feature known to confer high potency and selectivity towards HDAC6 [42]. Both MC2625 and MC2780 molecules were tested *in vitro* on epithelial (KB31), fibroblast (3T3-J2), and myogenic (C2C12) cells to evaluate their cytotoxicity by cell viability test and their capability to modulate mRNA expression of *IL-1β*, *IL-6*, and *IL-10* genes. Moreover, histological and immunohistochemical analysis were performed to study fibrous capsular reaction and pro- (IL-1β and IL-6) and anti-inflammatory (IL-10) cytokines-mediated immune response to silicone implants, adsorbed with HDACi and placed in C57BL/6J female mice.

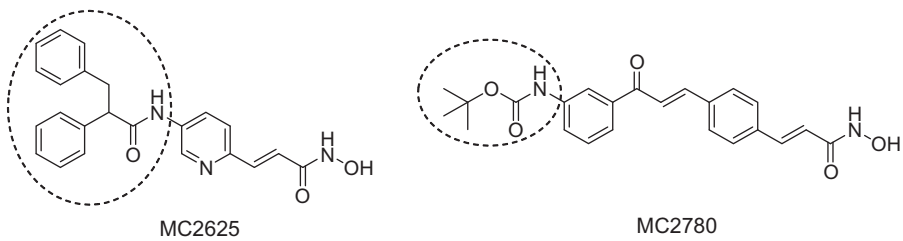

Figure 1. HDACi described as anti-inflammatory agents. Structures of the HDACi MC2625 and MC2780 studied in this work.

Materials and methods

Chemistry and biochemistry

The synthetic routes for the preparation of MC2625 (racemic mixture) and MC2780 have been recently described by our group [43,42]. Individual IC_{50} values for each HDAC isozyme were measured with the homogeneous fluorescence-release HDAC assay. Purified recombinant enzymes were incubated with serial diluted inhibitors at the indicated concentration, in 10-dose IC_{50} mode with three-fold serial dilution starting from 50 μM solutions. The deacetylase activities of HDACs 1–11 were measured by assaying enzyme activity using AMC-K(Ac)GL (classes I, IIb, and IV HDACs) or AMC-K(TFA)GL (class IIa HDACs) substrate. Deacetylated AMC-KGL was sensitive toward lysine peptidase, and free fluorogenic 4-methylcoumarin-7-amide (MCA) was generated, which can be excited at 355 nm and observed at 460 nm (Reactive Biology Corp., Annapolis, MD). Data were analyzed on a plate-to-plate basis in relationship to the control and imported into analytical software (GraphPad Prism, San Diego, CA).

In vitro studies of cytotoxicity and biological activity of HDACi

Cytotoxic effects and biological activity of MC2625 and MC2780 molecules were studied *in vitro* using epithelial (KB31), fibroblast (3T3J2), and myogenic (C2C12) cell lines. Cells were seeded (20×10^3 cells/cm^2) in six-well tissue culture dishes (BD Falcon, Milan, Italy) and cultured with Dulbecco's Modified Eagle's Medium (DMEM low glucose, Euroclone, Milan, Italy) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Milan, Italy), and 1% penicillin/streptomycin (Invitrogen, Darmstadt, Germany).

Cell viability assay

When populations reached 70% confluency, HDACi were added to the cell cultures at different concentrations ranging from 30 ng/mL to 300 μg/mL. After 24 h of incubation, cytotoxicity of MC2625 and MC2780 molecules was assessed by flow cytometry using BD Cell Viability Kit (BD Biosciences, Milan, Italy). The detection of injured, dead, and viable cells was performed by staining with thiazole orange (TO), a nucleic-acid-specific dye and propidium iodide (PI). Aliquots of 2.0 μL of TO and 1.0 μL of PI were added to 1 mL of cell suspensions containing $\sim 5 \times 10^5$cells, to obtain the final staining concentrations of 84 nM for TO and 4.3 μM for PI. After incubation for 5 min at room temperature, the samples were loaded on BD FACSCanto™ II system (Becton Dickinson, San Jose, CA) equipped with BD FACSDiva software. Data were acquired as a percentage of dead, injured, and live cells discriminated using TO and PI mean fluorescence intensity (MFI). According to the instructions of the manufacturer, high MFI value of TO combined with negative expression of PI identified living cells, while injured and dead cells were detected due to a decreased TO fluorescence intensity and an intermediate or high MFI value of PI, respectively. For analysis, MFI values of TO and PI from HDACi-untreated samples were used as references for each cell line. Statistical significance was calculated by Student's *t*-test comparing MC2625- and MC2780-treated cultures to untreated cells.

Gene expression study

KB31, C2C12, and 3T3J2 cells were treated *in vitro* for 24 h with MC2625 and MC2780 at concentrations of 30 ng/mL and 300 ng/mL. In order to evaluate the anti-inflammatory activity of HDACi, we added to culture media 5 μg/mL lipopolysaccharide (LPS) (Sigma-Aldrich, Milan, Italy). In parallel, samples untreated (negative controls) or treated with LPS alone (LPS-controls) were used as a reference. Total cellular RNA was extracted using TRIzol® (Invitrogen™ Life Technologies, Grand Island, NY) and quantified by NanoDrop 2000 (Thermo Fisher Scientific, Inc., Waltham, MA). All samples were reverse transcribed with Thermoscript™ RT-PCR System kit (Invitrogen™ Life Technologies, Grand Island, NY) and an iCycler iQ™ (Bio-Rad Laboratories, Hercules, CA). The amplification reaction was carried out using specific oligo primers (Table S1) (Invitrogen™ Life Technologies, Grand Island, NY), Platinum® SYBR® Green qPCR SuperMix UDG kit (Invitrogen™ Life Technologies, Grand Island, NY), and a DNA Engine Opticon® Real Time Thermal Cycler (MJ Research, St. Bruno, QC, Canada). The amount of gene products was calculated with a linear regression analysis from standard curves, demonstrating amplification efficiencies ranging from 95% to 100%. Data were reported as a fold increase of target gene expression defined as a complementary DNA (cDNA) ratio between target gene and hypoxanthine–guanine phosphoribosyltransferase (HPRT) housekeeping gene. Statistical significance was calculated by Student's *t*-test comparing with negative controls (asterisk: p value ≤ 0.05) or to LPS-controls (one black triangle: p value ≤ 0.05).

In vivo biological activity of HDACi

Ethics statement

C57BL/6J wild-type female mice (2 weeks old) ($n = 18$) were provided by the Animal House facility of Padova University, Italy. The use and care of animals as well as the surgical procedures were approved and supervised by the local Ethic committee (protocol 32132/2011 CEASA, University of Padova, Padova, Italy). For subcutaneous implantation of texturized silicone (Allergan, Inc., Irvine, CA), the animals were anesthetized by isofluorane (Forane®, Abbott S.P.A., Campoverde, Italy) (3% oxygen with the flow of 1 L/min) in a transparent Plexiglas box. After 30 d, the animals were sacrificed by cervical dislocation and the silicone patches along with surrounding tissues were removed.

In vivo implantation of silicone patches adsorbed with HDACi

Round-shaped silicone implants were prepared using a 6-mm biopsy punch (Kai Medical, Solingen, Germany) and then sterilized at 120 °C for 20 min. The patches were pre-treated overnight at 37 °C with MC2625 and MC2780 solutions (3 μg/mL in PBS/DMSO), obtaining a final adsorbed concentration of 60 ng/kg. HDACi-untreated silicone was prepared and implanted as control. After a skin incision on the back side, a patch (6 mm diameter) of texturized silicone was

subcutaneously implanted in mice. After 30 d, the animals were sacrificed by cervical dislocation and silicone along with surrounding tissues was explanted for histological evaluation.

Histology

Explants were fixed overnight in 10% formalin solution, washed in PBS, and embedded in paraffin (Carlo Erba Reagents S.r.l., Milan, Italy). Sections of 4 μm thickness were prepared (Histoslide 2000 microtome, Leica Reichert Jung, Leica Camera Inc., Allendale, NJ) and then stained with conventional hematoxylin–eosin (H&E) according to standard protocols. In parallel, the analysis of capsular collagen was performed using Trichrome Stain (Masson) Kit (Sigma-Aldrich, Milan, Italy) according to the instructions of the manufacturer.

Immunohistochemistry

Explants were washed in PBS, fixed in 10% formalin, and embedded in paraffin [44]. Serial 3-μm thick sections were cut using a rotative microtome, mounted on gelatin-coated slides, and then processed for immunohistochemical analysis by ABC/HRP (avidin–biotin complex/horseradish peroxidase) technique. After deparaffinization in xylene and rehydration, sections were immersed in citrate buffer (pH 6) and thus subjected twice to microwave irradiation for 5 min. All sections were treated for 30 min with 0.3% hydrogen peroxide in methanol for quenching endogenous peroxidase activity. Thereafter, the samples were incubated overnight at 4 °C with the following antibodies: (i) rabbit anti-IL-1β polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA); (ii) rabbit anti-IL-6 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA); (iii) rabbit anti-IL-10 polyclonal antibody (Immunological Sciences, Rome, Italy). The immunodetection of target proteins was performed by incubation at room temperature with secondary biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) for 1 h and, at a later phase, with peroxidase-conjugated avidin (Vectastain Elite ABC Kit Standard, Vector Laboratories, Burlingame, CA) for 35 min. The immunoreactive sites were revealed using 0.1% H_2O_2 and 0.05% 3,3-diaminobenzidine (DAB) (Vector Laboratories Burlingame, CA). Finally, sections were counterstained with Mayer's hematoxylin and observed using Leica DFC480 microscope (Leica Camera Inc., Allendale, NJ). Negative control experiments were carried out: (i) by omitting the primary antibody; (ii) by substituting the primary antibody with an equivalent amount of non-specific immunoglobulins; (iii) by pre-incubating the primary antibody with the specific blocking peptide (antigen/antibody = 5 according to supplier's instructions). Data were collected by two experienced observers in light microscopy. The intensity of immunoreactivity was evaluated microdensitometrically using an IAS 2000 image analyzer (Delta Sistemi, Rome, Italy) connected to a microscope via TV camera. The calibration of system was made taking as zero the background obtained in sections incubated with non-immune serum. For analysis, 10 regions (100 μm^2 area) per section were delineated within fibrous capsule by a measuring diaphragm and data were expressed as a fold increase of intensity value detected for each target protein in HDACi-treated samples compared with controls. Significant differences were statistically defined using the analysis of variance (ANOVA) followed by Duncan's multiple range test as a post hoc test.

Results

HDAC inhibition data

Both MC2625 (racemic mixture) and MC2780 molecules were tested against all the HDAC1-11 isoforms in 10-dose IC_{50} mode with three-fold serial dilution starting from 50 μM solutions, and the relative inhibition data are recapitulated in Table 1 [43,42]. From these data, MC2625 behaved as a specific HDAC3/HDAC6/HDAC8 inhibitor, because it is able to inhibit these isoforms at nanomolar (HDAC3 and HDAC6) or submicromolar (HDAC8) concentration, while the remaining HDAC enzymes are inhibited in the range 1.4–11.7 μM. Differently, MC2780 emerged as a potent and selective HDAC6 inhibitor, displaying an IC_{50} value of 0.01 μM against this isoform and being from 120- to >5000-fold less potent versus the other HDAC enzymes.

In vitro citotoxicity study of HDACi

Epithelial KB31, fibroblast 3T3-J2, and myogenic C2C12 cell lines were employed to assess potential citotoxic effects of MC2625 and MC2780 molecules at concentrations ranging from 300 μg/mL to 30 ng/mL. Based on the fluorescence intensity values of TO and PI, flow cytometrical analysis evidenced that 300 μg/mL MC2625 drastically affected the viability of 3T3-J2 (0% viable cells) while exerted less cytotoxicity in C2C12 (66% viable cells) (Figure 2). At the same concentration, MC2780 caused 100% cell death in 3T3-J2 and C2C12 populations (Figure 3). When added at lower doses (≤30 μg/mL), a higher percentage of viable cells ranging from 85 to 96 % in 3T3-J2 cells and 90–96% in C2C12 samples was observed, hence suggesting the suitability of these HDACi solutions for *in vitro* and *in vivo* evaluation of biological activity. The viability of KB31 cells was not affected by MC2625 and MC2780 molecules, in accordance with published data reporting that mechanisms of resistance to HDAC inhibitors are active in tumor cells [45].

Table 1. Inhibition of HDAC1–11 isoforms by MC2625 and MC2780.

	IC_{50} against HDAC isoforms, μM										
Cpd	1	2	3	4	5	6	7	8	9	10	11
MC2625	1.42	1.77	0.08	11.7	9.37	0.01	8.77	0.61	10.6	1.8	10.2
MC2780	2.9	2.1	10.8	3.2	>50	0.01	>50	1.2	68.8	5.1	12.0

Evaluation of *in vitro* biological activity of HDACi

Originally described by its ability to inhibit T-helper (Th) 1 activation and Th1 cytokine production, interleukin 10 plays an essential part in controlling inflammation and instructing adaptive immune responses [46]. Besides immune cells [47], keratinocytes [48], muscle cells [49], and fibroblasts [50] have shown to express IL-10. Consistent with these evidences, the analysis of qPCR products (Figure 4) evidenced a basal expression of IL-10 in KB31, C2C12, and 3T3J2 cells (Figure 4). As previously demonstrated for other HDAC

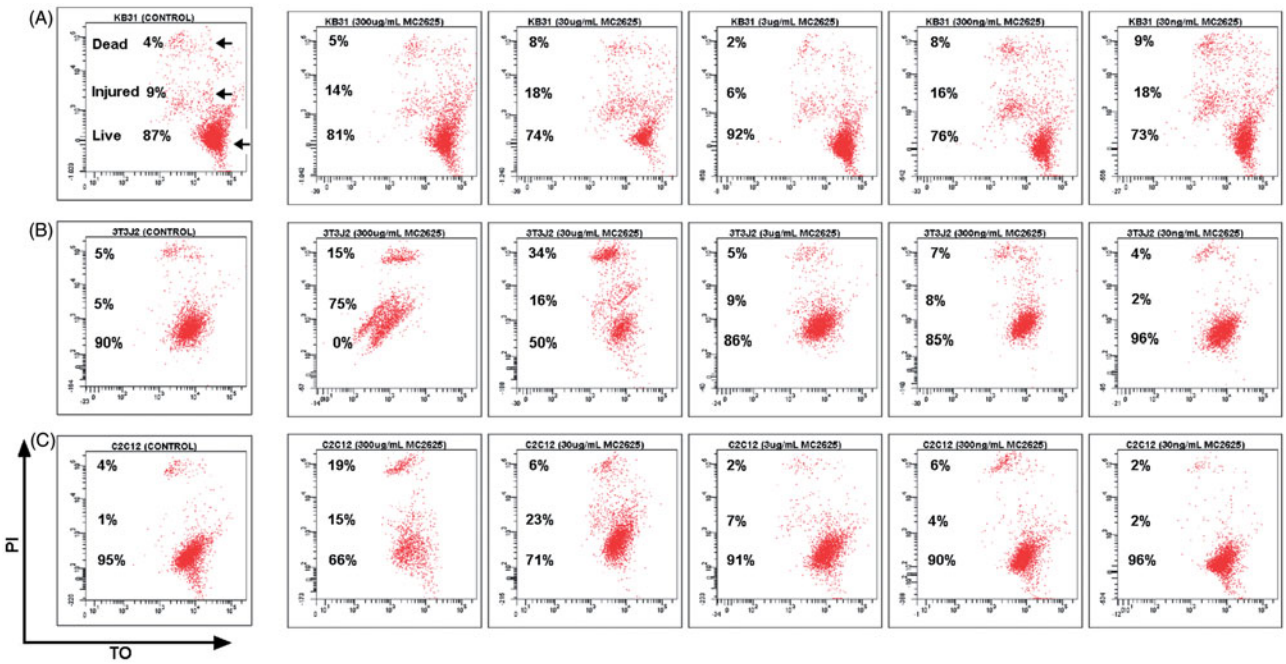

Figure 2. Cell viability in cell lines treated with MC2625. Cell viability test on KB31 (A), 3T3J2 (B), and C2C12 cells (C) treated with MC2625 solutions (from 300 µg/mL to 30 ng/mL) for 24 h at 37 °C and measured by flow cytometry with thiazole orange (TO) and propidium iodide (PI). Data were acquired as a percentage of dead, injured and live cells discriminated using TO and PI mean fluorescence intensity (MFI). According to instructions of the manufacturer, high MFI value of TO combined with negative expression of PI identified living cells, while injured and dead cells were detected due to a decreased TO fluorescence intensity and an intermediate or high MFI value of PI, respectively. For analysis, MFI values of TO and PI from HDACi-untreated samples were used as references for each cell line. Statistical significance was calculated by Student's *t*-test comparing MC2625- and MC2780-treated cultures to untreated cells (p value<0.05).

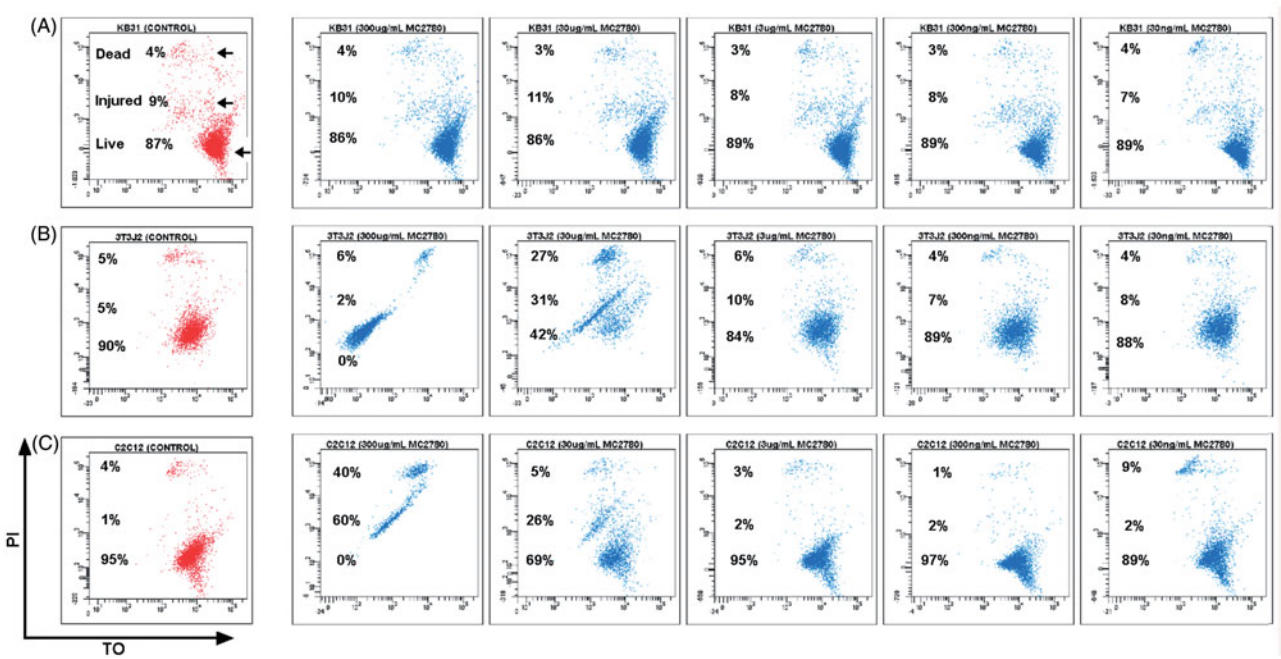

Figure 3. Cell viability in cell lines treated with MC2780. Cell viability test on KB31 (A), 3T3J2 (B), and C2C12 cells (C) exposed to MC2780 solutions (from 300 µg/mL to 30 ng/mL) for 24 h at 37 °C and measured by flow cytometry with thiazole orange (TO) and propidium iodide (PI). Data were reported as a percentage of dead, injured, and live cells measured by flow cytometry with BD Cell Viability Kit (BD Biosciences, Milan, Italy) containing thiazole orange (TO) solution to stain all cells and propidium iodide (PI) to stain dead cells (p value < 0.05).

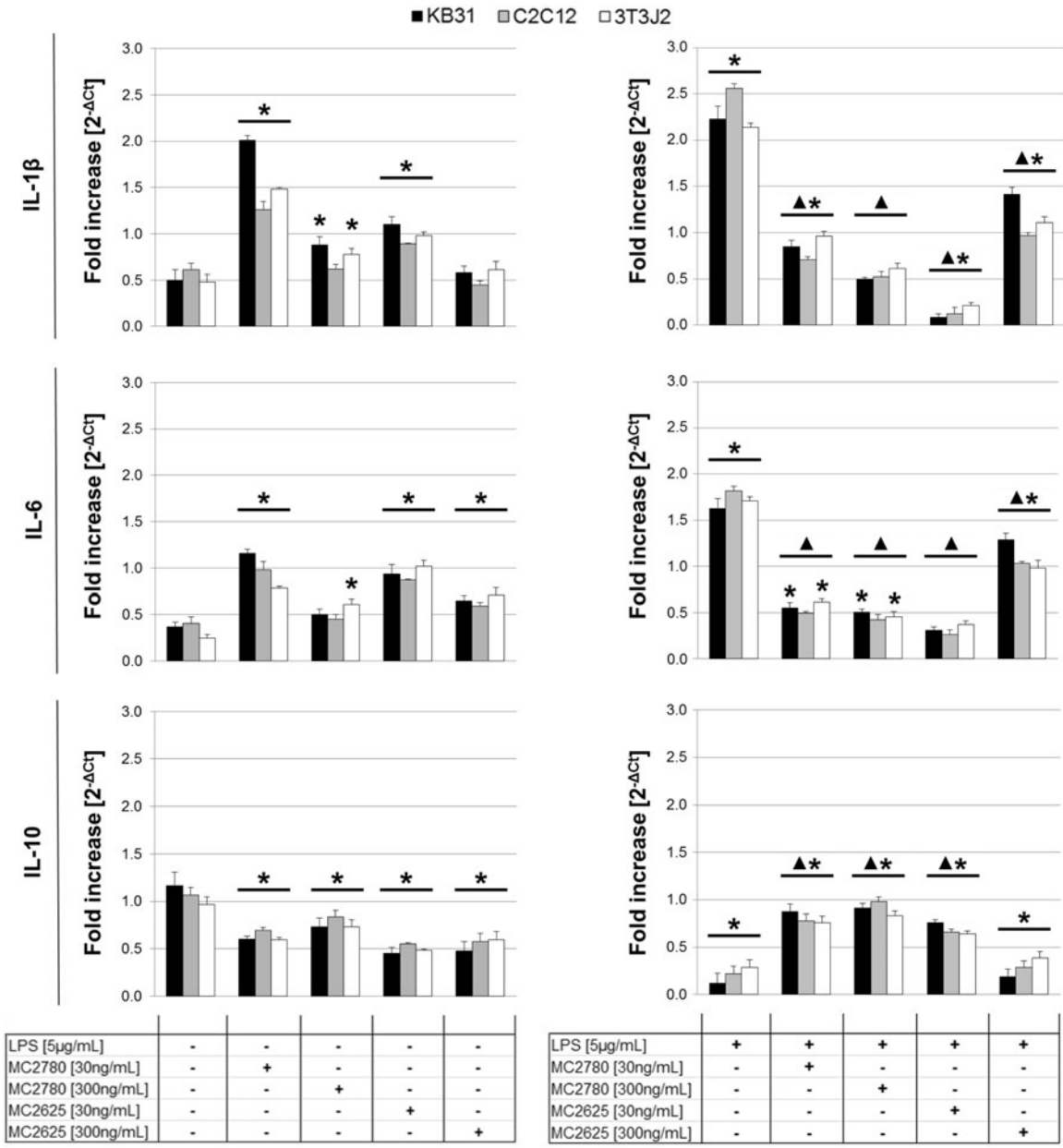

Figure 4. Expression of *IL-1β, IL-6*, and *IL-10* genes is modulated by MC2625 and MC2780 in LPS-mediated *in vitro* inflammation. KB31, C2C12, and 3T3J2 cultures were treated *in vitro* for 24 h with MC2780 and MC2625 used both at 300 ng/mL and 30 ng/mL, in the presence or absence of LPS (5 µg/mL). In parallel, samples untreated (negative controls) or treated only with LPS (LPS-controls) were prepared. To prove the anti-inflammatory activity of our HDAC inhibitors, the analysis of *IL-10* gene expression was performed by qPCR. The amount of gene products was calculated using a linear regression analysis from standard curves, demonstrating the amplification efficiencies ranging from 95% to 100%. We reported the fold increase of gene expression that was defined as the cDNA ratio between target gene and reference gene (HPRT). Statistical significance was calculated using Student's *t*-test, comparing with negative controls (asterisk: p value < 0.05) or to LPS-controls (black triangle: p value < 0.05).

inhibitors [51], MC2625 and MC2780 solutions with higher (300 ng/mL) and lower (30 ng/mL) concentration downregulated ($p \leq 0.05$) the expression of IL-10 while stimulating the transcription of IL-1β and IL-6. These data are in accordance with other studies reporting that HDAC inhibitors deacetylate MAPK phosphatase-1 (MKP-1) increasing TLR signaling-mediated inflammation [52]. When the cells were cultured with LPS alone, all samples demonstrated a significant reduction of IL-10 transcription activity, probably due to TLR signaling activation following MKP-1 acetylation by LPS [52,53]. Interestingly, the co-treatment of cell cultures with LPS and MC2780 (300 ng/mL or 30 ng/mL) or MC2625 (30 ng/mL) showed to restore ($p \leq 0.05$) IL-10 gene expression and to downregulate the transcription of pro-inflammatory cytokines (IL-1β and IL-6). Taken into consideration these *in vitro* findings, using an *in vivo* mouse model, we, therefore, investigated the potentiality of MC2625 and MC2780 to inhibit the inflammatory process triggered by silicone subcutaneous implantation.

In vivo anti-inflammatory activity of MC2625 and MC2780

Round-shaped silicone implants pre-adsorbed or not with HDACi were placed subcutaneously in 2 weeks old C57BL/6J female mice. After 30 d from implantation, silicone patches

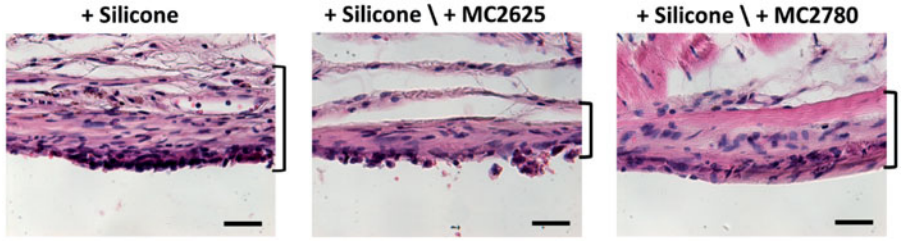

Figure 5. H&E staining. Histological analysis by hematoxilin/eosin on silicone patches and surrounding tissues excised from C57BL/6J mice. HDACi-untreated animals (+Silicone); MC2625-treated animals (+ Silicone\+ MC2625); MC2780-treated animals (+ Silicone\+MC2780). Capsule thickness was marked by square brackets. Scale bar: 25 μm.

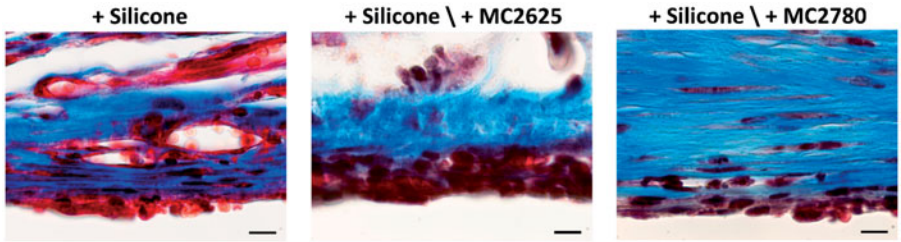

Figure 6. Masson's trichrome staining. Histological analysis by Trichrome (Masson) Stain on silicone patches and surrounding tissues excised from C57BL/6J mice. HDACi-untreated animals (+Silicone); MC2625-treated animals (+ Silicone\+ MC2625); MC2780-treated animals (+ Silicone\+MC2780). Cytoplasm and muscle fibers (red (in web)/black (in print)); collagen (blue (in web)/black (in print)). Scale bar: 10 μm.

along with surrounding tissues were extracted and submitted to histological evaluation.

The most common complications in silicone mammary implants is the development of capsular fibrosis and capsular contracture resulting from a silicone-mediated activation of fibroblasts [54], macrophages, and T cells [55]. As reported by several studies, human fibrous capsule is characterized by three layers known as *intimal*, *intermediate,* and *outer layers* where the *intimal zone* is the inner compartment composed of fibrocytes and histiocytes forming an epithelial-like single layer, synovial-type metaplasia, the *intermediate layer* contains smaller fibrils in loose connective tissue, and the *outer layer* is the transition zone characterized by densely packed collagen fibers and blood vessels [56]. In response to silicone implants, activated inflammatory cells secrete pro-inflammatory cytokines, recruiting fibroblasts and promoting collagen deposition. IL-1β [57] and IL-6 [58] are demonstrated to promote the progression of inflammation and fibrosis. In contrast, IL-10 exerts anti-inflammatory activity and regulates collagen synthesis [59,60]. In our work, a fibrous capsule was detected in all animals around the silicone patch, showing a bilayered structure with cells located predominantly in the inner layer while extracellular matrix largely expressed in the outer layer (Figure 5). Morphometric analysis of silicone specimens demonstrated a reduced capsule thickness in samples treated with HDACi, suggesting that MC2625 and MC2780 modulated *in vivo* the inflammatory response acting on fibroblast populations and down-regulating collagen deposition. Moreover, a significant difference in the orientation of capsular collagen fibers was observed among samples treated with HDAC inhibitors compared with controls, demonstrating that MC2625 and MC2780 differently affected the process of fibrillogenesis or collagen assembly. The analysis by Masson's trichrome staining (Figure 6) evidenced that the fibers were randomly distributed in silicone patches adsorbed with MC2625. In contrast, controls and samples treated with MC2780 showed a fibrotic capsule with bundled parallel fibers. The evidences collected were consistent with the study reported by Laitung et al. [54], who found that lower capsular formation and contracture is related to less number of myofibroblasts in the tissue around expanding devices. The severity of capsule formation and contracture has a positive linear correlation with the degree of local inflammation [18], that is largely regulated by macrophage-derived growth factors and fibrocyte-stimulating cytokines, such as IL-6, TNF-a, IL-1β, and TGF-β [61]. Compared with controls, HDACi-treated samples showed a significant ($p < 0.05$) lower immunoreactivity for IL-1β and IL-6 (Figure 7) besides an increased expression of anti-inflammatory IL-10. These data were consistent with results from our *in vitro* studies demonstrating that MC2625 or MC2780 would contribute to inhibit local inflammation in silicone surrounding tissue regulating the expression of IL-1β, IL-6, and IL-10 from inflammatory and not inflammatory cells.

Discussion and conclusion

Histone deacetylases balance the acetylation activities of histone acetyltransferases on chromatin remodeling and regulate gene transcription. Accumulated evidence indicates that HDAC activity is also associated with the development and progression of some chronic fibrotic diseases [62]. Thus, a growing interest is recently focused on the potential applications of HDAC inhibitors in the treatment of disorders arising from unbalanced fibroblast activation and proliferation. Several studies report that HDACi exerts anti-inflammatory effects downregulating the expression of TNF-α, TGF-β, IL-1β, and IL-6 under *in vitro* and *in vivo* experimental conditions. Moreover, it is demonstrated that HDAC inhibitors regulate the immune response through the inhibition of HDAC6 and the acetylation of non-histone proteins (i.e., p53, GATA1-3, STAT3, STAT5, Foxp3, and NF-κB) [63]. In our study, using *in vitro* and *in vivo* settings, two inhibitors of HDAC, called MC2625 and MC2780, were tested at not cytotoxic concentrations for anti-inflammatory and anti-fibrotic activities. Both MC2625, an inhibitor of HDAC3/6/8, and MC2780, a selective inhibitor of

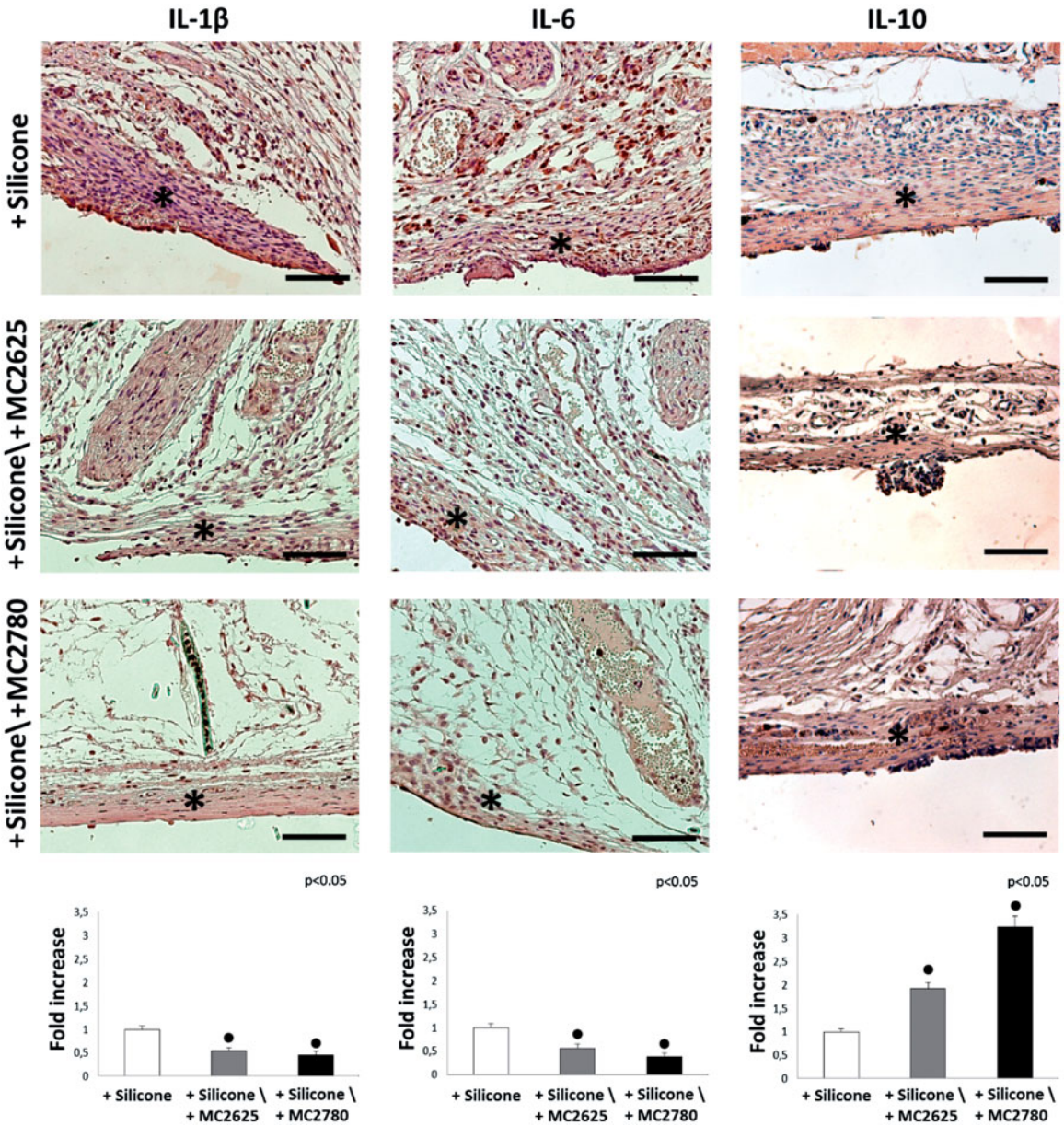

Figure 7. Silicone implantation-induced inflammation is reduced *in vivo* by MC2625 and MC2780. Immunohistochemical analysis of *IL-1β*, *IL-6*, and *IL-10* genes in explants from mice transplanted with silicone alone (*group:* + Silicone), silicone adsorbed with MC2625 (*group:* + Silicone\+ MC2625) or MC2780 (*group:* + Silicone\+ MC2780). Immunoreactive sites (brown) were revealed by exposure to DAB substrate. The intensity of immunoreactivity was evaluated microdensitometrically using an IAS 2000 image analyzer (Delta Sistemi, Rome, Italy) connected to microscope via TV camera. The calibration of system was made taking as zero the background obtained in sections incubated with non-immune serum. For analysis, ten regions (100 μm^2 area) per section were delineated within fibrous capsule (black asterisk) by a measuring diaphragm. In histograms, data were expressed as a fold increase of intensity value detected for each target protein in HDACi-treated samples compared to controls. Significant differences were statistically defined using the analysis of variance (ANOVA) followed by Duncan's multiple range test as a post hoc test (black circle: p value < 0.05).

HDAC6, demonstrated comparable anti-inflammatory effects downregulating IL-1β, IL-6, and stimulating the expression of IL-10 but diverged in the anti-fibrotic abilities. The differential biological response to MC2625 and MC2780 well correlated with their distinct inhibition profile and were consistent with published evidences reporting the ability of HDACi to regulate the innate immune response inhibiting inflammatory cells and stimulating conventional and regulatory T lymphocytes [64]. It is reported that the inhibition of HDAC6 is essential for the stabilization of Foxp3, the winged-helix family transcription factor involved in the differentiation of Treg cells from $CD4^+$ naïve T cells. Taken into consideration that IL-10 is expressed by Treg and IL-10 suppresses the production of pro-inflammatory cytokines by dendritic cells and macrophages [65], the comparable anti-inflammatory activity of MC2625 and MC2780 could be dependent on the inhibition that both molecules exerted on HDAC6. The reduction of fibrous capsule thickness observed in mice treated with HDAC inhibitors further confirmed the ability of both MC2625 and MC2780 to modulate the inflammatory response, although with a different grade. The implantation of biomaterials is

known to trigger a classical foreign body response and leads to a chronic inflammation [66] driven by monocytes and macrophages recruited by chemokines and chemo attractants such as transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), and interleukin (IL-1). After chronic inflammation is resolved, fibroblast cells activate and differentiate into myofibroblasts due to the stimulation of TGF-β, a key regulator of extracellular matrix formation and remodeling [67]. Fibroblasts and myofibroblasts secrete collagen types I and III to form a fibrous capsule around the implant and contract the wound edges. Several studies demonstrated that TGF-β-inducible genes, including collagen and smooth muscle actin are suppressed by HDAC inhibitors [68,69] and the extracellular matrix remodeling is regulated by the inhibition of class I HDAC3 [70] Moreover, also the process of collagen assembly or fibrillogenesis has been demonstrated to be affected by HDAC inhibitors [71]. In our study, although both HDAC inhibitors promoted a reduction of fibrous capsular thickness, only MC2625 showed to exert an anti-fibrotic activity interfering with the parallel assembly of collagen, a predisposing condition to the development of capsular contracture. Consistent with data above reported, this biological effect was hypothesized to be mediated by the inhibition of class I HDAC3 and 8.

Further studies on MC2625 and MC2780 will explore their potential therapeutical applications in other inflammation-related diseases especially those well responding to the administration of recombinant IL-10 [72].

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Declaration of interest

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Supplementary material available online

Supplementary Table S1