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RESEARCH ARTICLE

ANTI-INFLAMMATORY ACTIVITY OF NOVEL HDAC8 INHIBITORY 2,5-DISUBSTITUTED-1,3,4-OXADIAZOLES WITH GLYCINE/ALANINE HYBRIDS

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ABSTRACT

Histone deacetylases (HDACs) are the regulators of inflammation and HDAC inhibitors are shown to be anti-inflammatory agents. Previously, we designed and synthesized a series of novel glycine and alanine hybrids of 2,5-disubstituted 1, 3, 4-oxadiazoles as class I HDAC inhibitors. All the compounds synthesized (10a-j) showed moderate HDAC8 selectivity and anti-tumor activity. Here we evaluated the anti-inflammatory potency of the compounds on *E. coli*-infected mouse macrophage, RAW 264.7, cells. Among the 10 compounds (10a-j), compounds 10f and 10h alone did not inhibit the macrophage proliferation. Further studies using 10f and 10h showed reduced colony-forming units (CFU) of bacteria isolated from the infected macrophage and inhibition of the COX-2 inflammatory protein levels along with inhibition of the pro-inflammatory cytokines (IL-12, TNF-α, IFN-γ). Further *in vivo* studies using carrageenan-induced paw edema in rat model demonstrated a significant reduction in the paw edema at 20 mg/kg body weight when compared to untreated control. The study results thus signify that compounds 10f and 10h with moderate HDAC8 inhibitory activity also possess potent anti-inflammatory activity.

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INTRODUCTION

Acetylation of histone and non-histone proteins regulates diverse cellular processes including cell proliferation, inflammatory and immune response, DNA repair etc (Buuh *et al*., 2017). A recent study showed that more than 1750 proteins in human cells are acetylated on their lysine amino acid side chains suggesting that this post-translations modification regulates the function of several proteins involved in cellular physiology. The acetylation status of proteins is maintained by two groups of antagonizing enzymes - Histone acetyltransferases (HATs) and Histone deacetylases (HDACs) (Downe and Baetz, 2016). HDACs are one of two key epigenetic regulators involved chromatin condensation and gene repression. In humans, there are 18 HDACs that are identified and classified in to four classes based on their homology to yeast proteins (Yoshida *et al*., 2017).

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Overexpression of HDACs has been linked to several pathologies including cancer, neurodegenerative disorders, metabolic disorders etc and pharmacological inhibition of HDACs (HDACi) was effective in treatment of these diseases (Dunn and Rao, 2017). Inflammation is an integral part of innate immune response and uncontrolled inflammatory gene expression is the underlying cause of several human pathologies including caner (Colotta *et al*., 2009). Recently it is demonstrated that HDACs play an important role in Toll-like receptor (TLR) and interferon (IFN) signalling pathways in innate immunity (Grabiec *et al*., 2011). Also specific HDACs play a major role in differentiation and function of several immune cells such as macrophases, B cells, T cells and also T regs (Shakespear *et al*., 2011). Among Class I HDACs, HDAC1 and HDAC3 are involved in represion of Hypoxia induced factor 1 alpha (HIF-1a)-dependent inflammatory response (Kim *et al*., 2007) and HDAC2 in inhibition of B-cell proliferation-associated inflammation. HDAC1 is also responsible for inhibiting IFN-mediated inflammation. Recently, it was demonstrated that speific inhibition of HDAC8

reduces pro-inflammatory cytokine production without affecting the cell viability (Li *et al*., 2015). We, previosly, synthesized, characterized and determined the HDAC8 selectivity of a series of 10 novel 2,5-disubstituted, 1,3,4, oxadiazoles containing alanine and glycine hybrids (10a-10j) (Pidugu *et al*., 2016). All the 10 compounds showed moderate selectivity towards HDAC8 and were very potent in inhibiting breast cancer cell proliferation (Pidugu *et al*., 2016). Since inflammation is one of the hallmarks of cancer and HDAC8 inhibition reduced the inflammation, we believed these compounds (10a-10j) with moderate HDAC8 selectivity might also have anti-inflammatory activity. The present study was thus carried out using *E.coli*-infected mouse macrophase cells, RAW 256.7, to evaluate the anti-inflammatory activity of the 10 compounds and the two potent molecules, 10f and 10h, were further evaluated using *in vivo* rat model of carrageenan-induced paw edema

MATERIALS AND METHODS

Cell culture and MTT assay

RAW 264.7, mouse macrophage cells, cells were obtained from NCCS, Pune and cultured in DMEM medium supplemented with 10% Fetal Bovine serum and 1x Penicillin-streptomycin solution. The cells were incubated in 5% CO_2 at 37 °C. The cytotoxicity of the compounds (10a-10j) was dteremined using MTT assay as described earlier (Kalle *et al*., 2010) The *E.coli* bacteria was cultured in LB medium.

Phagocytosis and intracellular bacterial viability assay

Phagocytosis of *E.coli* by RAW 264.7 was carried out as described previously (Annamanedi and Kalle, 2014). Growing the cells in gentamycin for 24 h eliminated the unphagocytosed, extracellular bacteria. The cells were then treated with or without compounds (10f and10h) at 0.1 μM along with ampicillin (10 μg/ml) for 12 h in DMEM medium without antibiotics. The bacterial cell viability of the intracellular bacteria isolated by lysing the cells in distilled water was assessed by counting the number of the colony forming units (CFU) on LB agar plates using 100-fold serially diluted cell lysates.

HDAC8 activity assay

The HDAC8 inhibitory activity of 10f and 10h was determined using the immunoprecipitated HDAC8 from total lysate of *E.coli*-phagocytosed cells treated with ampicillin and/or 10f and 10h as described earlier (Annamanedi and Kalle, 2014).

Immunoblot analysis of COX-2

The protein levels of COX-2 inflammatory protein in *E.coli*-phagocytosed macrophage cells treated with ampicillin and/or 10f and 10h are determined by immunoblot analysis as described earlier (Arunasree *et al*., 2008). β-actin was used as loading control.

Cytokine analysis

The levels of pro-inflammatory cytokines (TNF-α, IFN-γ, IL-12 & IL-6) and anti-inflammatory cytokine (IL-2) levels was determined by ELISA using Quantikine immunoassays kits (R&D Systems, USA) according to manufacturer's instructions.

Carrageenan-induced rat paw edema model

Albino Wister rats (150-200 g) of either sex and of approximate same age were procured from Sri Venkateswara Enterprises, Bangalore, India. The animals were fed with standard pellet diet (Hindustan lever Ltd. Bangalore) and water ad libitum. All the animals were housed in polypropylene cages. The animals were kept under alternate cycle of 12 hours of darkness and light. The animals were acclimatized to the laboratory condition for 1 week before starting the experiment. The animals were fasted for at least 12 hours before the onset of each activity. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC No.-GU/GIS/IAEC-2013/P.Col.03/2013) after scrutinization. Injecting 0.1 ml of 1 % carrageenan in 0.9 % saline into sub-planter area of right hind paw subcutaneously induced paw edema. The paw thickness was measured before carrageenan injection and then at hourly intervals up to 5 hrs with plethysmometer (Anilkumar *et al*., 2017). All the animals with induced paw edema were divided into six groups consisting of six animals in each group. Group I served as vehicle-treated control with normal saline (5 ml/kg, p.o), Group II, standard, was given Ibuprofen (10 mg/kg) remaining groups received 10 and 20 mg/kg of compound 10f and compound 10h. All the drugs were given by oral gavage one hour prior to carrageenan injection. The percentage inhibition of edema was calculated for each group with respect to the vehicle treated control group.

% Inhibition of edema = 100(1-(Vt/Vc))

Where Vt and Vc are volume of carrageenan-injected paws of drug treated group and control group respectively.

Statistical analysis

The statistical significance was determined using two-way ANOVA Bonferroni post test using GraphPad prism software . $p<0.05$ was considered significant.

RESULTS

Compounds 10f and 10h were not cytotoxic to RAW 264.7 macrophages

To evaluate the anti-inflammatory activity of the synthesized glycine and alanine hybrids of 2,5 disubstituted 1,3,4, oxadaiazoles (10a-10j), first the cytotoxicity of the compounds on RAW 264.7 macrophages was determined by MTT assay. The results clearly demonstrated that only 10f and 10h were not cytotoxic even at 100 μM concentration. All other compounds inhibited macrophage cell proliferation (Fig. 1A). Therefore further experiments were carried out with 10f and 10h compounds. Next, we evaluated the anti-inflammatory effect of 10f and 10h on bacteria-phagocytosed macrophages in combination with antibiotic, ampicillin. The CFU counts clearly showed that 10f and 10h were able to lower the bacterial viability inside the macrophages (Fig. 1B). Further, to confirm the anti-inflammatory effect of 10f and 10h is by inhibition of HDAC8, the HDAC8 was immunoprecipitated from the *E.coli*-phagocytosed RAW 264.7 cells and activity assay was carried out. The assay results indicated a significant

increase in the HDAC8 activity in *E.coli*-phagocytosed RAW 264.7 cells. This activity was significantly lowered by 10f and 10h (Fig. 1C).

10f and 10h inhibit COX-2 and inflammatory cytokines

Further, to assess the anti-inflammatory activity of 10f and 10h, during bacterial infection, the protein levels of COX-2 were determined by Western blot analysis and the results showed a significant decrease in the COX-2 protein levels indicating resolution of inflammation (Fig. 2A) by HDAC8 inhibitors and inhibition of bacterial infection by ampicillin. The regulation of inflammation by the compounds was demonstrated by significant decrease in the pro-inflammatory cytokine (IL-6, IL-12, TNF-α, IFN-γ) (Fig. 2B) levels released into the culture supernatant and increase in the anti-inflammatory cytokine, IL-2, levels (Fig. 2C).

In vivo anti-inflammatory activity of 10f and 10h in carrageenan-induced rat paw edema

Next, we assessed the anti-inflammatory effects of 10f and 10h in carrageenan-induced rat paw edema model and compared the results with known anti-inflammatory drug, Ibuprofen. The animals were pretreated with the compounds before injecting carrageenan into the paw. Analysis of the data clearly showed a decrease in the paw edema volume in animals pre-treated with the compounds (Table 1). The compound 10f showed more significant effect when compared to 10h (Fig. 3A & 3B). However, both the compounds did not show significant anti-inflammatory activity when compared to the standard ibuprofen.

Table 1. Time-dependent decrease in Paw volume (ml) of carrageenan-induced paw edema in rats treated with 10f and 10h

	Paw volume (ml) (% inhibition)					
	0hr	1 hr	2 hr	3 hr	4 hr	5 hr
Control	0.276±0.012	0.466±0.019	0.600±0.018	0.632±0.015	0.620±0.015	0.570±0.015
Ibuprofen (10 mg/kg)	0.270±0.009	0.384±0.010$^{\$}$ (17.39%)	0.456±0.018$^{\$}$ (25.00%)	0.462±0.018$^{\$}$ (26.98%)	0.416±0.016$^{\$}$ (33.87%)	0.306±0.016$^{\$}$ (47.36%)
10f (10 mg/kg)	0.264 ±0.012	0.450±0.011^{ns} (2.13%)	0.552±0.017^{ns} (8.33%)	0.560±0.014$^{\#}$ (11.11%)	0.522±0.009$^{\$}$ (16.12%)	0.406±0.020$^{\$}$ (29.82%)
10f (20 mg/kg)	0.280±0.007	0.398±0.018^{ns} (15.21%)	0.498±0.009$^{\#}$ (18.33%)	0.514±0.013$^{\$}$ (19.04%)	0.472±0.008$^{\$}$ (24.19%)	0.338±0.010$^{\$}$ (42.10%)
10h (10 mg/kg)	0.262 ±0.012	0.478±0.014^{ns} (0.8%)	0.568±0.017^{ns} (1.77%)	0.566±0.011$^{\#}$ (11.11%)	0.528±0.015$^{\$}$ (14.83%)	0.422±0.017$^{\$}$ (26.31%)
10h (20 mg/kg)	0.258±0.012	0.440±0.017^{ns} (4.34%)	0.548±0.014$^{\#}$ (8.47%)	0.542±0.016$^{\$}$ (14.28%)	0.502±0.008$^{\$}$ (19.35%)	0.354±0.005$^{\$}$ (38.59%)

$p>0.05^{ns}$, $P<0.001^{\$}$, $p<0.01^{\#}$, $p<0.05$* Significance followed by 2 way ANOVA Bonferroni post test compared with control group

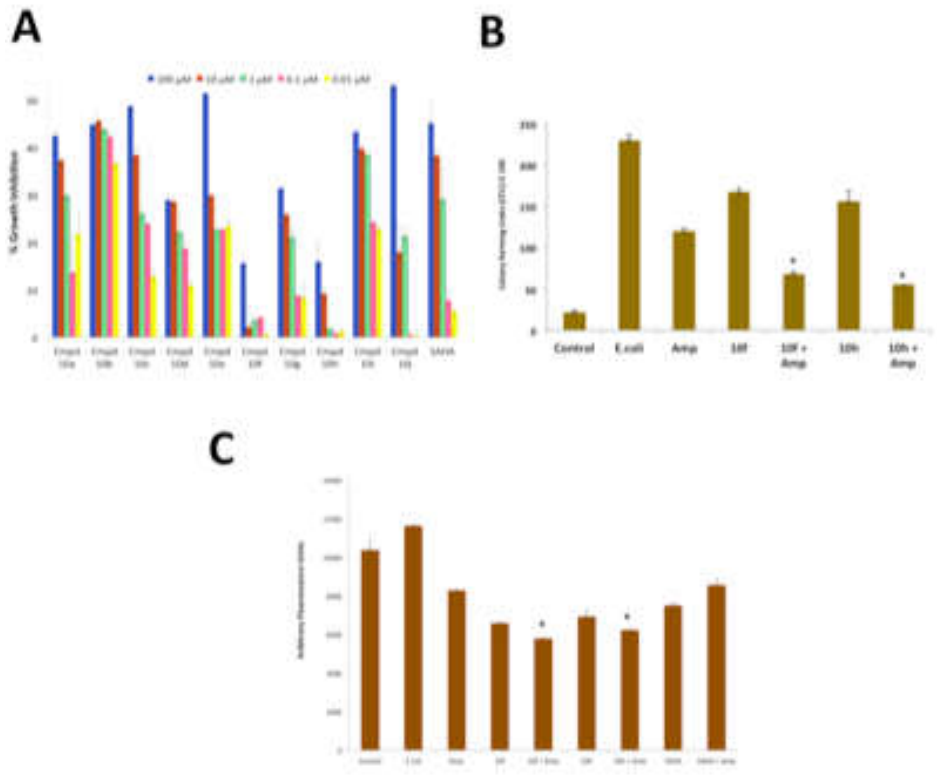

Figure 1. Compounds 10f and 10h inhibited *E.coli* growth in RAW 264.7 macrophages

A. Bar graphs showing the % growth inhibition of RAW 264.7 macrophages in presence of Compounds **10a-10j**. **B**. Graph showing the number of colony forming units (CFU) of *E. coli* survived after phagocytosis by RAW 264.7 cells and treated with **10f** and **10h**. C. HDAC8 activity assay using immunoprecipitated HDAC8 from cell lysates of *E.coli*-phagocytosed RAW 264.7 cells treated with or without **10f** and **10h.** * denotes $p<0.05$.

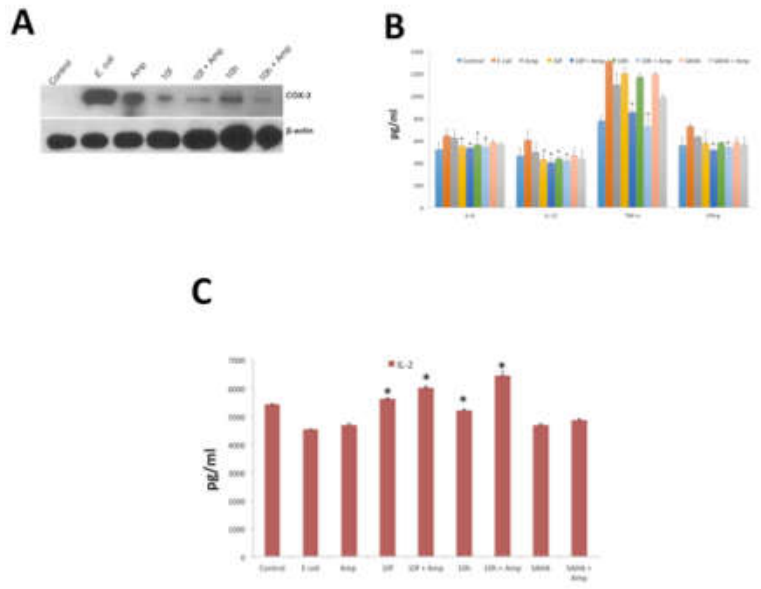

Figure 2. 10f and 10h inhibited COX-2 and inflammatory cytokines

A. Immunoblot showing the proteins levels of COX-2 in *E.coli*-phagocytosed RAW 264.7 macrophages treated with or without ampicillin, **10f/10h** or in combination. **B.** The levels of pro-inflammatory cytokines (IL-6, IL-12, TNF-**α**, INF-γ) released into culture supernatant by *E.coli*-phagocytosed macrophages. **C.** The levels of anti-inflammatory cytokine (IL-2) released in response to **10f** and **10h** treatment.

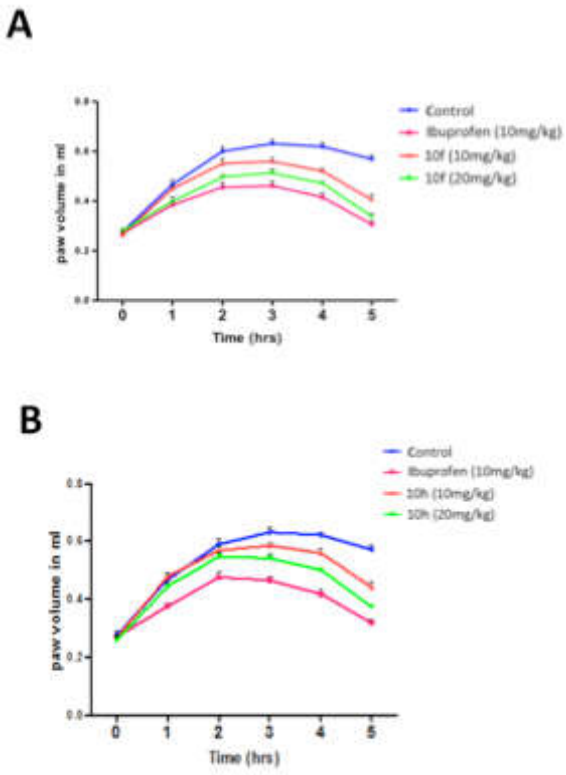

Figure 3. 10f and 10h inhibited the carrageenan-induced paw edema volume in rat. Graph showing time-dependent decrease in the paw edema volume (ml) in rats pretreated with 10f (A) or 10h (B)

DISCUSSION

Epigenetic gene regulation is key for maintaining cellular homeostasis (Matilainen *et al*., 2017). Histone modifications, such as acetylation and deacetylation, is one of the three epigenetic mechanisms involved in gene regulation (Egger *et al*., 2004). Although HDACs were known to initially deacetylate only histone proteins, it is now very well established that HDACs also have non-histone protein substrates and therefore are now termed as lysine deacetylases (KDACs) (Van Dyke, 2014). Inhibition of overexpression of HDACs is considered as a better treatment strategy in several diseases (Xu *et al*., 2007). It is well demonstrated that during bacterial infection, the inflammatory signaling pathway is activated and epigenetic mechanisms also play a major role in inflammatory gene expression (Man *et al*., 2017; Ciarlo *et al*., 2013). Several HDAC inhibitors were shown to be effective in inhibiting pathogens such as bacteria (Tegtmeyer *et al*., 2017),

virus (Lu *et al*., 2017), parasites such as schistosoma (de Oliveira *et al*., 2017) etc. However, due to the functional overlapping among the different classes of HDACs, pan-HDAC inhibition is undesirable demanding selective isoform-specific HDAC inhibitors (Qin *et al*., 2017). HDAC8 belongs to class I HDACs and is the only HDAC to be crystalized in full length to date (Hu *et al*., 2000). HDAC8 is well characterized in terms of catalytic activity and structural features (Vannini *et al*., 2004). It is known to be involved in progression of several cancers, specifically female-specific cancers due to its X-chromosome genomic localization (Thakur *et al*., 2015). Previously, we designed and synthesized a series of 10 molecules of 2,5, disubstituted, 1,3,4 oxadiazoles with glycine and alanine hybrids and were shown to be moderate HDAC8-selective inhibitors. The anti-cancer efficacy of these compounds was also demonstrated in breast cancer cells (Pidugu *et al*., 2016). Recently, the role of HDAC8 in inflammation was demonstrated (Meng *et al*., 2016; Orlikova *et al*., 2012) Since inflammation is the underlying cause for several diseases including bacterial infection and cancer, we evaluated the anti-inflammatory efficacy of the compounds (10a-10j) using *E.coli*-phagocytosed RAW 264.7 macrophages. The study results showed that among the 10 compounds, compounds 10f and 10h did not inhibit macrophage proliferation and thus help in phagocytic activity. Also, 10f and 10h inhibited HDAC8 activity and thus inflammation *via* inhibition of COX-2 expression and pro-inflammatory cytokine release. The results are in agreement with previous studies (Aung *et al*., 2006).

Carrageenan-induced paw edema is suitable model to the acute inflammation (Salvemini *et al*., 1996) and the paw edema is associated with the release of inflammatory mediators histamine, serotonin, bradykinin, proteases and prostaglandins (Hajhashemi *et al*., 2011) (Rosa D*et al*., 1971). To further assess the anti-inflammatory effects of the compounds 10f and 10h, the animals were pre-treated with the compounds and then induced paw edema using carrageenan. The compounds showed significant inhibition of carrageenan induced paw edema compared with control. The activity might be due to inhibition of the inflammatory mediators responsible for inflammation *via* HDAC8 inhibition. Similar results were obtained with HDAC8 inhibition in LPS-induced inflammatory mice model (Li *et al*., 2015). In conclusion, the present study demonstrates that 2,5-disubstituted 1, 3, 4-oxadiazoles of glycine and alanine hybrids as potent and promising anti-inflammatory compounds. The study thus signifies the use of HDAC8 selective inhibitors as anti-inflammatory agents.

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