Biochemical Pharmacology 102 (2016) 130–140

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

The histone acetyltransferase p300 inhibitor C646 reduces pro-inflammatory gene expression and inhibits histone deacetylases

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ARTICLE INFO

Article history:
Received 16 October 2015
Accepted 14 December 2015
Available online 21 December 2015

Chemical compounds studied in this article:
C646 (PubChemCID: 1285941)
SAHA (PubChemCID: 5311)

Keywords:
C646
Inflammation
NF-κB
Macrophages
Acetylation
Histones

ABSTRACT

Lysine acetylations are reversible posttranslational modifications of histone and non-histone proteins that play important regulatory roles in signal transduction cascades and gene expression. Lysine acetylations are regulated by histone acetyltransferases as writers and histone deacetylases as erasers. Because of their role in signal transduction cascades, these enzymes are important players in inflammation. Therefore, histone acetyltransferase inhibitors could reduce inflammatory responses. Among the few histone acetyltransferase inhibitors described, C646 is one of the most potent (K_i of 0.4 μM for histone acetyltransferase p300). C646 was described to affect the NF-κB pathway; an important pathway in inflammatory responses, which is regulated by acetylation. This pathway has been implicated in asthma and COPD. Therefore, we hypothesized that via regulation of the NF-κB signaling pathway, C646 can inhibit pro-inflammatory gene expression, and have potential for the treatment of inflammatory lung diseases. In line with this, we demonstrate here that C646 reduces pro-inflammatory gene expression in RAW264.7 murine macrophages and murine precision-cut lung slices. To unravel its effects on cellular substrates we applied mass spectrometry and found, counterintuitively, a slight increase in acetylation of histone H3. Based on this finding, and structural features of C646, we presumed inhibitory activity of C646 on histone deacetylases, and indeed found inhibition of histone deacetylases from 7 μM and higher concentrations. This indicates that C646 has potential for further development towards applications in the treatment of inflammation, however, its newly discovered lack of selectivity at higher concentrations needs to be taken into account.

1. Introduction

Lysine acetylations are reversible posttranslational modifications that frequently occur on histones and other cellular proteins. These modifications have been recognized to play important regulatory roles in signal transduction cascades and gene expression [1,2]. Protein lysine acetylations are regulated by histone acetyltransferases (HATs) as writers and histone deacetylases (HDACs) as erasers. In the past decades many different isoforms of HATs and HDACs have been discovered [3,4], and

Abbreviations: HAT, histone acetyltransferase; HATi, HAT inhibitor; HDAC, histone deacetylase; PCAF, P300/CBP-associated factor; MOZ, monocytic leukemic zinc finger; COX-2, cyclooxygenase-2; NF-κB, nuclear factor kappa B; COPD, chronic obstructive pulmonary disease; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; SEAP, secreted embryonic alkaline phosphatase; HDACi, HDAC inhibitor; SAHA, suberanilohydroxamic acid; LDH, lactate dehydrogenase; PCLS, precision-cut lung slices; LPS, lipopolysaccharide; IFNγ, interferon gamma; TNF-α, tumor necrosis factor alpha; iNOS, inducible nitric oxide synthase; IL-1β, interleukin 1 beta; IL-12b, interleukin 12 subunit beta; IL-6, interleukin 6; IL-8, interleukin 8; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; AMC, 7-amino-4-methylcoumarin; KAT8, lysine acetyltransferase 8; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; DMF, dimethylformamide; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; LC, liquid chromatography; MS/MS, tandem mass spectrometry; TSA, trichostatin A; siRNA, small interfering RNA.

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http://dx.doi.org/10.1016/j.bcp.2015.12.010
0006-2952/

aberrations in their activity have been associated with diseases such as inflammatory disorders or cancer [5,6].

For this reason, the development of small molecule inhibitors of these enzymes and their applications in therapeutic areas, such as the treatment of inflammatory diseases, have received considerable attention. This has resulted in the development of small molecule HAT inhibitors (HATi) [7]. Virtual screening enabled the identification of the small molecule inhibitor C646 as a potent and cell-permeable p300 HATi (K_i 0.4 μM) which is selective for p300 among HAT isoenzymes such as PCAF, GCN5 and MOZ [8].

C646 has shown interesting effects in disease models. For example, C646 has successfully been applied in an animal model of neuropathic pain. In this study, C646 was administered in rats via a lumbar intrathecal catheter, demonstrating the feasibility of local administration of C646 in animals. It was found that C646 treatment diminished both the p300 promoter binding and the expression of COX-2 [9]. In another study on prostate cancer cell lines it was found that C646 mediated inhibition of p300 increased apoptosis, which was, among others, caused by inhibition of the androgen receptor and the NF-κB pathway [10]. These studies indicate that C646 influences signaling cascades such as the NF-κB pathway.

The effects of C646 on the NF-κB pathway could be explained by the role for p300 that has been described in the regulation of the NF-κB pathway [5]. For instance, acetylations of the p65 NF-κB subunit on lysines 218, 221 and 310 are mediated by the HATs p300 and PCAF [11] and increase transcriptional activity. In contrast, acetylations on lysines 122 and 123 decrease transcriptional activity and are also mediated by p300 [12]. Furthermore, upon DNA binding, the transcriptional activation domain of p65 interacts with the HATs p300 and CBP as co-activators of gene transcription [13]. Thus, p300 plays a crucial role in the regulation of acetylation of specific lysine residues of NF-κB, which then determine NF-κB transcriptional capacity, DNA binding and duration of action.

This suggests that inhibition of the HAT p300 using small molecule inhibitors such as C646 may allow for regulation of gene expression via the NF-κB transcription factor [7]. Since the NF-κB pathway is a key factor in inflammatory responses, this calls for investigation of the potential of C646 to suppress these responses. Of particular interest may be applications in inflammatory lung diseases such as asthma and chronic obstructive pulmonary disease (COPD), which still pose a major health problem. Interestingly, a role for the NF-κB pathway has been described in these diseases, as reviewed [14]. Increased NF-κB activity has been observed in the bronchial epithelium and peripheral blood mononuclear cells of asthmatic patients [15,16]. Increased nuclear localization of p65 was observed in sputum macrophages during exacerbations of COPD [17] and also in bronchial biopsies of stable COPD patients [18]. Several lines of research have focused on modulating NF-κB activity as a novel therapeutic strategy for the treatment of asthma and COPD [14]. We hypothesize that pharmacological inhibition of the HAT p300 by small molecule inhibitor C646 will result in inhibition of pro-inflammatory gene expression via inhibition of the NF-κB signaling pathway.

For these reasons, we set out to explore the p300 HATi C646 in a model system for inflammatory lung diseases. We observed decreased NF-κB reporter gene activity in RAW-Blue murine macrophages, and decreased pro-inflammatory gene expression in RAW264.7 murine macrophages as well as in murine lung tissue slices. Next to this we set out to quantify changes in lysine acetylation in the cellular substrates of p300. Acetylation of histone H3 and histone H4 was quantified using mass spectrometry in RAW264.7 macrophages. Counterintuitively, C646 increased acetylation on histone H3 residues 18–26, containing H3 K18 and H3 K23. Based on these findings and the structural properties of C646 we investigated its inhibitory potential on recombinant HDACs and found inhibition from 7 μM and higher concentrations for type I and II HDACs. Importantly, these findings call for further optimization of the selectivity profile of the p300 HATi C646 and its derivatives.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents were purchased from Sigma–Aldrich (Zwijndrecht, the Netherlands) unless otherwise stated. C646 was purchased from Axon Medchem (Groningen, the Netherlands) and SAHA from Selleckchem (Munich, Germany). The purity of C646 was assessed by HPLC, MS, and NMR by Axon Medchem, and the same was done for SAHA by Selleckchem.

2.2. Cell culture and viability assay

RAW264.7 macrophages were purchased from American Type Culture Collection (Manassas, Virginia, USA), and cell culture reagents were purchased from Life Technologies (Bleiswijk, the Netherlands). RAW-Blue macrophages were purchased from InvivoGen (Toulouse, France). RAW264.7 macrophages were cultured as described previously [19]. RAW-Blue macrophages were cultured in the same manner, with the addition of Zeocin (200 μg/mL) to the culture medium according to the manufacturer's instructions. For the experiments, cells were used until passage 18. Viability of C646 or HDAC inhibitor (HDACi) suberanilohydroxamic acid (SAHA) treated RAW264.7 was assessed by CellTiter 96 Aqueous One Solution Cell Proliferation Assay (# G3581, Promega, Leiden, the Netherlands). For the assay, cells were seeded at 7500 cells per well in 96 wells plates. On the following day, medium was replaced with medium containing C646 or SAHA at the appropriate concentrations. After 20 h of incubation, the CellTiter reagent was added to the wells. After 1 h of incubation with this reagent, the absorbance at 490 nm was measured using a Synergy H1Hybrid Multi-Mode Microplate Reader (BioTek, Vermont, USA). The amount of absorbance at this wavelength is indicative of mitochondrial conversion of the reagent, which is linked to cell viability.

2.3. Precision-cut lung slices

Precision-cut lung slices (PCLS) were prepared as described previously [19]. All experiments were performed according to national guidelines and upon approval of the experimental procedures by the local Animal Care and Use committee of Groningen University, DEC number 6962A. Viability of C646 treated PCLS was assessed by the amount of lactate dehydrogenase (LDH) released by the tissue slices into the culture medium. The measurements were performed as described previously [19]. Viability of SAHA treated PCLS was not determined.

2.4. Treatment of RAW264.7, RAW-Blue and PCLS with inhibitors and LPS IFNγ

Cells or PCLS were pre-treated with C646 at 1, 5, 10, 15, 20, 25 or 30 μM (for PCLS the 30 μM concentration was not included due to decreased viability), or with the HDACi SAHA at 0.41 μM for 16 h. Inhibitor stocks were prepared in dimethylformamide (DMF) and were further diluted in DMEM culture medium. Vehicle treatment constituted of pre-treatment with 0.3% DMF for the cells (corresponding to the same final DMF% as for 30 μM C646), or with 0.2% DMF for PCLS (corresponding to the same final DMF% as for 20 μM C646), for 16 h. Subsequently, cells were stimulated with 10 ng/mL lipopolysaccharide (LPS, *Escherichia coli*, serotype 0111:B4; Sigma–Aldrich) and 10 ng/mL interferon gamma (IFNγ, cat.#