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The PDE4 inhibitor CHF6001 affects keratinocyte proliferation via cellular redox pathways

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ABSTRACT

Psoriasis is a skin disease characterized by abnormal keratinocyte proliferation and inflammation. Currently, there are no cures for this disease, so the goal of treatment is to decrease inflammation and slow down the associated rapid cell growth and shedding. Recent advances have led to the usage of phosphodiesterase 4 (PDE4) inhibitors for treatment of this condition. For example, apremilast is an oral, selective PDE4 inhibitor that is able to reduce skin inflammation and is Food and Drug Administration (FDA)-approved to treat adults with moderate to severe psoriasis and/or psoriatic arthritis. However, common target-related adverse events, including diarrhea, nausea, headache, and insomnia limit the usage of this drug. To circumvent these effects, the usage of PDE4 inhibitors specifically designed for topical treatment, such as CHF6001, may combine local anti-inflammatory activity with limited systemic exposure, improving tolerability. In this study, we showed that CHF6001, currently undergoing clinical development for COPD, suppresses human keratinocyte proliferation as assessed via BrdU incorporation. We also observed decreased re-epithelialization in a scratch-wound model after CHF6001 treatment. At the molecular level, CHF6001 inhibited translocation of phosphorylated NF-κB subunit p65, promoting loss of nuclear cyclin D1 accumulation and an increase of cell cycle inhibitor p21. Furthermore, CHF6001 decreased oxidative stress, measured by assessing lipid peroxidation (4-HNE adduct formation), through the inactivation of the NADPH oxidase.

These results suggest that CHF6001 has the potential to treat skin disorders associated with hyperproliferative keratinocytes, such as psoriasis by targeting oxidative stress, abnormal re-epithelization, and inflammation.

Key words: NOX, NF-kB, cyclin D1, skin, p21

INTRODUCTION

Psoriasis is a chronic, inflammatory skin disease that affects 1% to 3% of the population worldwide and involves keratinocyte hyperproliferation in the epidermis, inflammatory infiltrates, and aberrant differentiation [1]. The phenotypic appearance of psoriasis is characterized by red lesions covered by silvery scale-like plaques. The intensity of psoriatic lesions can vary from a few random spots to a massive outbreak that covers the entire body. There is no cure for this disease; treatments are primarily focused on reducing or clearing plaques as well as preventing remission by modulating inflammation and slowing down psoriasis-associated rapid cell growth and shedding.

In recent years, an improved understanding of the pathomechanisms underlying this disease has lead to the developments of new treatments. Recent studies showed that in psoriatic patients, expression of all phosphodiesterase 4 (PDE4) isoforms (A-D), part of a family of enzymes known to regulate cyclic adenosine monophosphate (cAMP) levels and immune homeostasis, is elevated compared to healthy controls [2]. cAMP is a second messenger involved in regulating a wide array of immune-related genes such as IL-2, IL-6, IL-10, and Tumor Necrosis Factor alpha (TNFα) through cAMP-responsive elements located in gene promoters, as well as through crosstalk with Nuclear Factor-κB (NF-κB) signaling [3]. Several PDE4 inhibitors have recently been evaluated in clinical trials for treating various skin inflammatory conditions, including apremilast, roflumilast, and crisabolore, with apremilast approved for the treatment of psoriasis and crisaborole for the treatment of atopic dermatitis [4]. In particular, aprelimilast exhibits pleiotropic, synergistic, and attenuating effects on a key group of cytokines involved in the pathogenesis of psoriasis, most notably IL-17A/F, IL-22, and TNF-α, and these effects correlate with reduced skin manifestations of the disease [2]. However, common target-related adverse events including diarrhea, nausea, and headache are associated with apremilast usage [4]. Therefore, alternative routes of administration could be explored with the goal of increasing tolerability by utilizing drugs designed for topical treatment and reducing systemic effects.

CHF6001 is an inhaled selective inhibitor of PDE4 isoforms A-D [5], which is endowed with anti-inflammatory properties in several *in vitro* models, involving lymphocytes, monocytes, macrophages, and dendritic cells [6-11]. Clinical studies have shown that topical administration of CHF6001 is well-tolerated [12] and reduces the allergen challenge response in asthmatic patients [13]. CHF6001 is currently in clinical development for the treatment of Chronic Obstructive Pulmonary Disease (COPD) (Clinical trials.gov Identifier NCT02986321).

In the present study, we investigated whether the PDE4 inhibitor CHF6001 could be used as an agent to modulate proliferation and inflammation in keratinocytes *in vitro*. Since psoriasis is characterized by distinct histological features of the skin, including keratinocyte hyperproliferation,

we evaluated the effect of CHF6001 on keratinocyte (HaCaT) proliferation and re-epithelialization in a scratch-wound model, along with crucial molecules involved in cell cycle progression, i.e. cyclin D1, and key players in redox-inflammatory crosstalk, such as NF-κB and NADPH oxidase.

METHODS

Cell culture and treatments

HaCaT keratinocytes (a gift from Dr. F. Virgili), were grown in Dulbecco's modified Eagle's medium High Glucose (Lonza, Milan, Italy) at 37□ in 95%air/5%CO_2 , as previously described by Sticozzi *et al*. 2012. [16].

HaCaT cells were treated with different doses (0.1 – 10 μM) of the specific PDE4 inhibitor CHF6001 for 24 hrs. This compound was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM as a stock solution. The stock was then diluted to the required concentrations directly in the medium. The final concentration of DMSO in culture medium during treatment did not exceed 0.1% (v/v). For glucose oxidase (GO) experiments, cells were pre-treated with 0.1-100 nM concentrations of CH6001 for 24 hrs and then treated with 1 U/mL GO for 1 hr.

Cellular viability

Viability studies were performed 24h after treatment using the Lactate Dehydrogenase (LDH) release assay as previously described by Sticozzi *et al*. 2013 [17]. Prior to each assay, the cells were lysed by adding Triton X-100 to the culture media for 30 min at 37□ to obtain a representative maximal LDH release as the positive control with 100% toxicity. The amounts of LDH in the supernatant were determined and calculated according to kit instructions (EuroClone, Milan, Italy). All tests were performed in triplicate, and the assay was repeated at least three times independently.

Cellular proliferation

Cells were seeded in a 96-well plate and grown until 50% confluent. Cells were then treated with various doses of CHF6001, ranging from 0.1 to 1000 nM, for 24 hrs. Cellular proliferation was evaluated using a 5-bromo-20-deoxyuridine (BrdU) assay kit (BrdU test kit, Roche, Milan, Italy) [14]. This kit detects BrdU incorporated into cellular DNA during cell proliferation, utilizing an anti-BrdU antibody. Absorbance was measured at λ 450 nm (reference wavelength: 690 nm).

In vitro wound healing assay

HaCaT cells were seeded in 60 × 15 mm Petri dishes and grown until confluent. Cells that were 90% confluent were then treated with different doses of CHF6001. The cellular monolayers were scratched by using a 200 μl pipette tip to generate a cell-free zone (0.8 – 1 mm in width), as previously described [15]. Cells were next incubated for 36 h at 37°C and photographed at different time points. The wounded area was marked on the base of the Petri dish, and the same field was

photographed at the different time points. Images were acquired on a Leica imaging microscope [15].

Western blot analysis

Total cell lysates were extracted in RIPA Buffer with protease and phosphatase inhibitor cocktails (Sigma–Aldrich), as previously described [16]. Cells were harvested by centrifugation, and protein concentration was determined using Bradford assays (Bio-Rad Protein Assay; Bio-Rad, Milan, Italy). Samples were loaded onto 10% SDS–polyacrylamide electrophoresis gels, separated by molecular size, and then electro-blotted onto nitrocellulose membranes. After blocking with Tris-buffered saline, pH 7.5, containing 0.5% Tween 20 and 5% milk, membranes were incubated overnight at 4°C with primary antibodies (listed in Table 2), washed, and then incubated with horseradish peroxidase-conjugated secondary antibodies for 1hr. The bound antibodies were detected using chemiluminescence on the Bio-Rad ChemiDoc Imaging System. β-actin (Cell Signaling, Celbio, Milan, Italy) was used as loading control. Densitometry of the bands was calculated using ImageJ.

Cytoplasmic and nuclear isolation

To assess p47 translocation to the plasma membrane, cells were treated with CHF6001 for 24 hrs with and without 1 U/mL GO and then homogenized on ice in Tris-HCl buffer, pH 7.4, containing 1mM EGTA, 1mM EDTA, protease, and phosphatase inhibitors. Samples were then separated via centrifugation (100,00 x g) at 4□ for 30 min. Cytosolic fractions were solubilized in lysis buffer containing 1% Triton X-100 and then homogenized with a 25-gauge needle. Equal amounts of proteins were loaded onto SDS-PAGE gels and then transferred to nitrocellulose membranes. Western blots were performed as described above.

Immunofluorescence

Briefly, HaCaT cells were grown on coverslips in medium with 10% Fetal Bovine Serum (FBS), and, after treatment, were fixed in 4% paraformaldehyde in Phosphate Buffered Saline (PBS) for 30 min at 4□ as described previously by Sticozzi *et al*., 2012 [17]. After permeabilization and blocking, coverslips were then incubated for 1hr with primary antibody, washed, and then incubated with secondary antibodies for 1hr (see antibodies table). Nuclei were stained with 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) (Sigma Aldrich, Milan, Italy) for 1 min after removal of secondary antibodies. Coverslips were then mounted onto glass slides using with anti-fade 1,4-diazabicycloctane in glycerine (DABCO) mounting medium and examined using a Leica light

microscope equipped with epifluorescence at 40x magnification. Negative controls were performed by omitting primary antibodies. Images were acquired and analyzed using Leica software.

Oxyblot analysis of 4-HNE (4-Hydroxynonenal)

We used the OxyBlot Protein Oxidation Detection Kit (Chemicon, USA) to detect the carbonyl groups of oxidized proteins. Briefly, after derivatization of carbonyl groups to 2,4-dinitrophenylhydrazone (DNP-hydrazone) via reaction with 2,4-dinitrophenylhydrazine (DNPH), the DNP-derivatized protein samples were separated by polyacrylamide gel electrophoresis, and we performed western blotting as described previously [17].

Quantitative real-time PCR

Total RNA was extracted using an Aurum Total RNA Mini Kit with DNase digestion (Bio-Rad) and reverse- transcribed using the iScript cDNA Synthesis Kit (Bio-Rad), as previously described [18]. The primer pairs (Table 1) capable of hybridizing with unique regions of the appropriate gene sequence were obtained from the Real-Time PCR Gen Bank Primer and Probe Database: RTPrimerDatabase [19]. Ribosomal Protein L13a (RPL13a), RPL11a, and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were employed as reference genes. After normalization, the fold increase or decrease was determined with respect to control, using the formula $2^{-\Delta\Delta CT}$, wherein ΔCT is (gene of interest CT) (reference gene CT), and $\Delta\Delta CT$ is (ΔCT experimental)(ΔCT control).

Table 1. Primers used in this study

Gene	Primer sequence	Ta (1C)	Product length (bp)	QPCR amplification efficiencya (%)	Cycles (n)	GenBank Accession No./Ref.
CYCLD1	F: 5′- TGGAGCCCCTGAAGAAGAG -3′ R: 5′ - TGGAGCCCCTGAAGAAGAG G-3′	60.1	236	96.2	39	BC112037.1
MMP2	F: 5′- GCAGTGGGGGCTTAAGAAGA-3′ R: 5′- AGCCGTACTTGCCATCCTTC -3′	60.0	380	97.0	39	NM_001127891.1
MMP9	F: 5′- CATCCGGCACCTCTATGGTC-3′ R: 5′- CATCGTCCACCGGACTCAAA-3′	59.9	230	96.8	39	NM_001127891.1
RPL13A	F: 5′ -CCTAAGATGAGCGCAAGTTGAA-3′ R: 5′ -CCACAGGACTAGAACACCTGCTAA-3′	60.2	203	97.3	39	[18]

RPL11A	F: 5′ -TGCGGGAACTTCGCATCCGC-3′ R: 5′ -GGGTCTGCCCTGTGAGCTGC-3′	60.1	108	96.5	39	NM 000975.2
GAPDH	F: 5′ -TGACGCTGGGGCTGGCATTG-3′ R: 5′ -GGCTGGTGGTCCAGGGGTCT-3′	60	134	94.6	39	NM 002046.3

Table 2. Antibodies used in this study

Product	**Catalog No.**	**Company**
p47 phox	4312	Cell Signaling
β-actin	4970	Cell Signaling
p-NF-κB p65	3033	Cell Signaling
p21	HUGO291	Abcam
Cyclin D1	MCA1756	Bio-Rad

Statistical Analysis

All data were analysed using two-way analysis of variance (ANOVA). Results were considered to be significant when the P-value was <0.05. Data are expressed as means ± SD of triplicate determinations obtained in five separate experiments.

RESULTS

Effect of CHF6001 on cellular toxicity

The first step of the present study was to determine whether CHF6001 affected keratinocyte (HaCaT) viability. Cellular cytotoxicity of CHF6001 in concentrations ranging from 0.1 to 10000 nM was analyzed by using an LDH release assay. As shown in Fig. 1, after 24 hrs of treatment with CHF6001, no cytotoxic effect was detected in HaCaT cells, with respect to control (DMSO 0.1%). Cells treated with Triton X-100 represent the positive control (i.e. 100% of mortality) (Fig. 1).

Effect of CHF6001 on cellular proliferation

Based on our viability results, we tested whether treatment with the three highest non-toxic doses of CHF6001 affected cellular proliferation, using 5-Bromo-2'-deoxyuridine (BrdU) assays. As shown in Fig. 2, we observed that treatment with CHF6001, ranging from 100 nM to 1000 nM concentrations, significantly decreased cell proliferation by 25%, although no dose-dependent effect was detected (Fig.2).

Effect of CHF6001 on keratinocyte migration

Besides proliferation, keratinocytes also migrate during wound healing, so we next performed an *in vitro* wound-scratch assay to assess the effects of CHF6001 treatment on keratinocyte migration/proliferation. As shown in Fig. 3, we observed that CHF6001 significantly decreased the ability of HaCaT cells to closure the *in vitro* wounds after 24 hrs of treatment. Interestingly, the inhibitory “wound closure” effect persisted, even at later time points (30 and 36 hrs).

Effect of CHF6001 on proteins involved in the cell cycle

Since CHF6001 clearly decreased keratinocyte proliferation, we analysed the effect of CHF6001 treatment on levels of cyclin D1, which is a protein that promotes cellular proliferation through interaction with cyclin-dependent kinases (CDKs) [20]. As shown in Fig. 4, CHF6001 treatment reduced cyclin D1 mRNA (A) and protein (B) levels. Since the cell-cycle regulatory activity of cyclin D1/CDK complexes requires nuclear translocation [21], we performed nuclear fractionation experiments to determine whether CHF6001 treatment affected cyclin D1 localization. As shown in Fig. 4C, CHF6001 treatment decreased cyclin D1 nuclear translocation.

Since we observed decreased proliferation (Fig. 2) and cyclin D1 nuclear translocation (Fig. 4A and 4C) in response to CHF6001 treatment, we next wanted to determine whether CHF6001 treatment altered levels of p21, since this protein is able to inhibit the activity of CDKs [22] and

promotes G1 cell cycle arrest [23]. As showed in Fig. 4D, CHF6001 treatment significantly increased p21 protein levels at a concentration of 1000 nM. Treatment with lower doses of CHF6001 (1000 and 10000 nM) did not significantly affect p21 levels (Fig. 4D). No changes in p27, another cell cycle inhibitor, were observed (data not shown).

Effect of CHF6001 on NF-κB subunit p65 translocation

NF-κB has been previously shown to promote cell cycle progression and stimulate transcription of cyclin D1 through via binding to κB sites located in the cyclin D1 promoter [24]. Since we observed decreased proliferation and cyclin D1 levels in response to CHF6001 treatment (Fig. 2 and 4), we decided to investigate whether CHF6001 treatment could affect the nuclear translocation of NF-κB subunit p65. As shown in Fig. 5, 24 hrs after CHF6001 treatment, we observed a significant decrease in the translocation of phosphorylated NF-κB subunit p65 (red colour) as shown by immunofluorescence (A) and nuclear fractionation experiments (B).

Effect of CHF6001 on NADPH oxidase (NOX) activation

Previous literature has shown a potential role for PDE4 inhibitors in modulating oxidative stress pathways by decreasing the formation of reactive oxygen species (ROS) [25-30]. In addition, CHF6001 has been previously reported to inhibit the activation of oxidative burst in neutrophils and eosinophils [7]. In support of this idea, we also observed decreased nuclear translocation of phosphorylated NF-κB subunit p65 in keratinocytes, in response to CHF6001 treatment (Fig. 5). Since NF-κB activation is known to be regulated by oxidative stress [31, 32], we investigated whether CHF6001 treatment could influence NAPDH oxidase (NOX) activation, an endogenous source of cellular ROS [33]. In resting cells, the subunits of the NOX complex are separated between membranes and the cytosol to ensure dormancy; for instance, p47 resides in the cytosol in resting cells [34, 35]. However, upon stimulation, p47 translocates to the plasma membranes and transports other cytosolic complex components to a docking site to promote NOX activation [33]. To determine whether CHF6001 treatment affected NOX activation, we measured p47 membrane levels via immunofluorescence (Fig. 5A) and western blot (Fig. 5B) analysis. As shown in Fig. 6, treatment with glucose oxidase (GO), an enzyme that catalyzes production of H_2O_2 [36, 37], induced p47 membrane translocation; however, in cells pre-treated with CHF6001, GO failed to induce p47 translocation in a dose-dependent manner (Fig.6).

Effect of CHF6001 on formation of 4-HNE protein adducts

Since we observed that the translocation of both NF-κB subunit p65 and NOX subunit p47 were altered by CHF6001 treatment (Fig. 5 and 6), we wanted to determine whether treatment with CHF6001 also affected lipid peroxidation by measuring the levels of 4-hydroxy-2-nonenal (4-HNE), which is routinely used as a readout of altered redox homeostasis [38].

As depicted in Fig.7, treatment with glucose oxidase (GO), which generates H_2O_2, clearly increased 4-HNE levels, and this effect was suppressed in a concentration-dependent manner by pre-treatment with CHF6001.

DISCUSSION

Our study provides a rationale for testing the effects of topical application of PDE4 inhibitor CHF6001 in inflammatory skin disorders, such as psoriasis. Indeed, the rationale for the use of PDE4 inhibitors for treatment of psoriasis has been recently reinforced by the approval of the oral PDE4 inhibitor apremilast for moderate-to-severe plaque psoriasis. The mechanism of action of apremilast has been associated with its anti-inflammatory properties [39].

First, we performed cellular viability experiments to exclude any cytotoxic effects of CHF6001 on keratinocytes. Next, we investigated the effects of CHF6001 on *in vitro* models of keratinocyte proliferation and wound repair. In many common skin diseases, such as psoriasis, cell-cycle control mechanisms can go awry, resulting in pathological epidermal hyperplasia (thickening). Here we observed that CHF6001 treatment decreased keratinocyte proliferation and inhibited re-epithelization in a wound-scratch assay, which could be due to effects on keratinocyte proliferation and/or migration. In accordance with reduced proliferation, we also observed decreased nuclear cyclin D1 levels in response to CHF6001 treatment and increased levels of cell cycle inhibitor p21. We also tested the effects of CHF6001 on NF-κB via assessing nuclear translocation of phosphorylated NF-κB subunit p65, since NF-κB transcriptionally regulates cyclin D1 [24] and is inhibited by cAMP activation [3]. Since NF-κB is regulated by the redox status of keratinocytes [31, 32], we also tested the effects of CHF6001 on oxidative stress. PDE4 inhibitors have been shown to decrease ROS in multiple models [25-30]. CHF6001, in particular, was shown to inhibit the activation of oxidative burst in neutrophils and eosinophils [7]. Therefore, we tested the effects of CHF6001 on NOX activation and observed decreased translocation of NOX regulatory subunit p47 in response to CHF6001. We also observed decreased levels of lipid peroxidation product 4-HNE in response to CHF6001, suggesting that CHF6001 decreases levels of oxidative damage in keratinocytes, inhibiting nuclear translocation of phosphorylated NF-κB subunit p65 and keratinocyte proliferation.

These findings are of interest to the field, since oxidative stress has been linked to psoriasis [40]. Although, there are controversial opinions on this topic, due to the often unsuccessful results from trials investigating the effectiveness of topical applications of antioxidants for treatment of psoriasis [41]. However, more recent studies have confirmed the presence of altered redox homeostasis in the blood and skin of psoriatic patients [42]. Numerous studies have observed significantly increased levels of oxidative stress markers in psoriatic patients and decreased levels of antioxidants. In addition, lipid peroxidation levels in psoriatic lesional skin were significantly higher than those in non-lesional cutaneous tissue [43]. For instance, there are several reports demonstrating a positive correlation between oxidative damage and psoriasis in patients with

increased pathological severity; furthermore, increasing severity of pathology is negatively correlated with antioxidant levels [44].

The ability of ROS to modulate redox sensitive transcription factors has been demonstrated in a variety of inflammatory signaling pathways, and NF-kB has been shown to be one of the main players in the inflammatory conditions found in psoriasis [45]. The potential ability of CHF6001 to prevent NF-kB activation in keratinoctyes could result in a potential anti-inflammatory effect in cutaneous tissues and ameliorate the presentation of the clinical features found in psoriatic patients.

As previously mentioned, our study provides a rationale for testing the efficacy of topical application of CHF6001 for treating psoriasis in other models, although we did not measure the effects of CHF6001 on changes in inflammatory status in the present *in vitro* work. It should be mentioned that another PDE4 inhibitor, apremilast, was approved by the Food and Drug Administration in 2014 for the treatment of psoriasis and was the first oral drug of its class. In two clinical studies involving 352 and 844 patients with active psoriasis, apremilast was found to be effective in treating moderate to severe plaque psoriasis [46, 47]. In another small clinical study, the PDE4 inhibitor roflumilast, which is an oral drug approved for the treatment of COPD, reduced severity of psoriatic lesions [48]. Interestingly, data from Moretto *et al*. 2015 suggested that CHF6001, which is currently undergoing clinical trials as an inhaled agent in COPD, is about 10-fold more potent in inhibiting all four isoforms of PDE4 than roflumilast and in eliciting anti-inflammatory effects as well [7]. In addition, Schafer *et al*. 2014 demonstrated that roflumilast is more potent than apremilast in increasing cellular cAMP levels [49]. Indeed, here we show that CHF6001 is effective in modulating keratinocyte hyperproliferation and re-epitheliazation. Therefore, CHF6001, formulated as a topical agent for the treatment of psoriasis and other hyperproliferative skin diseases, could prove to be as or more efficacious than current therapies, reducing the side effects that are normally associated with systemic exposure to PDE4 inhibitors.

Interestingly, in a recent patent (WO 2018/0017713- https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2018017713&tab=PCTBIBLIO), CHF6001 was claimed to be superior, compared to other PDE4 inhibitors, in ameliorating inflammation in dermal and ocular models of inflammation. We suggest that future studies should focus on comparing CHF6001 with other PDE4 inhibitors in more complex models of hyperproliferative skin diseases.

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Figure Legends:

Fig. 1. **Effect of CHF6001 on cellular toxicity.** After 24 hrs of treatment with 0.1 to 10000 nM concentrations of CHF6001, we evaluated the cytotoxicity of the drug in HaCaT cells by measuring lactate dehydrogenase (LDH) release. As a positive control, we measured LDH release from HaCaT cells treated with Triton X-100 for 30 min. Data are normalized with respect to values from the samples treated with Triton X-100 (average of six experiments ± SEM *p< 0.05).

Fig. 2. Effect of CHF6001 on cellular proliferation. In order to assess the effects of CHF6001 treatment on proliferation, we performed a BrdU incorporation assay after HaCaT cells were treated with various concentrations of CHF6001, ranging from 10nM to 1000 nM, for 24 hrs. Data are expressed as percentages of the mean of the control samples (average of six experiments ± SEM *p< 0.05).

Fig. 3. Effect of CHF6001treatment on HaCaT cells in "*in vitro*" wound closure. Confluent HaCaT cells were wounded using a 200 uL pipette tip, washed, and allowed to regenerate in normal conditions (control=DMSO) or after CHF6001pretreatment (100nM or 1000nM concentrations) for 24 hrs. Multiple photographs of the wound were obtained at 40x magnification (left panel), and the distance between cell borders (after wound) represent 0% recovery. Using image analysis software (see Methods), we calculated the percentage of cellular recovery from the distance of wound closure edges. Cellular recovery was measured at 1, 4, 6, 10, 12, 24, 30, and 36 hrs post-wounding. Quantification of the area is shown in the right panel. Data shown are the averages of results from three independent experiments. *p< 0.05

Fig. 4. Effect of CHF6001 on cyclin D1 levels. Transcript (A) and protein levels (B) of cyclin D1 were measured in HaCaTs treated with CHF6001 for 24 hrs. Nuclear translocation of cyclin D1 in HaCaTs treated with CHF6001 (concentrations ranging from 0.1 to 100 nM) for 24 hrs was assessed by performing nuclear fractionation followed by western blotting. Quantification of a representative blot is shown in (C). Protein levels of cell cycle regulator p21 were also assessed using western blotting, and quantification is shown in (D). Data are expressed as arbitrary units (AU) (averages ± SEM of five independent experiments, *p< 0.05).

Fig. 5. CHF6001 pre-treatment decreased translocation of phosphorylated NF-κB subunit p65 in a dose-dependent manner. (A) Nuclear translocation of phosphorylated p65 (red staining) was measured in HaCaT cells treated with dimethyl sulfoxide (DMSO) and 10 or 100 nM concentrations

of CHF6001 using immunofluorescence. Nuclear staining was detected using DAPI (blue staining). Quantification of fluorescence intensity is depicted in panel (B). Data are expressed as arbitrary units (averages ± SEM of five independent experiments, *p< 0.05).

Fig. 6. CHF6001 pre-treatment decreased GO-induced NOX activation. HaCaT cells were pretreated with 0.1-100 nM concentrations of CH6001 for 24 hrs and then treated with 1 U/mL glucose oxidase (GO) for 1 hr to induce oxidative stress. Cytosolic translocation of p47 was measured via immunofluorescence (red staining). Representative image is shown in (A). Membrane levels of p47 were also assessed via cytosolic fractionation followed by western blotting, and a representative image is shown in (B). Quantification of band intensities using ImageJ, after normalizing to beta-actin. Data are expressed as arbitrary units, as shown in the lower panel of (B) (averages ± SEM of five independent experiments, *p< 0.05).

Fig. 7. CHF6001 pre-treatment decreased GO-induced formation of 4HNE protein adducts. In this experiment, keratinocytes were pretreated with 0.1-100 nM concentrations of CH6001 for 24 hrs and then treated with 1 U/mL glucose oxidase (GO) for 1 hr to induce oxidative stress. Levels of 4HNE protein adducts in keratinocytes were measured using immunoblotting, and a representative blot is shown in the upper panel. Quantifcation of band intensities using ImageJ, after normalization to beta-actin, is shown in the lower panel. Data are expressed as arbitrary units (averages ± SEM of 3 independent experiments, *p< 0.05).

Fig. 1

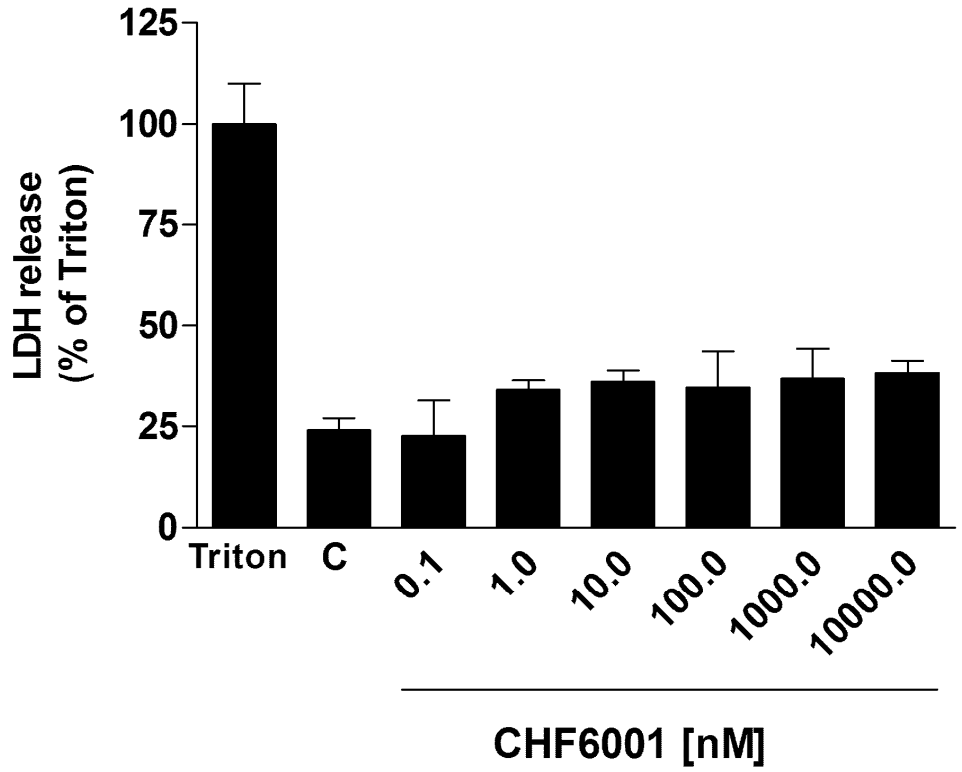

LDH release
(% of Triton)
125
100
75
50
25
0
Triton
C
0.1
1.0
10.0
100.0
1000.0
10000.0
CHF6001 [nM]

Fig. 2

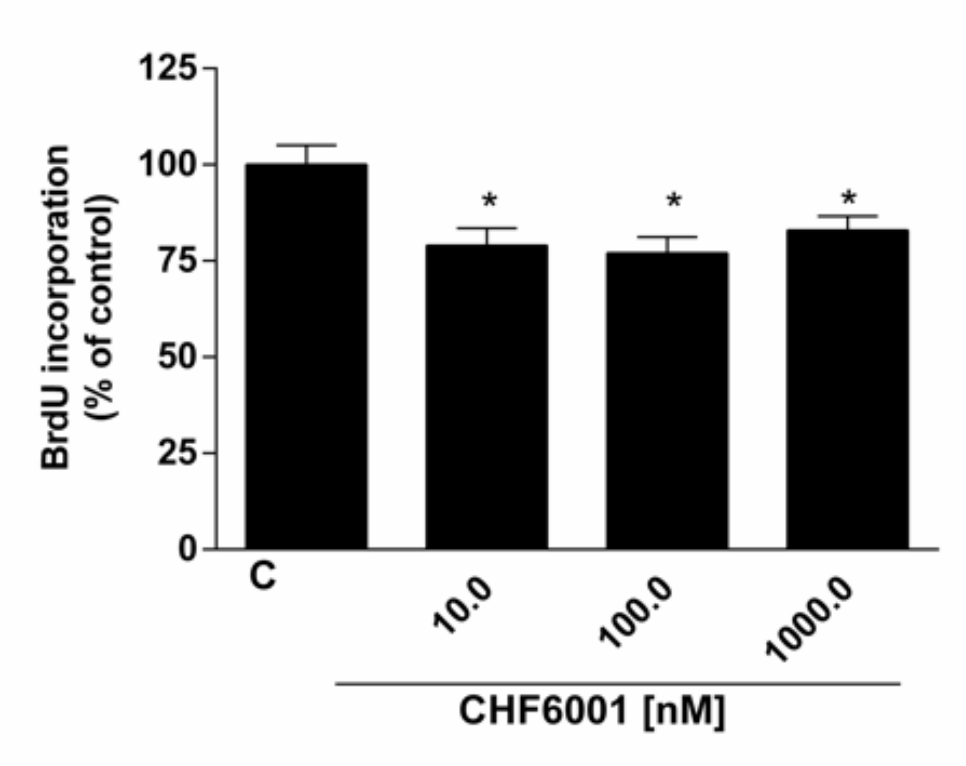
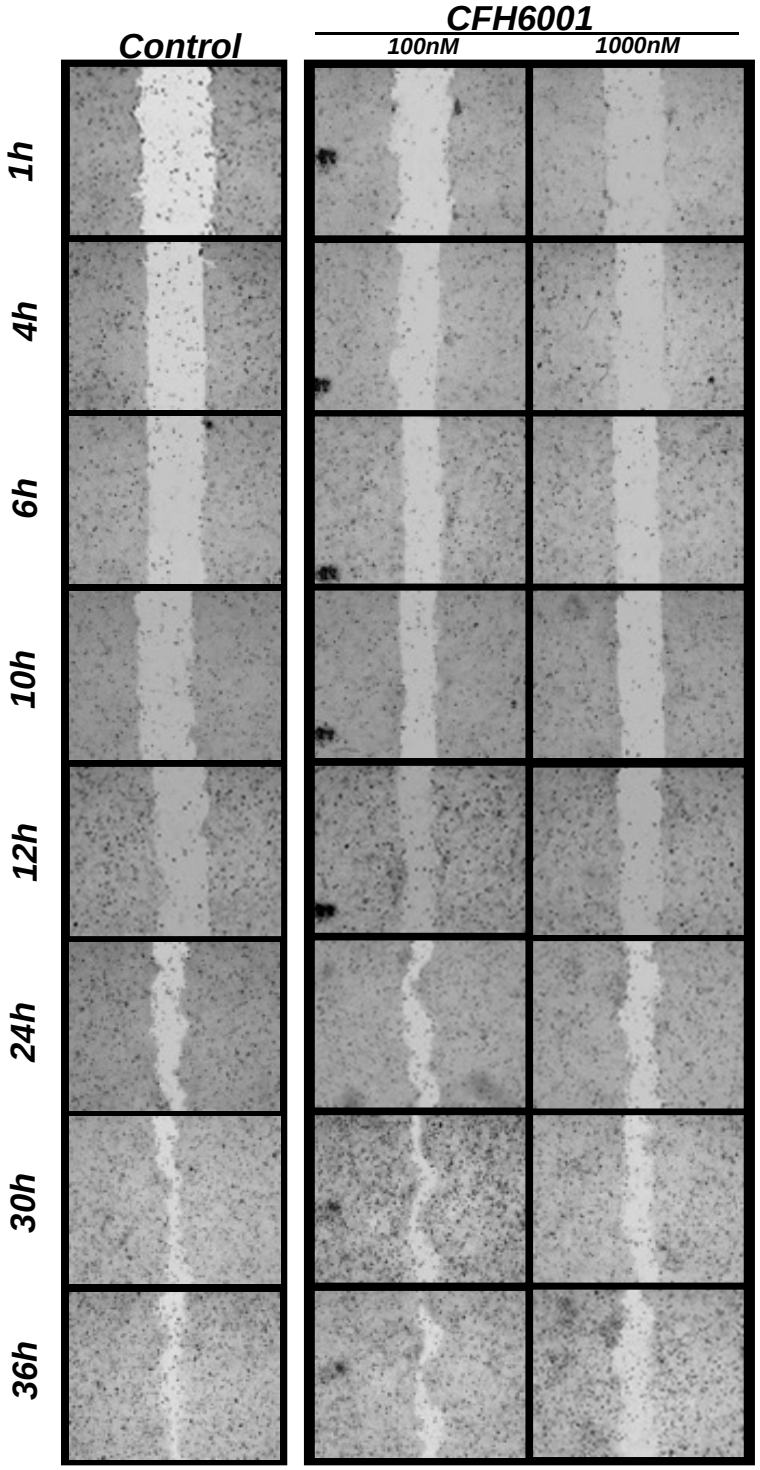

BrdU incorporation
(% of control)
125
100
75
50
25
0
*
*
*
C
10.0
100.0
1000.0
CHF6001 [nM]

Fig. 3


Control
CFH6001
100nM
1000nM
1h
4h
6h
10h
12h
24h
30h
36h

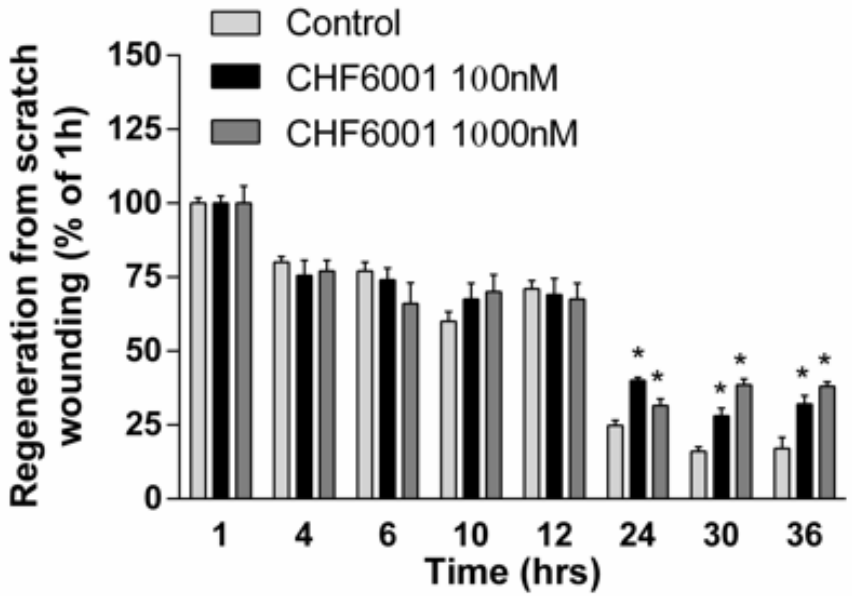
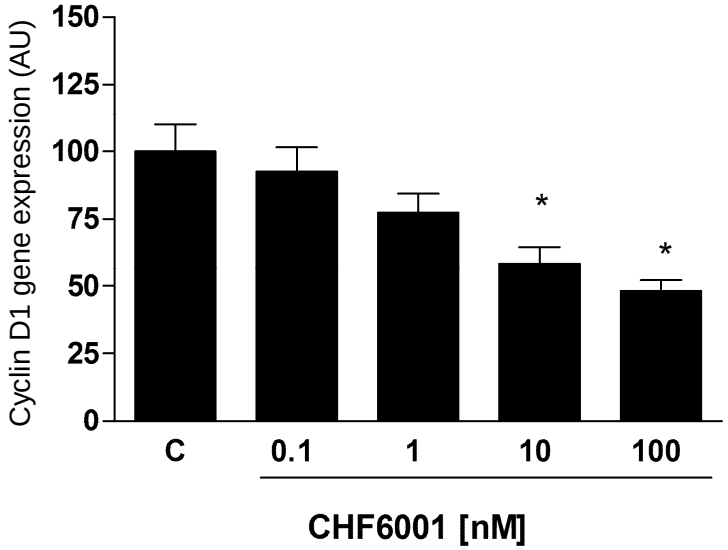
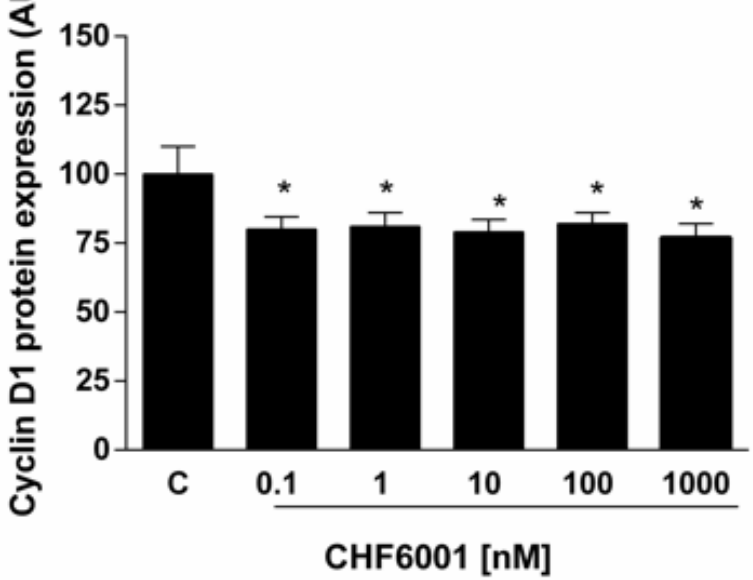

Control
CHF6001 100nM
CHF6001 1000nM
Regeneration from scratch wounding (% of 1h)
150
125
100
75
50
25
0
1
4
6
10
12
24
30
36
Time (hrs)
*

Fig. 4

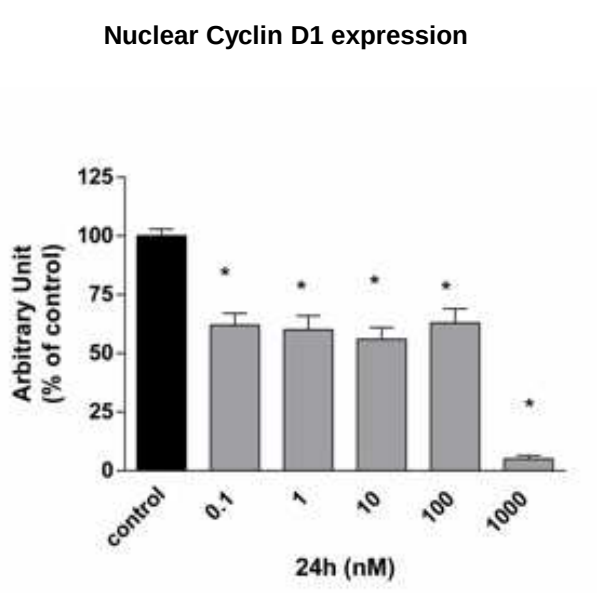
A


Cyclin D1 gene expression (AU)
150
125
100
75
50
25
0
C
0.1
1
10
100
CHF6001 [nM]

B


Cyclin D1 protein expression (AU)
150
125
100
75
50
25
0
C
0.1
1
10
100
1000
CHF6001 [nM]

C


Nuclear Cyclin D1 expression
Arbitrary Unit
(% of control)
125
100
75
50
25
0
control
0.1
1
10
100
1000
24h (nM)

D

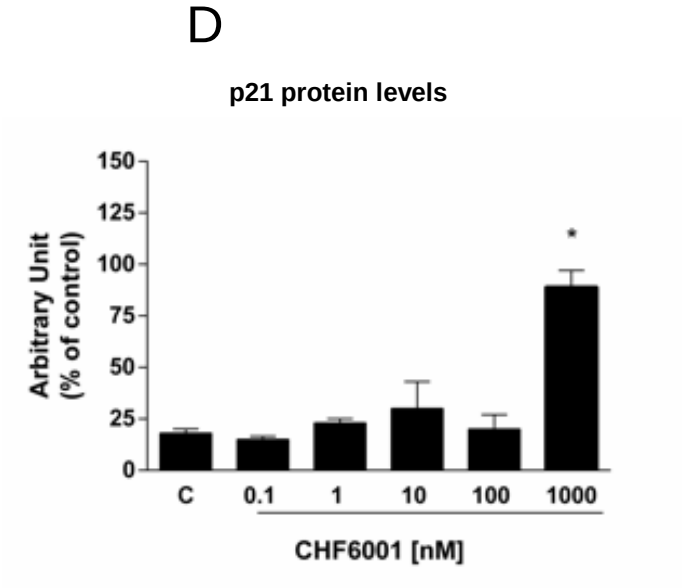
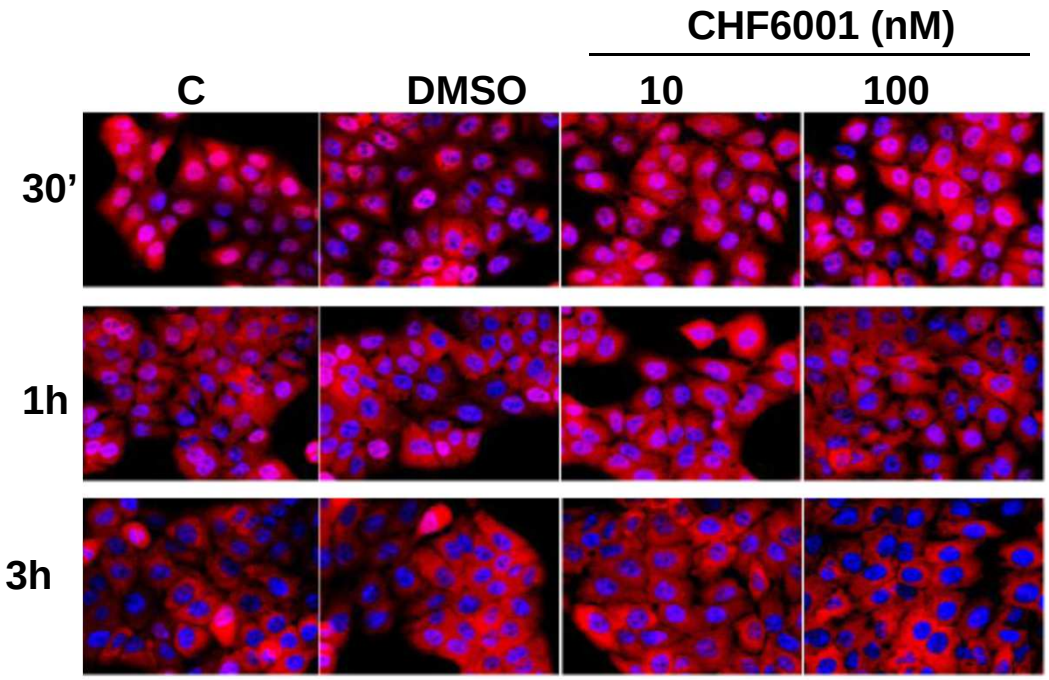

p21 protein levels
Arbitrary Unit
(% of control)
150
125
100
75
50
25
0
C
0.1
1
10
100
1000
CHF6001 [nM]

Fig. 5

A


CHF6001 (nM)
C
DMSO
10
100
30’
1h
3h

B

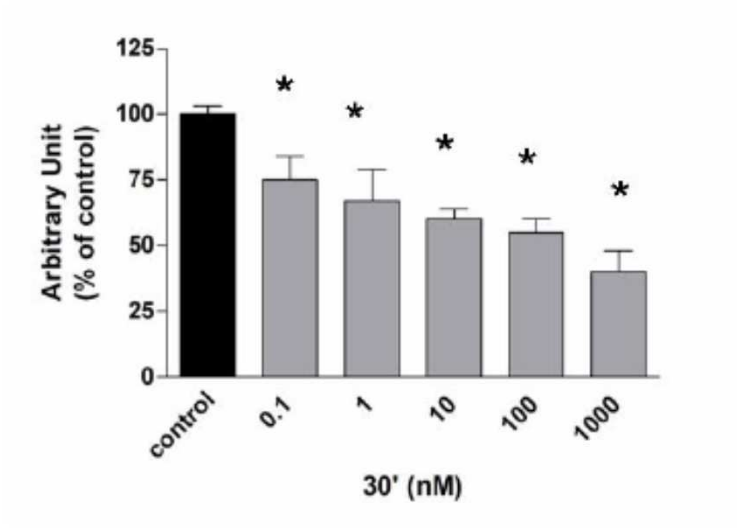
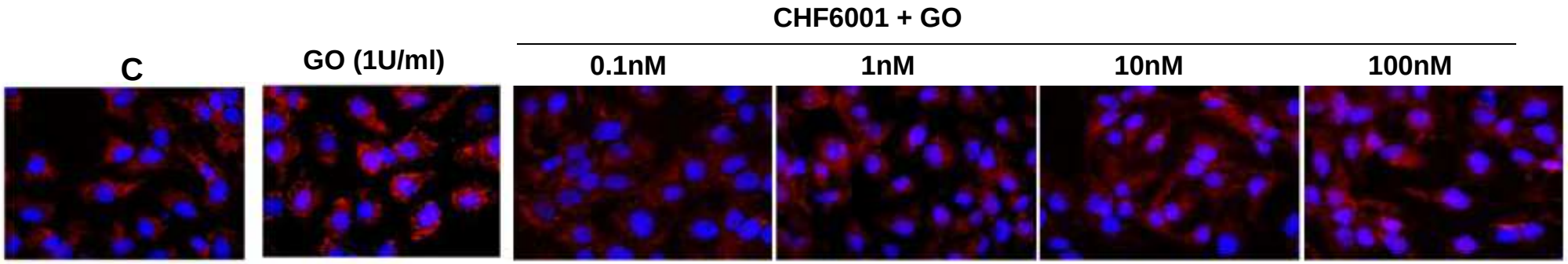
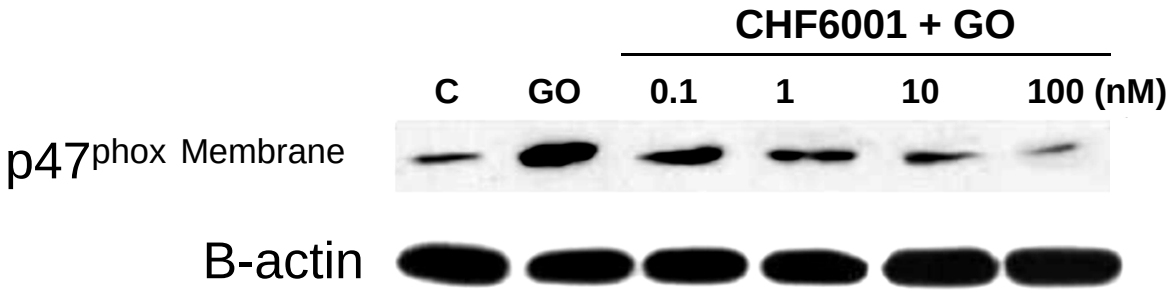

Arbitrary Unit
(% of control)
125
100
75
50
25
0
control
0.1
1
10
100
1000
30' (nM)

Fig. 6

A


CHF6001 + GO
C
GO (1U/ml)
0.1nM
1nM
10nM
100nM

B


CHF6001 + GO
C
GO
0.1
1
10
100 (nM)
p47phox Membrane
B-actin

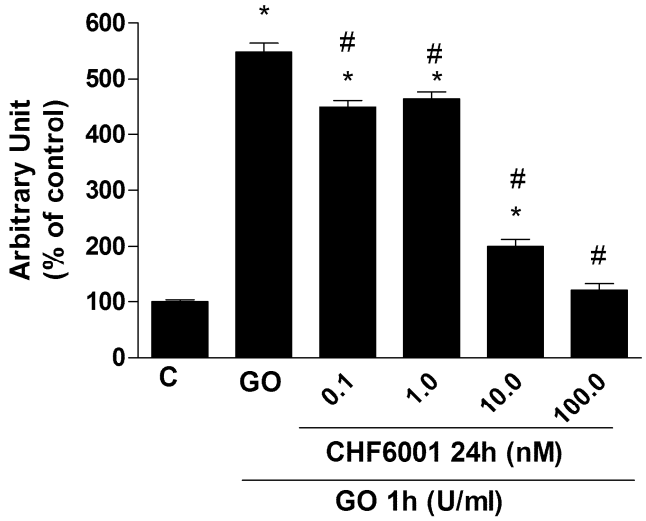
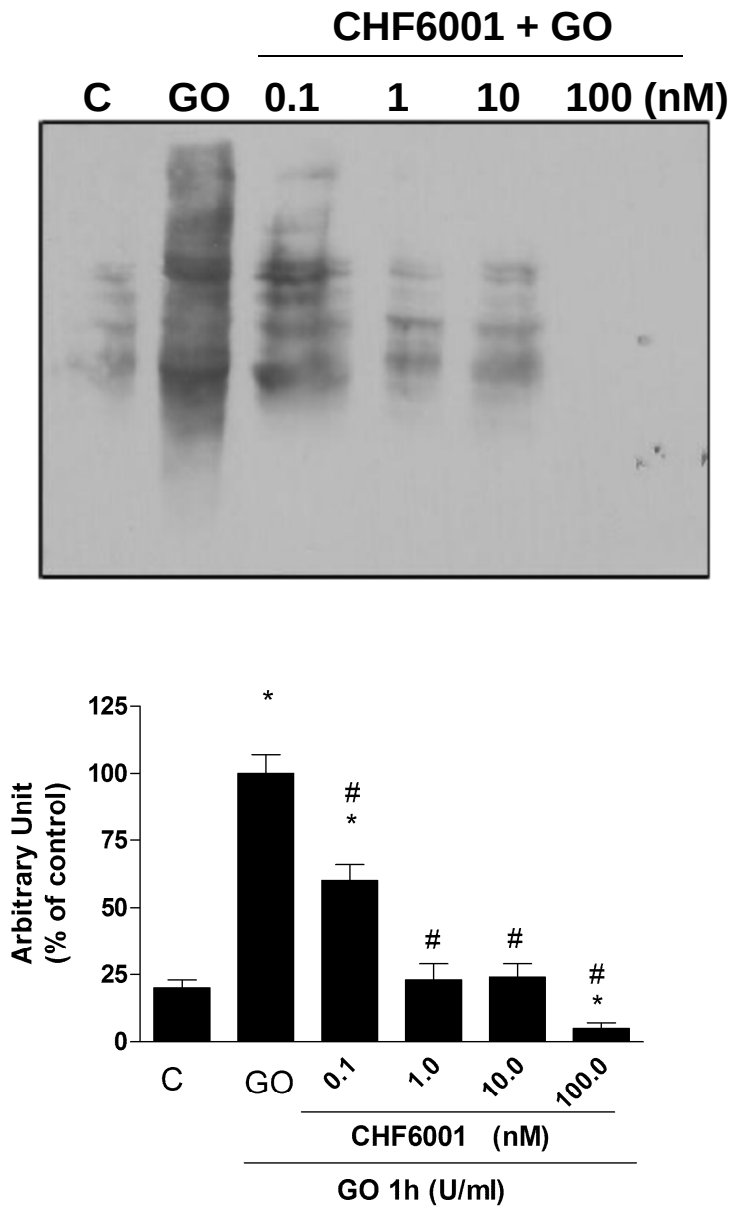

Arbitrary Unit
(% of control)
600
500
400
300
200
100
0
C
GO
0.1
1.0
10.0
100.0
CHF6001 24h (nM)
GO 1h (U/ml)

Fig. 7


CHF6001 + GO
C
GO
0.1
1
10
100 (nM)
Arbitrary Unit
(% of control)
125
100
75
50
25
0
*
#
*
#
#
#
*
C
GO
0.1
1.0
10.0
100.0
CHF6001 (nM)
GO 1h (U/ml)