

# A novel macrolide/fluoroketolide, CEM-101, reverses corticosteroid insensitivity under oxidative stress via PI3K pathway inhibition

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## Abstract

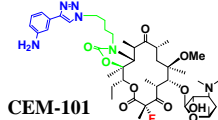
**Rationale** We have recently demonstrated that the reduction of histone deacetylase (HDAC)2 activity via PI3K activation causes corticosteroid (CS) insensitivity in COPD (ATS 2007). Here we show that CEM-101, a novel macrolide/fluoroketolide, which has potent activities against many bacteria causing pneumonia (starting Phase 2), reverses oxidative stress-dependent CS insensitivity via inhibition of PI3K signaling. **Methods:** CS sensitivity was determined by calculation of IC<sub>50</sub> value of dexamethasone on TNF $\alpha$ -induced IL-8 production in U937 monocytic cell line. HDAC activity and phosphorylation level of Akt as a marker of PI3K activation were measured by fluorescence based activity assay and western blotting, respectively. HDAC2 mRNA expression were also determined in A549 type II alveolar epithelial cell line. Cells were exposed to oxidative stress such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) or cigarette smoke extract (CSE) to induce steroid insensitivity. **Results:** Oxidative stress decreased CS sensitivity with concomitant down-regulation of total HDAC activity/HDAC2 expression and increased Akt phosphorylation. Treatment with CEM-101 (10  $\mu$ M) restored CS sensitivity in H<sub>2</sub>O<sub>2</sub> exposed cells. In addition, CEM-101 (10 - 100  $\mu$ M) restored HDAC activity and HDAC2 expression reduced under oxidative stress and also inhibited Akt phosphorylation, which effects were more potent than those of any macrolides currently used clinically. **Conclusion:** CEM-101 restores CS sensitivity in oxidative stress-dependent CS insensitive model via enhancement of HDAC activity/expression due to PI3K signaling inhibition. This novel fluoroketolide, CEM-101, has potential for a novel treatment of COPD or severe asthma, which are steroid insensitive.

## Introduction

•Chronic obstructive pulmonary disease (COPD) is characterized by progressive inflammation in the peripheral lung and oxidative stress. Down-regulation of histone deacetylase 2 (HDAC2) expression and activity by activation of PI3K $\delta$  causes corticosteroid insensitivity in COPD [1-4].

• Erythromycin has been shown to increase HDAC2 levels by activating the HDAC2 promoter [5].

• A novel macrolide/fluoroketolide, CEM-101 (Cempra Pharmaceutical, Inc.) has more potent anti-bacterial effect than other macrolides currently used [6]. We confirmed whether CEM-101 restores oxidative stress-induced corticosteroid sensitivity, HDAC2/PI3K dependently



## Methods

**Cells:** The human monocytic U937 cells and the human type II alveolar epithelial carcinoma A549 cells were treated with CEM-101 or other macrolides (erythromycin, clarithromycin and azithromycin) prior to stimulation with H<sub>2</sub>O<sub>2</sub> or cigarette smoke extract. U937 were differentiated into an adherent macrophage-like morphology by exposure to PMA as needed.

**Cytokine ELISA:** TNF $\alpha$ -induced IL-8 concentrations were determined by sandwich ELISA (R&D Systems Europe). IC<sub>50</sub> values for dexamethasone on IL-8 production were calculated using Prism 4.0 (GraphPad Software Inc.) as a marker for steroid sensitivity.

**HDAC activity:** HDAC activity was measured with HDAC Fluorescent Activity Assay kit (BIOMOL<sup>®</sup> International, Inc.).

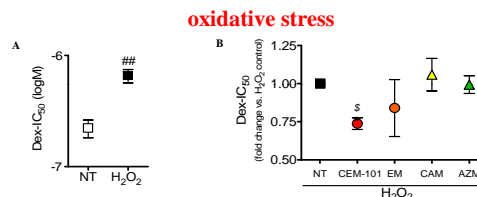
**Cell lysis & Western blotting:** Whole cell extraction was performed using modified RIPA buffer. Proteins were size-fractionated and transferred on nitrocellulose membranes and phospho Akt and Akt band intensities were detected by chemiluminescence.

**RNA extraction & Real-time PCR:** RNA was extracted using the RNeasy Mini kit (Qiagen) and reverse transcribed using the High Capacity cDNA reverse transcription kit (Applied Biosystems). HDAC2 and GNB2L1 mRNA expression levels were quantitated by real-time PCR using the TaqMan<sup>®</sup> Gene Expression Master Mix (Applied Biosystems) on a Rotor-Gene 6000 (Corbett Research).

## Aim

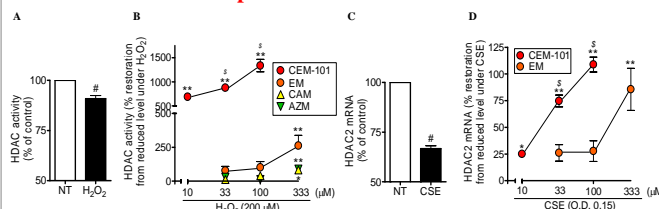
To explore whether a novel macrolide/fluoroketolide, CEM-101, restores corticosteroid sensitivity compared to other macrolides currently used clinically.

## Results 1: Effects of macrolides on corticosteroid sensitivity under oxidative stress



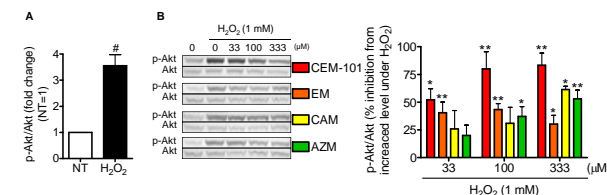
U937 cells stimulated with H<sub>2</sub>O<sub>2</sub> (200  $\mu$ M) overnight were pretreated with macrolide compounds (CEM-101; 10  $\mu$ M, Erythromycin (EM), Clarithromycin (CAM), and Azithromycin (AZM); 100  $\mu$ M) for 30 min. The cells were treated with dexamethasone (10<sup>-11</sup> to 10<sup>-6</sup> M) for 45 min, followed by the TNF $\alpha$  stimulation overnight. TNF $\alpha$ -induced IL-8 release was evaluated by ELISA and IC<sub>50</sub> values for dexamethasone on IL-8 production were calculated using Prism. Data are expressed as fold changes against non-treatment (NT) under H<sub>2</sub>O<sub>2</sub>. Values represent means of five (A) or four (B) experiments  $\pm$  SEM. \*  $p < 0.01$  (vs. non-treatment control; NT), \*  $p < 0.05$  (vs. treatment with H<sub>2</sub>O<sub>2</sub> only), <sup>§</sup>  $p < 0.05$  (vs. CAM and AZM).

## Results 2: Effects of macrolides on total HDAC activity and HDAC2 mRNA expression under oxidative stress



(A)(B) Effects of macrolides on total HDAC activity in PMA-differentiated U937 cells. Cells were pre-treated with macrolides (10 to 330  $\mu$ M) for 20 min. After H<sub>2</sub>O<sub>2</sub> (200  $\mu$ M) stimulation for 4 hrs, total HDAC activity was assayed. Data are expressed as % of non-treatment control (NT) (A) or % restoration from reduced level under H<sub>2</sub>O<sub>2</sub> (B). (C)(D) Effects of macrolides on HDAC2 mRNA in A549 cells. Cells pre-treated with macrolides (10 to 330  $\mu$ M) for 30 min were exposed to cigarette smoke extract (CSE; 0.15 O.D.) for 1 hr. The cell were washed twice and incubated with medium for 4 hrs. Data are expressed as % of non-treatment control (NT) (C) or % restoration from reduced level under CSE (D). Values represent means of four (A and B) or three (C and D) experiments  $\pm$  SEM. \*  $p < 0.01$  (vs. NT), \*  $p < 0.05$ , \*\*  $p < 0.01$  (vs. treatment with H<sub>2</sub>O<sub>2</sub> or CSE only), <sup>§</sup>  $p < 0.05$  (vs. EM, CAM and AZM).

## Results 3: Effects of macrolides on phosphorylation levels of Akt under oxidative stress



Effects of macrolides on H<sub>2</sub>O<sub>2</sub>-induced phosphorylation of Akt in PMA-differentiated U937 cells. Cells were pre-treated with macrolides (33 to 330  $\mu$ M) for 20 min. After H<sub>2</sub>O<sub>2</sub> (1 mM) stimulation for 30 min, cells were lysed. Phosphorylation levels of Akt were measured by western blot. Data are calculated relative to total protein, and expressed as fold change against non-treatment control (NT) (A) or % restoration from increased level under H<sub>2</sub>O<sub>2</sub> (B). Values represent means of four (A) or three (B) experiments  $\pm$  SEM. \*  $p < 0.01$  (vs. NT), \*  $p < 0.05$ , \*\*  $p < 0.01$  (vs. treatment with H<sub>2</sub>O<sub>2</sub> only).

## Summary/ Conclusion

- Oxidative stress impaired corticosteroid sensitivity in parallel with reduction of total HDAC activity/HDAC2 mRNA expression and elevation of Akt phosphorylation.
- CEM-101 (10  $\mu$ M) significantly restored corticosteroid sensitivity in our oxidative stress model. On the other hand, other macrolides did not improve sensitivity even at 10 times higher concentration.
- CEM-101 (10 to 100  $\mu$ M) reversed total HDAC activity and HDAC2 mRNA expression dose-dependently under oxidative stress. Other macrolides restored them only at higher concentration (333  $\mu$ M).
- CEM-101 concentration-dependently inhibited H<sub>2</sub>O<sub>2</sub>-induced Akt phosphorylation up to 80 %. Although other macrolides inhibited it, the efficacies were less than that of CEM-101.
- This study shows that a novel macrolide, CEM-101 restored corticosteroid sensitivity through PI3K pathway dependent-enhancement of HDAC activity/expression under oxidative stress. CEM-101 might be a viable option for the treatment of COPD or severe asthma, which has less response to corticosteroid.

## References

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