



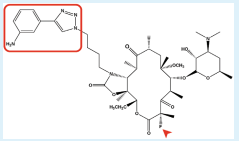
### Abstract (revised)

**Background:** The novel fluoroketolide Solithromycin (CEM-101) accumulates to high levels in cells (AAC 53:3734-43, 2009). Based on previous observations with azithromycin (AZM) and gentamicin (GEN), we have examined whether CEM-101 is sequestered in lysosomes, causes phospholipidosis, induces apoptosis, and interferes with reactive oxygen species (ROS) production.

**Methods:** (i) cell fractionation of cells incubated with [<sup>14</sup>C]-labeled CEM-101; (ii) assay of cell phospholipids and *in vitro* measurement of inhibition of lysosomal phospholipase A1 (PaseA1); (iii) DAPI-detected apoptosis in incubated and electroporated cells; (iv) increase in the fluorescence intensity of CM-H<sub>2</sub>DCFDA (ROS detection). For incubated cells, CEM-101 and AZM were used at 0-100 mg/L, whereas GEN was used 0-900 mg/L to compensate for its low uptake by cultured cells.

**Results:** (i) distribution: [<sup>14</sup>C]-labeled CEM-101 co-distributed for 50-70% in lysosomes in J774 macrophages and LLC-PK1 proximal tubular cells, with the remainder in cytosol; (ii) total phospholipids: ~1.5-fold increase for fibroblasts incubated 2-3 days with CEM-101 or AZM (10 mg/L) or with GEN (0.9 g/L); IC<sub>50</sub> towards PaseA1: CEM-101 and GEN: ~50 μM, AZM: ~100 μM; (iii) Apoptosis (LLC-PK1 cells) (a) incubated: < 5% with CEM-101 or AZM vs. ~15% with GEN; (b) electroporated: no change over control for CEM-101 vs. ~15% for GEN (both 0.3 mM); (iv) ROS production: no constant change in H<sub>2</sub>DCFDA fluorescence (up to 50 mg/L) for CEM-101 and AZM in LLC-PK1 cells.

**Conclusions:** Like AZM and GEN, CEM-101 causes lysosomal phospholipidosis in relation to its accumulation in lysosomes and its inhibitory activity towards PaseA1. This alteration, however, is not associated with apoptosis and causes no constant change in ROS production. This suggests that phospholipidosis induced by CEM-101 is unlikely to cause marked cell toxicity or antimicrobial defense impairment at microbiologically meaningful concentrations.



Solithromycin (CEM-101)

The molecule possesses a 11,12-carbamate-butyl-[1,2,3]-triazolo-aminophenyl side chain (square) and a fluorine atom (arrowhead). It is probably monocationic at neutral or moderately acidic pH

### References

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### Background and Aim

CEM-101 (see structure on the left) is a novel ketolide with activity against erythromycin- and telithromycin-resistant pneumococci. It also shows significant intracellular activity against phagocytized *S. aureus*, *L. monocytogenes*, and *L. pneumophila*. CEM-101 accumulates in eukaryotic cells, which may account for this useful intracellular activity (1).

We previously described that azithromycin, which also accumulates to a large extent in cells, induces a conspicuous overloading of lysosomes with phospholipids related to its preferential accumulation in these organelles (2). We also know that aminoglycosides, like macrolides, accumulate in lysosomes, and induce a phospholipidosis associated with apoptosis and necrosis (3,4). Both drugs have been shown to inhibit lysosomal phospholipase A1 *in vitro* (2,6,7).

The aim of the present study was to examine how CEM-101 would behave in this context, using various types of cultured cells and an *in vitro* model for phospholipase inhibition studies.

### Methods

#### Cell fractionation:

Cells (J774 macrophages) were incubated with [<sup>14</sup>C]-labeled CEM-101 (10 mg/L) for 24h, collected, and homogenized in 0.25 M sucrose. After removal of unbroken cells and nuclei, the cytoplasmic extract was subjected to isopycnic centrifugation in a sucrose gradient (25,000 RPM, 16h; 4°C). Fractions were collected and assayed for marker enzymes and for radioactivity (5).

#### Assay of cell phospholipids:

Cell homogenates were extracted with acidified chloroform-methanol, and lipid phosphorus assayed by reduction of phosphomolybdate complex after mineralization (6).

#### *In vitro* measurement of the activity of lysosomal phospholipase A1:

Liposomes containing 1-palmitoyl-2-[<sup>14</sup>C]oleoyl-phosphatidylcholine were exposed to a soluble extract of purified liver lysosomes and the release of [<sup>14</sup>C] lysoderivative quantified by thin layer chromatography (6, 7).

#### Measurement of apoptosis:

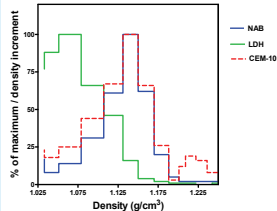
Apoptosis (J774 macrophages, fibroblasts, LLC-PK1 cells) was detected in both incubated and electroporated cells by staining with DAPI and counting of fragmented nuclei (4, 8).

#### Detection of reactive oxygen species (ROS):

This was assessed by the increase in fluorescence intensity of CM-H<sub>2</sub>DCFDA, a commonly used tracer for production of ROS in cultured eukaryotic cells (9).

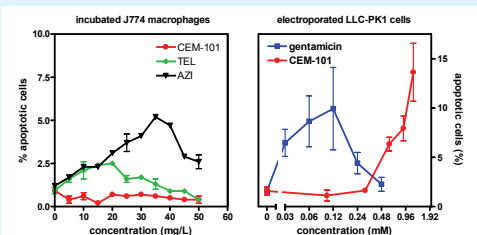
### Results

Distribution of <sup>14</sup>C-CEM-101 in cytoplasmic extracts of J774 cells incubated for 24h with 10 mg/L



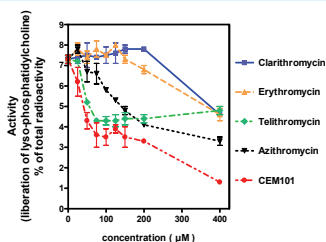
CEM-101 distributes largely like N-acetyl-β-hexosaminidase (NAB), an established marker of lysosomes, and is dissociated from lactate dehydrogenase (LDH), a cytosolic enzyme.

Apoptosis induced by CEM-101 in incubated cells (48 h; J774 macrophages) or after electroporation (LLC-PK1 cells): comparison with azithromycin, telithromycin (left) and gentamicin (right)



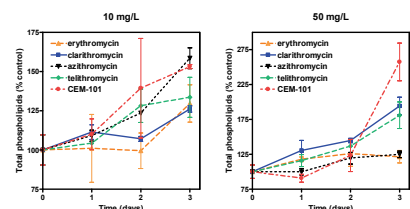
CEM-101 causes no detectable apoptosis in incubated cells and is considerably less potent than gentamicin in electroporated cells (the decline seen at large concentration for azithromycin [left] and gentamicin [right] is due to necrosis)

#### Inhibition of lysosomal phospholipase A1 by CEM-101: comparison with azithromycin, telithromycin, erythromycin and clarithromycin



CEM-101 causes a marked inhibition of lysosomal phospholipases that is more pronounced than that of azithromycin at equimolar concentrations

#### Accumulation of phospholipids in fibroblasts incubated for up to 3 days in the presence of 10 or 50 mg/L of CEM-101; comparison with erythromycin, clarithromycin, telithromycin, and azithromycin



CEM-101 causes a marked increase in total cell phospholipids, similar to that seen with azithromycin at 10mg/L. This increase was larger at 50mg/L (the lower accumulation seen with azithromycin at this concentration is probably related to cytotoxicity). All values are means ± standard deviations.

#### ROS production:

CEM-101 up to 80 mg/L caused no detectable change in ROS production in LLC-PK1 cells under conditions in which gentamicin caused a highly significant increase compared to control.

### Conclusions

- Like azithromycin and gentamicin, CEM-101 causes lysosomal phospholipidosis in relation to its accumulation in lysosomes and its inhibitory activity towards phospholipase A1. This is somewhat surprising given the monoclonal character of CEM-101. Further studies are needed to better understand structure-activity relationships in this context.

- The phospholipidosis induced by CEM-101, however, is not associated with apoptosis and causes no change in ROS production.

- This suggests that phospholipidosis induced by CEM-101 is unlikely to cause marked cell toxicity or antimicrobial defense impairment at microbiologically meaningful concentrations.

### Acknowledgements

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