Phospholipidosis and its Reversal Induced by Solithromycin and its Main Animal Metabolites (N-Acetyl-Solithromycin and Des-Aminophenyltriazol-Hydroxy-Solithromycin): Studies with Cultured Rat Fibroblasts

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Abstract (edited)

Background and Aims
Solithromycin (SOL) and other macrolides (e.g., azithromycin [AZM]), induce lysosomal phospholipidosis (due to their ability to concentrate in lysosomes where they inhibit the activity of phospholipases) but do not cause apoptosis. Our aim was to compare SOL to its two main animal metabolites (N-acetyl-solithromycin [NAS] and des-aminophenyltriazol-hydroxy-solithromycin [CEM-214]) and to AZM for development and reversibility of phospholipidosis, using a previously validated cell culture model.

Methods
Rat fibroblasts (primary cultures) were exposed to SOL, NAS, CEM-214 or AZM at both equimolar (0-25 µM) and equiweight (0-50 mg/L) concentrations for up to 5 days, after which cells were transferred to drug-free medium for 5 additional days. Cells were monitored for (i) drug accumulation (SOL and AZM; microbiological assay [disk diffusion; pH 7.4]), (ii) total phospholipids; (iii) morphological appearance (optic and electron microscopy).

Results
SOL and AZM accumulated rapidly in cells (up to approx. 300-fold at equilibrium. SOL, NAS and AZM caused (i) a marked and similar increase (up to 4-5-fold over controls) in cell total phospholipids (time and concentration dependent); (ii) the accumulation of dense (phase contrast) and highly stained (toluidine blue) cytoplasmic granules (optic microscopy), and of dense, lamellar material in lysosomes (electron microscopy). CEM-214 caused moderate phospholipid accumulation and minimal morphological alterations. Transfer of cells incubated with SOL or NAS to drug-free medium allowed for return of total phospholipids to normal values and disappearance of morphological alterations within 5 days in parallel with the almost complete release of the antibiotic (tested for SOL only). With AZM, only a partial decrease of total phospholipids (about 50%) and of lamellar material in lysosomes, and of the accumulated antibiotic was seen over the same period.

Conclusions
While SOL, NAS and AZM caused lysosomal phospholipidosis to the same extent, its rapid reversal with SOL and NAS, but not with AZM, together with the mild effects caused by CEM-214, suggest that this effect of macrolides on cell metabolism and structure will be more transient upon treatment with SOL than with AZM.

References

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Background

Solithromycin (SOL) is a novel fluoroketolide that shows enhanced potency, compared to other macrolides (e.g., azithromycin) against most susceptible strains and maintains low MICs against target organisms (e.g., *S. pneumoniae*) resistant to macrolides. In animals, SOL gives rise to two main metabolites, N-acetyl-solithromycin (NAS) and des-aminophenyltriazol-hydroxy-solithromycin (CEM-214). These metabolites make up <10% of total metabolites in humans.

Lysosomal phospholipidosis is a well known side effect of macrolides that has been repeatedly observed in both animals receiving these antibiotics and in cultured fibroblasts exposed to these drugs. Focusing on the latter model, we showed that macrolide-induced phospholipidosis is related to (i) the intralysosomal accumulation of these antibiotics [1], and (ii) their ability to bind to phospholipid bilayers, impairing the access of lysosomal phospholipases to their substrate [2;3].

In a cultured cell model (human THP-1 monocytes), solithromycin displays a fast and extensive cellular accumulation [3]. It also inhibits lysosomal phospholipase A1 in an a-cellular model [4].

Based on these considerations, our main objectives were to examine:

1. whether solithromycin accumulated by cells is concentrated in lysosomes of cultured fibroblasts;
2. whether SOL, NAS and CEM-214 cause the development of a lysosomal phospholipidosis in these cells (AZM as comparator) and to test for reversibility of this cellular alteration upon drug withdrawal.

Methods

1. ***Cells:*** all studies reported here used primary cultures of rat fetal fibroblasts (3^d to 5^{th} passage after primary culture)
2. ***Subcellular localization of solithromycin:*** cells were incubated with [^{14}C]-labeled solithromycin, harvested, homogenized, and fractionated using differential pelleting and isopycnic centrifugation as previously described to study the subcellular localization of macrolides (see [1] for a typical example with AZM).
3. ***Development of phospholipidosis and reversibility upon drug withdrawal:*** Total cell phospholipids were measured after extraction and mineralization as previously described [5]. Optic microscopy was performed on subconfluent cells (to allow for better visualization of the cytoplasm. Electron microscopy was performed on cell collected by scraping and embedded in agar to ensure random distribution (see [2;6-8] for details).
4. ***Accumulation and release of bioactive SOL and AZM from fibroblasts:*** The cellular contents of cells in SOL or AZM (after incubation with SOL or AZM, and after drug withdrawal) were measured using a microbiological assay (disc-diffusion method) using calibrated standards of each drug (normalized for cell protein content). Cellular apparent concentrations were calculated using a ratio of 5 µL of cell volume per mg of cell protein [8].

Main messages and conclusions

- SOL causes lysosomal phospholipidosis that is phenotypically and mechanistically (lysosomal accumulation [this poster]; impairment of phospholipase A1 [4]) similar to what has been reported for azithromycin [2,7].
- N-acetyl-solithromycin causes a similar and CEM-214 less phospholipidosis than solithromycin. Generation of these metabolites *in vivo* is, therefore, unlikely to cause more alterations than what will be caused by solithromycin itself.
- In contrast to azithromycin, solithromycin is rapidly released from incubated cells with a concomitant return of total cell phospholipid content to its control value (for both SOL and NAS) and a large disappearance of the lysosomal alterations typically observed with azithromycin. This could mitigate against long-term toxicity of solithromycin in relation to phospholipidosis.

Typical Results

1. Subcellular distribution of SOL

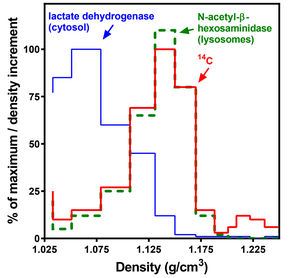

Figure 1: Distribution of ^{14}C and of marker enzymes of the cytosol and of lysosomes in a post-clear supernate of rat fetal fibroblasts incubated for 24h with [^{14}C]-SOL (10 mg/L) and subjected to isopycnic equilibration in a sucrose gradient

2. Development of phospholipidosis

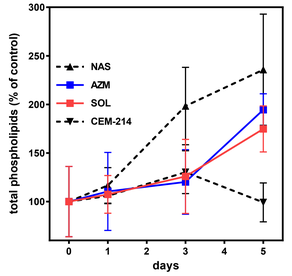

Figure 2: Phospholipidosis develoment in rat fetal fibroblasts exposed to 25 µM of SOL (21.1 mg/L), AZM (19.6 mg/L), NAS (22.1 mg/L) or CEM-214 (17.6 mg/L). Data area means with SD of 3 experiments (each with triplicate assays).

4. Reversibility (phospholipids, drug cell content, lysosomal alterations

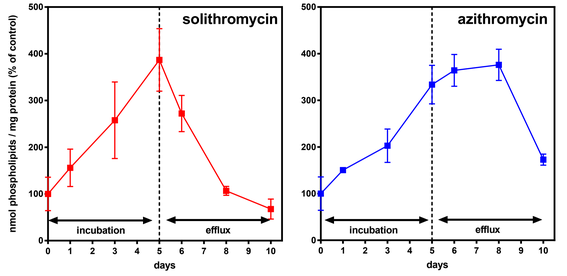

Figure 5: Development and reversibility of the phospholipidosis observed in rat fetal fibroblasts. Incubation: cells were exposed to 25 µM SOL or AZM. Efflux: cells were transferred to drug-free medium. Values are from 3 determinations with SD. Note that phospholipidosis developed to a larger extent in these experiments than in those shown in Figure 2.

3. Lysosomal alterations at day 3 (microscopy)

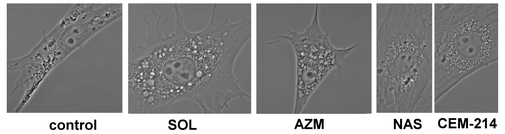

Figure 3: Phase contrast microscopy of rat fetal fibroblasts untreated (control) or exposed to 30 mg/L of SOL, AZM, NAS, or CEM-214 for 3 days. Cells were fixed with glutaraldehyde.

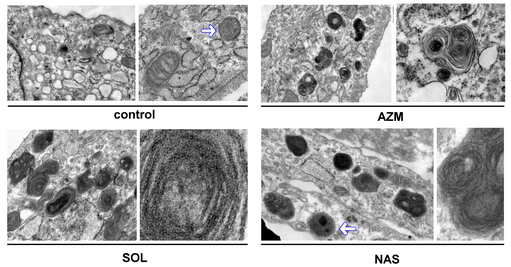

Figure 4: Electron microscopy of rat fetal fibroblasts untreated (arrow: normal lysosome) or exposed for 3 days to 10 mg/L of SOL, AZM or NAS. Upper row control: the white arrow points to a normal lysosome in control cells. Lower row NAS: the white arrow points to a lysosome with minimal accumulation of electron-dense material. AZM, SOL and NAS: high magnification shows the lamellar appearance of the material accumulated in lysosomes.

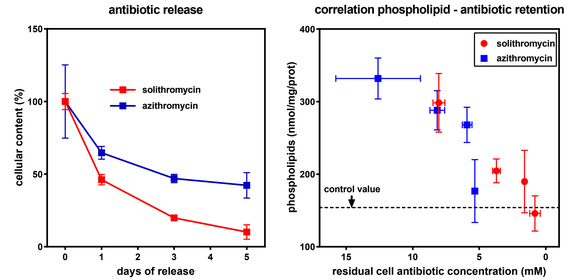

Figure 6: Release of SOL or AZM from rat fetal fibroblasts (left) and correlation between phospholipids and residual antibiotic content (right) in cells exposed for 3 days to 25 µM of SOL or AZM and thereafter transferred to drug-free medium.

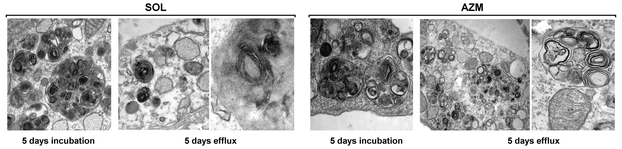

Figure 7: Electron microscopy of rat fetal fibroblasts exposed to 25 µM of SOL or AZM for 5 days ("5 days incubation") and thereafter transferred to drug-free medium for 5 additional days ("5 days efflux"). While both SOL and AZM caused massive accumulation of lamellar material after 5 days of incubation, much of it was disappearing for SOL after 5 days efflux but not for AZM.