Different approaches for studying anti-inflammatory mode of action of macrolides *in vitro*

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Introduction

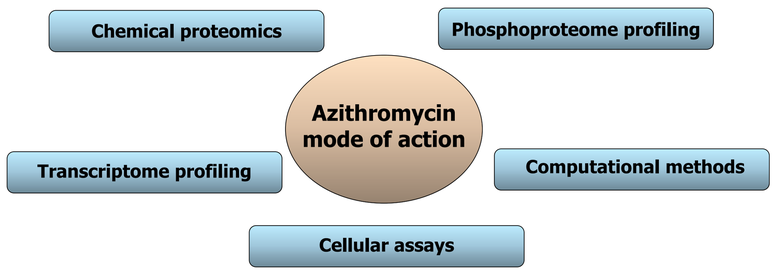
Macrolide antibiotics, in addition to their well established antimicrobial activity, show beneficial effects in various chronic inflammatory diseases such as chronic obstructive pulmonary disease, diffuse panbronchiolitis, cystic fibrosis and bronchiolitis obliterans syndrome. Despite long use of macrolide antibiotics and clear effects in clinical studies their anti-inflammatory mode of action is still unknown. Determination of mechanism of anti-inflammatory activity should accelerate discovery of new and more potent macrolides devoided of anti-bacterial activity. Our *in vivo* and *in vitro* data as well as literature data suggest monocytes and macrophages as an important target cells for anti-inflammatory activity of macrolide antibiotics. Therefore, we have studied mode of action of azithromycin (AZM), one of the most studied macrolide antibiotic, in murine monocyte/macrophage cell line J774A.1.

Objective

Determine the anti-inflammatory mode of action of azithromycin in monocytes/macrophages using different approaches.

Methodology

Azithromycin (AZM) inhibits production of IL-6, IL-1β, IL-12p40, CCL5 and PGE_2 without affecting production of TNFα in LPS-stimulated J774A.1 cells. The combination of multiple approaches and methods such as cellular assays, chemical proteomics, phosphoproteome and transcriptome profiling and computational methods were used in order to explain these activities.



Chemical proteomics: Aminopropyl linker was introduced on three different positions in AZM molecule. Derivatives were immobilized in various densities to solid support. Bound proteins from J774A.1 lysate were identified by mass spectrometry. VCP was fished out as potential target and validated using biochemical (binding and enzyme assay) and cellular assays reflecting function of VCP (unfolded protein response and NFκB pathway).
Phosphoproteome and transcriptome profiling: J774A.1 cells were treated with 50 μM of AZM and stimulated with LPS for 15-240 min for phosphoproteome and 45-360 min for transcriptome profiling. Phosphopeptides were isolated with various phospho-serine and phospho-tyrosine motif antibodies and analysed by mass spectrometry. Total RNA was isolated and hybridized to Affymetrix Mouse 430_2 arrays. Data analysis was performed using Ingenuity pathway analysis.
Computational methods: Principal component analysis (PCA) of the cellular and physico-chemical data and calculated 2D structural descriptors was performed using SIMCA-P software.
Cellular assays: Various cellular assays were performed in order to measure effect of AZM on lysosomal functions, vesicular trafficking, TLR4 recycling and signaling, eicosanoid production and $cPLA_2$ activity. Unless stated otherwise, cells were pretreated with compounds for 2h, LPS was used at concentration of 1 μg/ml and concentration of AZM was 50 μM.

References

- Bosnar M. et al. J Pharmacol Exp Ther, 2009; 331: 104-113
- Vrancic M. et al. British J Pharmacol, 2012; 165-1348-1360
- Munic V. et al. Pharmacol Res, 2011; 64:298-307
- Banjanac M. et al. Pharmacol Res, 2012; 66:357-362
- Nujic K. et al. Eur J Pharmacol,2012; 677:163-172
- Nujic K. et al. Cell Immunol, 2012; 279:78-86

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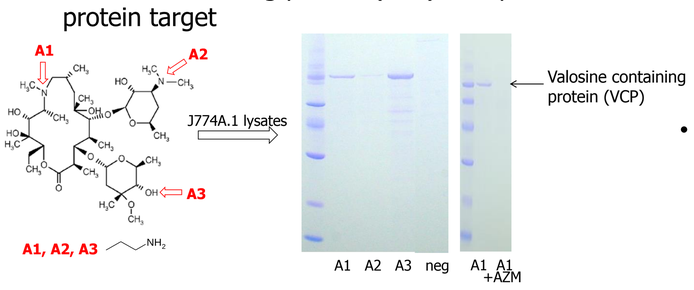
Chemical proteomics

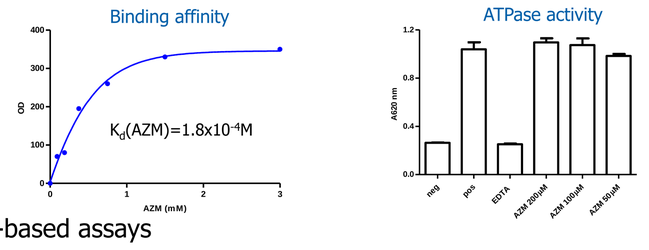
Target identification

- Immobilized AZM derivatives, J77A.1 cell lysates
- Valosine containing protein (VCP) as a potential AZM protein target

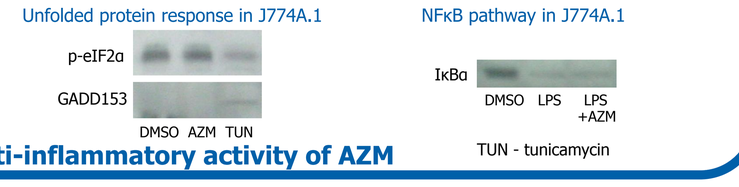


Target validation - VCP

- Biochemistry



- Cell-based assays

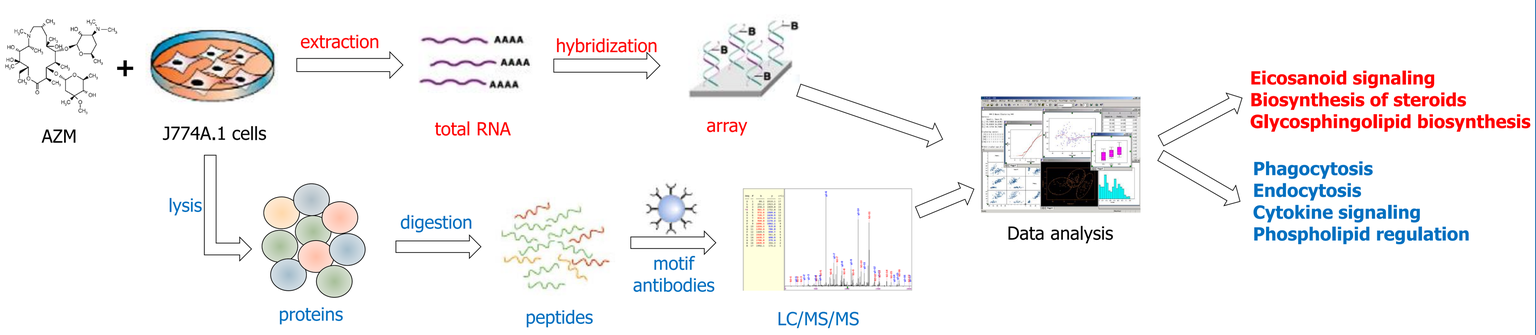


- **No high affinity protein target for anti-inflammatory activity of AZM**

Phosphoproteome and transcriptome profiling

Pathways and processes affected by AZM

- Phosphoproteome: 50 μM AZM, LPS 15-240 min, p-Tyr and p-Ser motif antibodies, Ingenuity pathway analysis
- Transcriptome: 50 μM AZM, LPS 45-360 min, total RNA, Affymetrix arrays, Ingenuity pathway analysis



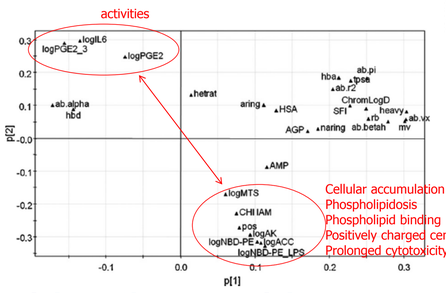
- **AZM affects vesicular trafficking, cytokine signaling and phospholipids**

Correlations

Correlations of activities and properties

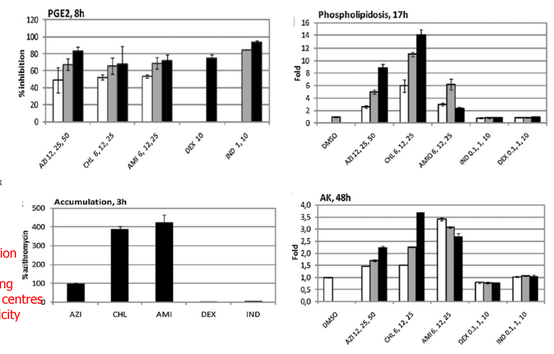
18 macrolides, measurements of IL-6, PGE_2, cellular accumulation, phospholipidosis, cytotoxicity and phospholipid binding (CHI AM)

	Anti-inflammatory						PK	Phospholipidosis				Cytotoxicity				PhysChem & mol prop	
	PGE2 / Fold			IL6 / Fold			Acc / % azi	NBD-PE ns / Fold		NBD-PE LPS / Fold		AK / Fold		MTS / Fract		CHI IAM	pos
c/ uM	50	25	12.5	50	25	12.5		50	25	50	25	50	25	50	25		
Azithromycin	0.17	0.33	0.51	0.42	0.49	0.64	100	7.94	4.86	9.30	6.62	3.35	1.97	0.28	0.10	74.48	2
Clarithromycin	0.74	0.94	1.04	0.84	0.98	1.03	42	1.35	1.09	2.20	1.39	1.99	1.26	0.12	0.00	51.77	1
Roxithromycin	0.94	0.89	0.94	0.97	0.95	1.08	21	1.30	1.16	1.63	1.23	1.22	1.16	-0.12	-0.07	51.33	1
Erythromycin	0.88	1.43	1.11	0.98	1.00	1.09	3	1.00	1.05	1.15	1.06	1.12	1.06	-0.06	-0.09	44.91	1
Tylosin	1.18	1.29	1.23	1.05	1.05	1.07	-1	1.09	0.95	1.12	1.04	0.90	0.87	-0.01	-0.07	34.14	1
Clari metab	1.21	1.18	1.11	1.04	1.06	1.14	7	1.10	0.99	1.14	1.07	1.08	1.05	0.01	-0.01	n.d.	1
Ery metab 1	1.12	1.23	1.29	1.06	1.01	1.06	17	1.08	1.14	1.27	1.23	1.10	1.20	-0.01	0.03	72.78	1
Ery metab 2	1.47	1.79	1.73	0.42	0.65	0.76	73	2.38	2.87	3.97	3.55	5.63	3.57	0.65	0.31	n.d.	1
Cpd 1	1.21	1.20	0.96	0.92	0.90	1.03	2	0.95	0.91	1.04	1.04	1.09	1.13	-0.05	-0.02	42.61	1
Cpd 2	0.89	1.23	0.97	0.89	0.99	1.05	22	1.12	1.09	1.58	1.16	0.97	0.93	-0.08	-0.06	54.41	1
Cpd 3	0.72	1.12	0.95	0.99	0.87	1.07	6	1.00	0.98	1.11	1.01	1.02	1.06	0.02	-0.01	27.16	1
Cpd 4	0.70	1.06	0.96	0.80	0.82	0.92	44	1.31	1.08	2.04	1.54	1.35	1.35	0.03	0.04	58.72	2
Cpd 5	0.32	0.56	0.61	0.55	0.72	0.86	49	3.56	1.86	5.59	3.40	1.87	1.42	0.07	0.08	55.95	2
Cpd 6	0.08	0.30	0.42	0.40	0.60	0.98	99	4.52	2.26	6.75	4.01	1.87	1.45	0.14	0.10	52.89	2
Cpd 7	0.26	0.16	0.33	0.29	0.57	1.05	95	4.58	2.78	7.50	4.87	2.11	1.68	0.16	0.14	51.37	2
Cpd 8	0.11	0.13	0.14	0.44	0.45	0.62	324	2.07	9.36	1.68	7.38	4.14	2.66	0.46	0.21	67.46	2
Cpd 9	4.53	4.29	2.89	0.91	0.95	0.92	31	1.17	0.99	1.03	0.96	1.82	1.63	0.39	0.37	51.37	1
Cpd 10	0.31	0.64	0.70	0.84	0.98	1.04	0	0.99	0.95	1.07	1.04	1.18	1.18	0.08	0.06	37.89	1



Correlations with other drugs

Chloroquine (CHL), amiodarone (AMI), dexamethasone (DEX) and indomethacine (IND)

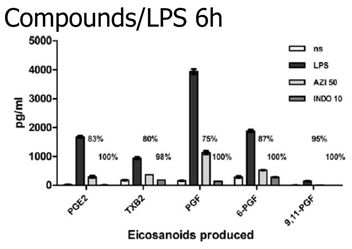


- **Anti-inflammatory activity of AZM correlates with accumulation, phospholipid binding, phospholipidosis, number of positively charged centres and cytotoxicity in prolonged cultures**
- **AZM activity comparable to activity of other cationic amphiphilic drugs (CHL, AMI), but not to DEX and IND**

Cellular assays

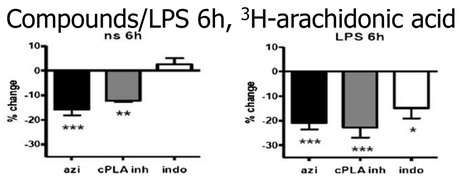
Eicosanoid production

Compounds/LPS 6h



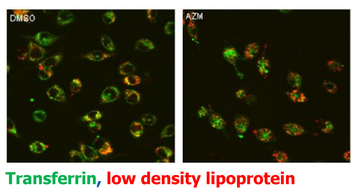
Arachidonic acid release

Compounds/LPS 6h, ^{3}H-arachidonic acid



Endocytosis

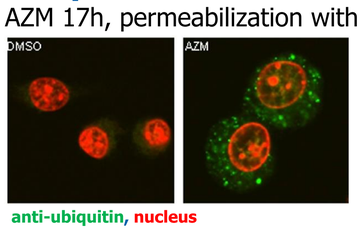
AZM 2h, transferrin and LDL 30 min



Transferrin, low density lipoprotein

Ubiquitination

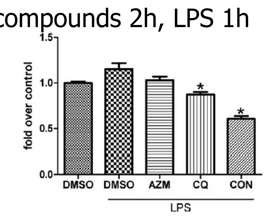
AZM 17h, permeabilization with acetone



anti-ubiquitin, nucleus

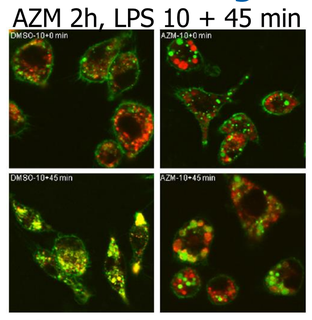
Surface TLR4

compounds 2h, LPS 1h



LPS trafficking

AZM 2h, LPS 10 + 45 min

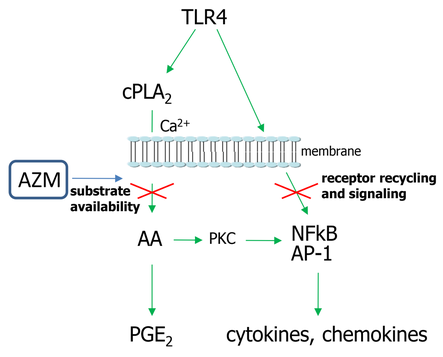


LPS, LysoTracker

- **AZM inhibits $cPLA_2$ activity and TLR4 recycling and signaling**

Conclusion

- No high affinity protein target for AZM
- Accumulation in acidic vesicles and membranes as main driver of anti-inflammatory activity *in vitro*
  - Activity of enzymes involved in phospholipid regulation and receptor recycling impaired – inhibition of production of lipid mediators and cytokines
  - Induction of phospholipidosis, impaired endocytosis and autophagy



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