Action of antibiotics characterized and predicted by NMR metabolomics

Verena Hoerr[1,2], Gavin E. Duggan[3], Lori Zbytnuik[4], Karen K.H. Poon[4], Bettina Löffler[2], and Hans J. Vogel[3]

[1]*Department of Clinical Radiology, University Hospital Muenster, Muenster, Germany,* [2]*Institute of Medical Microbiology, Jena University Hospital, Jena, Germany,* [3]*Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada,* [4]*Department of Physiology and Pharmacology, University of Calgary, Calgary, Alberta, Canada*

Target audience: Physicists, chemists and pharmacists with interest in ^{1}H NMR metabolomics and drug research.

Purpose: Bacterial resistance to commonly used antibiotics is continuously increasing. To counteract the remarkable adaptability of bacteria and to avoid the increasing spread of multi-drug-resistant pathogens, novel antibacterial targets are urgently needed.[1] To this end, drug discovery strategies have moved away from modifying the three main structural scaffolds of β-lactams, macrolides and fluoroquinolones to target- or whole cell based high-throughput screening (HTS) of large chemical libraries with the aim to identify novel targets. However these strategies have not led to any major success so far[2,3] due to a lack in integrated strategies which allow for target identification, evaluation and refinement in one overall concept. Recently novel prospects arose by the "omics" approaches as these techniques are able to detect all changes that occur in an organism in response to toxicological stimuli based on pattern recognition techniques.[4,5] Here we propose the ^{1}H NMR metabolomics approach as potential tool to identify and validate novel antibacterial targets.

Methods: Bacterial strains: Experiments were performed on the gram-negative wild type bacterial strain of *Escherichia coli K12*. Bacteria were cultured in 50 ml of defined medium. Antibiotics: ampicillin sodium, cefalexin, vancomycin hydrochloride, doxycycline hyclate, kanamycin sulfate, tetracycline, streptomycin sulfate, vancomycin hydrochloride and ciprofloxacin were tested. All antibiotics were dissolved in H_2O (pH=6.5) except for ciprofloxacin and cefalexin which were dissolved in an aqueous solution of pH=1.5. *E. coli* cultures incubated with 1% of the corresponding solvent served as controls. Fingerprinting: In an *E. coli* culture of 50 ml and an OD600 of 0.6 different antibiotics in a concentration of 100 µM were incubated for 30 min. The total amount of 50 ml was collected on separate 0.22 µm filters.[6] Immediately after the disappearance of the culture medium the unwashed filters were transferred to liquid nitrogen. Intracellular metabolites were extracted by cold methanol. Footprinting: Samples for footprinting were prepared simultaneously to intracellular metabolite extraction. After separating bacteria by filtration the filtrate was stored at -20 °C until NMR measurements. NMR-based metabolic profiling: For NMR profiling, one-dimensional nuclear Overhauser enhancement spectroscopy (NOESY) spectra were acquired on a 600 MHz Bruker Avance NMR spectrometer with a 5 mm TXI probe head equipped with z-gradient. Metabolomic profiles were measured using targeted profiling in Chenomx NMR Suite 4.6.[7] After normalizing the data, PLS-DA (Partial Least Squares Discriminant Analysis, n=6) was performed on the profiles using Umetrics' SIMCA-P software. Altogether 21 metabolites were profiled in bacterial fingerprints and 20 metabolites were identified in footprints.

Results and Discussion: Six antibiotics whose mode of action targets different cellular processes (cell wall synthesis: ampicillin, cefalexin, vancomycin; protein synthesis: doxycycline, kanamycin and DNA synthesis: ciprofloxacin) were characterized by their effect on intracellular fingerprints and extracellular footprints of an *E. coli* culture. PLS-DA analysis (Fig. 1 Fingerprinting: 4 components, R2=0.79, Q2=0.68) of metabolic fingerprint profiles clearly separated intra- from extracellular antibiotic action along the first component which explained 32% of the total metabolic variation. All extracts influenced by antibiotics with intracellular targets showed decreased TCA (tricarboxylic acid cycle)- metabolites in addition to specifically elevated/decreased metabolites observed for each antibiotic (Table 1). In addition, a PLS-DA model, based on the intracellular metabolite profiles obtained for doxycycline, kanamycin, and controls, was built to predict the response of the cells to tetracycline and streptomycin which are analogs of doxycycline and kanamycin, respectively (Fig. 2). The analysis showed an agreement of 88.7 % and 76.0 % between the mode of action of streptomycin and kanamycin and between that of tetracycline and doxycycline. In contrast to the fingerprint analysis, corresponding PLS-DA footprint analysis provided distinct profiles for antibiotics with extracellular antibiotic action and inhibition of cell wall biosynthesis (Fig.1 Footprinting: 7 components, R2=0.69, Q2=0.43). In cultures with cell wall affecting antibiotics, leakage of intracellular metabolites could be verified by FACS analysis.

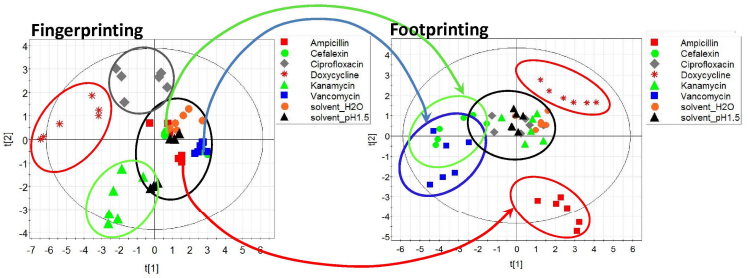

Fig.1: PLS-DA analysis of fingerprint (4 components, R2=0.79, Q2=0.68) and footprint (7 components, R2=0.69, Q2=0.43) profiles extracted from *E. coli* cultures after incubation with antibiotics with intra- (ciprofloxacin, kanamycin, doxycycline) and extracellular (ampicillin, cefalexin, vancomycin) action for 30 min.

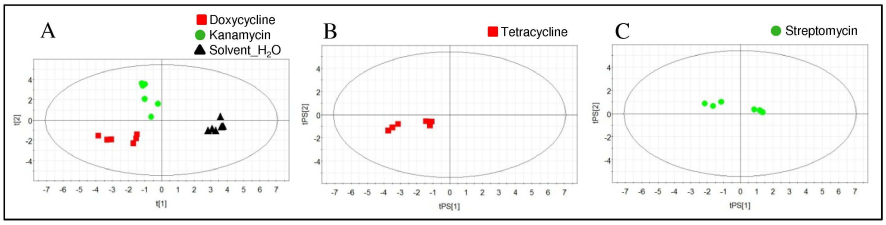

Fig.2: PLS-DA analysis of (A) fingerprints of *E. coli* cultures incubated with doxycycline, kanamycin and H_2O for 30 minutes (6 components, R2=0.99, Q2=0.90). Using this model as reference data set fingerprints of cultures incubated with (B) tetracycline and (C) streptomycin were predicted according to their mode of action. Streptomycin and tetracycline were predicted to have methods of action similar to kanamycin and doxycycline, respectively.

Table 1	doxycycline	kanamycin	ciprofloxacin
Elevated metabolites	choline, betaine, acetamide, glutamate	alanine	glutamate, betaine, glycin
Decreased metabolites	formate, ethanol	lysine, choline	-

Table 1: Significantly increased/decreased intracellular metabolite concentrations after treatment with doxycycline, kanamycin and ciprofloxacin.

Conclusion: Finger- and footprint metabolite profiles of *E. coli* extracted after treatment with different antibiotic classes can be separated based on the different antibiotic method of action and provide complementary information about intra- and extracellular processes. In addition, by using a training set of bacteria fingerprints obtained for different antibiotic classes, the mode of action of antibiotics can be predicted. Thus metabolomics may be a powerful tool in the field of integrated parallel drug research.

References: [1] JR Miller, et al. Expert Opin Drug Discov. 2010, 5(2), 145-154. [2] P Gribbon, et al. Drug Discov Today. 2005, 10(1), 17-22. [3] KM Overbye, et al. Drug Discov Today. 2005, 10(1), 45-52. [4] JC Lindon, et al. Pharm Res. 2006, 23(6), 1076-1088. [5] SC Booth, et al. Metallomics. 2011, 3(11), 1142-1152. [6] M Svensson, et al. Int J Food Microbiol. 2007, 113(2), 195-200. [7] AM Weljie, et al. Anal. Chem. 2006, 78(13), 4430-4442.