Searching for novel adjuvants of β-lactams antibiotics to contrast multidrug resistant Gram-negative bacteria

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β-lactams represent cornerstone antibiotics for the treatment of multi-drug resistant **Gram-negative bacteria** (MDR-GN), but resistance towards them is widespread and it is generally associated to the expression of **β-lactamases** [1,2]. To treat β-lactam-resistant MDR-GN, a relevant therapeutic option is the co-administration of a β-lactam and a **β-lactamase inhibitor** (BL-BLI) [3]. Unfortunately, resistance also to the most recent combination introduced in clinics (e.g., ceftazidime-avibactam) has been described [2], thus calling for the need to broaden the armamentarium of available BLIs to slow down the emergence of resistance and to support the clinical use of last generation β-lactams.

In the IN SIGNO project, we aim at discovering and developing new antibiotic adjuvants that could be used in combination with β-lactams against MDR-GN bacteria

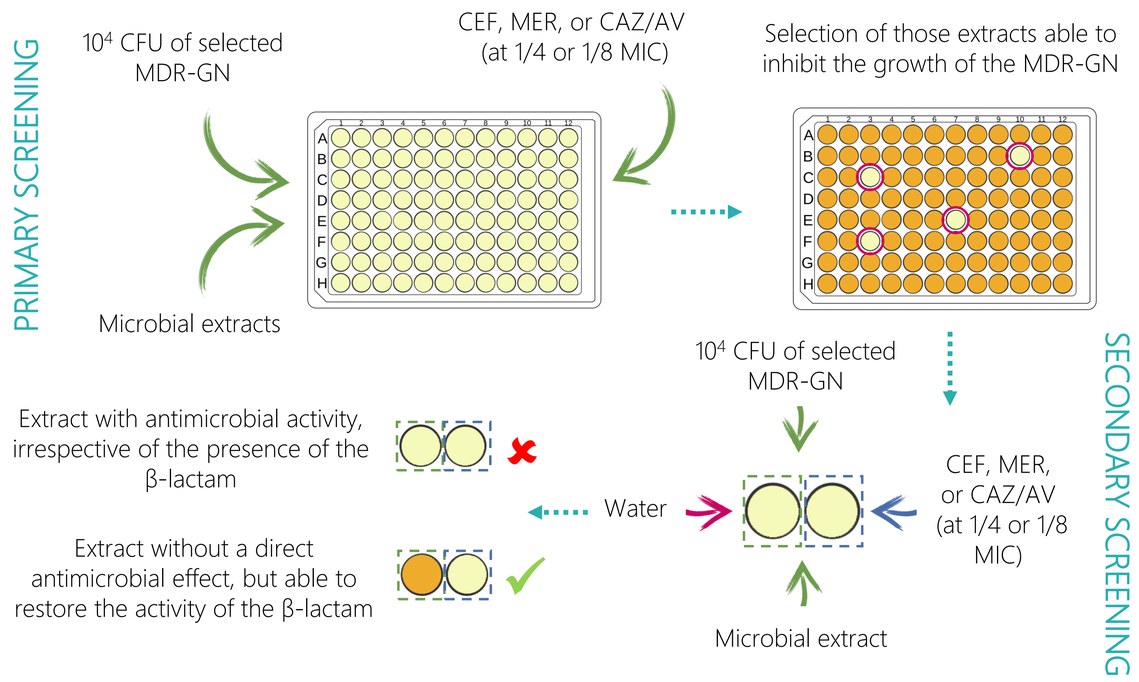
1 TARGET MODEL SELECTION

Five MDR-GN clinical isolates with different resistance profiles to β-lactams were selected and characterized. The minimal inhibitory concentration (MIC) of ceftriaxone (CEF), meropenem (MER), and ceftazidime-avibactam (CAZ/AV) toward them was estimated.

	Clinical isolate	Resistance determinant	MIC (μg/ml) CEF	MER	CAZ/AV
a)	*Escherichia coli*	CTX-M	256	≤0.25-0.5	1
b)	*Klebsiella pneumoniae*	KPC-3	512	64-128	8
c)	*Klebsiella pneumoniae*	KPC-31	64	1-2	32
d)	*Klebsiella pneumoniae*	VIM-1	1024	2-4	64
e)	*Pseudomonas aeruginosa*	VIM-2	256	32	32

2 NATURAL PRODUCT SCREENING

A 2-step, high-throughput screening assay to identify molecules able to restore the antimicrobial activity of CEF, MER, or CAZ/AV against clinical isolates (a), (b, d, e), or (c), respectively, was implemented and then used for screening ca. **39,000 crude extracts** mainly deriving from **filamentous actinomycetes**' and **filamentous fungi**'s culture broths.



>200 microbial extracts demonstrated the ability to restore the antimicrobial activity of CEF or MER against clinical isolates (a) or (b, d, e), respectively, whereas none of them succeeded in restoring the antimicrobial activity of CAZ/AV against model (c).

	Target	Antibiotic	HITs from primary screening	HITs from secondary screening
a)	*Escherichia coli* CTX-M	CEF	257	2
b)	*Klebsiella pneumoniae* KPC-3	MER	166	2
c)	*Klebsiella pneumoniae* KPC-31	CAZ/AV	90	0
d)	*Klebsiella pneumoniae* VIM-1	MER	772	173
e)	*Pseudomonas aeruginosa* VIM-2	MER	265	45

3 PURIFICATION AND CHARACTERIZATION

For the 161 producer strains of the putative **HITs** discovered from the screening, an **activity-guided purification process** was applied to isolate the bioactive compounds and proceed with their structural elucidation.

Through this process, the active peak was successfully identified in 22 active extracts: in the majority of cases, the observed bioactivities could be related to **siderophores**. Indeed, metallo-β-lactamases use zinc atoms to activate a nucleophilic water molecule that opens the β-lactam ring, being therefore susceptible to chelating agents.

All the identified compounds exhibited limited or none **antimicrobial effect** on their own. Three of them displayed none **cytotoxicity** towards rhabdomyosarcoma, HeLa, or AV3 cell lines, being therefore selected as the most promising **LEADs** to be further characterized.

Fermentation

Solid-phase extraction of supernatants and mycelial ethanolic extracts (mostly with Diaion HP-20 resin)

Chromatographic fractionation

Biological assay on all generated fractions

UV-MS analysis on active fractions

Chemical dereplication of isolated compounds

4 B-LACTAMSE PRODUCTION

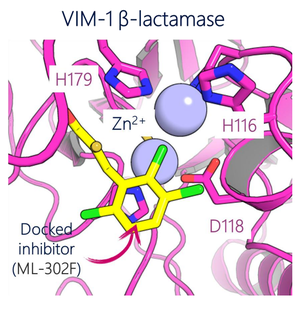
In parallel to the screening, *in silico*-optimized genes coding for the **five β-lactamases** determining the resistance phenotype of the selected clinical isolates were cloned in pET vectors and introduced into *Escherichia coli* BL21 cells.

Through expression and purification trials, optimal conditions for their production were identified: proteins were purified by affinity chromatography at >5 $mg_{prot}/l_{culture}$, with >90% purity.

Protein	Class	Purification yield ($mg_{prot}/l_{culture}$)	Purity (%)
KPC-3	Serine β-lactamase	29.6	>95%
KPC-31	Serine β-lactamase	6.3	>95%
VIM-1	Metallo β-lactamase	10.3	>90%
VIM-2	Metallo β-lactamase	9.0	>90%
CTX-M	Serine β-lactamase	9.6	>90%

Biochemical characterization of purified proteins (e.g., measurement of steady-state kinetic parameters on different potential substrates, characterization of protein stability etc.) was conducted as a pre-requisite of using them in *in vitro* assays for studying their interaction with the selected LEADs.

5 MOLECULAR DOCKING



Docking simulations were first performed with reference ligands reported in the literature, investigating software and process parameters for highlighting the interaction between β-lactamases and inhibitors in the presence of active site water molecules and metal ions. Tests with the meanwhile identified LEADs are on going.

As next steps, a further characterization of the selected molecules will be conducted, to better define their mode of action by *in vitro* and *in silico* tests, to evaluate their activity on a wider panel of MDR-GN clinical isolates, and to assess their cytotoxicity, ultimately paving the way for their possible preclinical development and future medical use.

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[3] doi: 10.1016/S0140-6736(21)02724-0.