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Commentary
Special Focus Issue – Part I: New Frontiers in Antibacterial Drug Discovery

Future Medicinal Chemistry

Chemical philanthropy: a path forward for antibiotic discovery?

"It remains to be seen if the premise adopted by the Community for Open Antimicrobial Drug Discovery will aid in reinvigorating the antibiotic pipeline."

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Antibiotic researchers, clinicians and regulators are all too familiar with the concept of antibiotic resistance, with scientific debate generating an ever-increasing number of annual publications on the topic. Despite overwhelming scientific evidence in support of an imminent 'superbug crisis' that may have catastrophic results for human health, the continued reporting of 'doomsday' messages by the media fails to convince the general public, in part due to a general misunderstanding of what antibiotic resistance actually means and how it relates to the individual. One common misconception is the belief that people, rather than the bacteria, become resistant to the antibiotics [1].

Public ignorance is a major driver of antibiotic resistance due to the significant overuse of antibiotics in both humans and animals. Concerning human use, it is ironic that clinicians, the very people who ought to know better, are often complicit in the overprescription of antibiotics [2]. On the other hand, misguided regulatory frameworks and commercial interests have led to the careless misuse of these precious drugs in agriculture, where pigs, poultry and fish are fed sub-therapeutic concentrations of last-resort antibiotics such as colistin to improve yields under intensive farming conditions [3]. If the emergence of antibiotic resistance was not already dire enough, this incredulous misuse of colistin, which represents the last line of defence against carbapenem-resistant *Enterobacteriaceae*, has led to the generation of heretofore never observed plasmid-mediated colistin resistance (*mcr-1)* in *Escherichia coli* (*E. coli*), initially in isolates derived from samples of raw meat, animals and humans in China [4], with subsequent identification from multiple sources worldwide [5–11].

Despite the vigorous rate of publications categorizing the spread of antibiotic resistance, the same cannot be said for the numbers of approved new drugs reaching the market [12], especially those that target antibiotic-resistant Gram-negative bacteria. A recent survey of investigational drugs targeting Gram-negative infections that have entered human clinical trials highlights 24 agents from 22 companies [13]. While it is encouraging to see such active company engagement, no doubt stimulated by numerous policy changes such as those made under the Obama administration [14], most of the antibiotics are improved iterations of legacy scaffolds for which there are already underlying resistance mechanisms (aminoglycosides, β-lactams, β-lactamase inhibitors, quinolones and tetracyclines). Although several novel non-β-lactam β-lactamase inhibitors also comprise the lineup (e.g., boronic acids and diazabicyclooctanes); their modes of action are not novel [13]. While there are clear benefits in making incremental improvements to compound classes that possess well-defined safety and pharmacological profiles, there remains a paucity of novel agents active

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against novel targets. Indeed, no drug with a novel mode of action against Gram-negative bacteria has been approved in the last 50 years.

There has been much debate over how to replenish the antibiotic pipeline [15]. The high attrition rates associated with the lead optimization and clinical development phases of antibiotic discovery necessitate a large number of high-quality leads from the outset. In turn, sources of significant chemical diversity are needed to generate such high-quality leads. Most antibacterial drugs in use today are derived from natural product origins. Historically, we have relied on Mother Nature to provide chemical scaffolds of sufficient complexity to enable the development of effective antibiotics optimized for target binding and bacterial penetration. Unfortunately, having mined natural product sources for over 50 years, it has become extremely challenging to identify new privileged scaffolds buried within an insurmountable background of known and/or nuisance compounds. Our once unfettered access to the rich sources of evolutionary chemical diversity that laid the foundations of the golden era of antibiotic discovery has seemingly come to an end.

With upper estimates of finding a novel secondary metabolite class sitting at a probability of around one in every 10^7 micro-organisms [16], a return to traditional natural product discovery remains a challenging task when coupled with conventional screening approaches, especially in the face of declining pharmaceutical R&D efficiency [17]. However, several new approaches to tackle these problems are gaining traction, such as interrogating 'unculturable' bacteria [18] and identifying novel targets by retro-biosynthetic analysis [19].

> "The lack of chemical diversity in modern-day synthetic compound libraries has been a contentious issue for some time."

The challenges of chemical diversity

If current technology limits our ability to further profit from Mother Nature, then the collective synthetic chemistry community provides an alternative source of molecular diversity. The pharmaceutical industry and commercial compound vendors have been dutifully amassing large screening collections full of novel chemotypes of broad utility in the drug discovery arena. Unfortunately, the vast bulk of these chemicals have been derived using high-throughput, but synthetically constrained combinatorial chemistry approaches. Technological advances in combinatorial chemistry have not been commensurate with pharmaceutical R&D productivity, and in the context of antibiotic discovery, collections derived from combinatorial efforts have been demonstrated to be ineffectual [20,21].

The lack of chemical diversity in modern-day synthetic compound libraries has been a contentious issue for some time. Man-made compound libraries cover an infinitesimally small area of infinitely large chemical space, and choosing the best regions to explore remains a significant challenge [22]. Before the advent of high-throughput screening (HTS), legacy drug discovery programs adopted a low-throughput approach, which was not driven by metrics inherent in an HTS discovery model. Comparatively small numbers of compounds possessing greater relative complexity were often made using a wealth of chemical reactions that did not benefit from the breadth of commercially available reagents of the modern era. Contrary to this approach, the modern day practice of outsourcing synthetic chemistry combined with the push to produce analogs using predictable chemical methodology has stifled the production of highly diverse screening libraries. A recent comparison [23] of past (1984) and present (2014) chemical reactions favored in medicinal chemistry versus natural products total synthesis revealed a startling observation: in the context of the types of reactions used to make molecules, the landscape of contemporary medicinal chemistry is essentially the same as it was 30 years ago, with a heavy bias toward amide bond formation, Suzuki-Miyaura coupling and S_NAr reactions. The poor uptake of modern synthetic innovations in industrial medicinal chemistry has led to compound collections rich in nitrogen but deficient in both chiral centers and oxygen atoms, resulting in a preponderance of lipophilic, planar scaffolds often overpopulated with a limited number of molecular shapes [24]. Such trends are at odds with the more polar chemistry space occupied by antibiotics [25], molecules that typically possess dense functionality and skeletal diversity, suggesting a limited capacity of *de novo* designed compound collections to modulate diverse binding targets [26]. Indeed, the common practice of curating libraries for 'rule of five' [27] compliance often selects for rod-like shaped molecules that preferentially bind to pocket and internal binding sites [28]. Molecules with structural and physicochemical characteristics beyond the 'rule of five' preferentially adopt disk- and sphere-like shapes, which increases the possibility of binding to more topographically difficult sites comprised of open, flat and grooved surfaces [28].

Two recent retrospective analyses of the antibiotic discovery efforts at Glaxo-SmithKline (GSK) and AstraZeneca highlight the above points in the context of antibiotic lead finding. Tommasi *et al.* [20] described the outcome of the discovery efforts from AstraZen-

eca's target-based and phenotypic antibacterial screening efforts between 2001 and 2010. Their commercial focus during this time was to develop agents with broad-spectrum activity against both Gram-positive and Gram-negative organisms. Notably, little difficulty was encountered in identifying hits against most targets during their HTS biochemical screens. However, by their own admission, their hit-to-lead triage strategy to prioritize synthetically tractable candidates devoid of risky chemical features may have led to the premature elimination of viable leads. Biochemical potency was often inextricably linked to hydrophobicity, resulting in lead compounds that were approximately 3–4 log units (clogP) more hydrophobic than typical antibacterial agents. Such compounds could not be engineered to display whole cell activity in Gram-negative bacteria, emphasizing the disconnect between the HTS active starting point and the physicochemical space necessary for efficient cell permeation.

In 2007, the team at GSK reflected on their antibacterial program between 1995 and 2001 [21]. Adopting a strong genomics/target focused approach, GSK devoted significant resources to validate numerous biochemical targets. Upward of 500,000 compounds from the SmithKline Beecham compound collection were screened over 67 HTS runs using a target-based approach, and three HTS runs using whole-cell screens. Although this led to five lead candidates against five distinct targets, none could be progressed to developmental candidate stage. Compounds identified during the biochemical screens lacked whole-cell activity following lead optimization, whereas those identified during the phenotypic screens possessed only Gram-positive activity, and were comprised predominantly of lipophilic nonspecific membrane-active agents. The team concluded that a paucity of biologically relevant chemical diversity in their screening set was a major contributor to the poor outcome.

Chemical philanthropy: CO-ADD

To address the issue of limited access to chemical diversity for generation of novel antimicrobial drug leads, we launched the Community for Open Antimicrobial Drug Discovery (CO-ADD) in early 2015 [29–31]. CO-ADD operates as an open-access facility within the University of Queensland (UQ), led by an academic research team with antibiotic R&D expertise. With financial support from both UQ and the Wellcome Trust, CO-ADD has two interdependent aims – to search for antimicrobial activity among compounds sourced from chemists anywhere in the world, and to generate a dataset from which it might be possible to examine the influence of chemotype on antimicrobial activity, cell penetration and drug efflux. Such data, used in combination with emerging methods to assay intracelullar drug penetration [32], might assist in the formulation of empirical rules to guide antibiotic design.

So, how does CO-ADD propose to address the chemical diversity problem? We believe that the broader chemical community may hold at least part of the answer. Globally, chemists are creating thousands of molecules each day, often constrained only by their imaginations. Without the commercial pressures often associated with drug discovery programs, academic chemists have a virtually unlimited synthetic toolbox; molecules with varying degrees of molecular complexity are being made to test new methodologies, address hypotheses concerning disease targets or to reach a total synthesis end game. Such molecules, once tested or analyzed, usually end up being stashed away in vials alongside the intermediates used to produce them. As the years go by, their final resting place is usually on a shelf in some freezer, and through the ebb and flow of projects in the research group, it is not uncommon for such compounds to eventually be forgotten or discarded. Importantly, very few of these compounds will have ever been tested for antimicrobial activity, as many groups have never considered this possibility, while those who may look to repurpose their compounds in some capacity may not have the resources and/or necessary collaborations to explore this option. It is our contention that such compound collections, unconstrained by metrics that drive commercial drug discovery programs, may have high-quality antimicrobial hits residing amongst them. Indeed, approximately one third [29] of the approximately 100 million unique compounds deposited in the Chemical Abstracts Service registry reside within the physicochemical space of antibacterial compounds (MW <1200 Da and log P between -10 and 2) [25].

In light of this, we ask chemists engaged in synthetic campaigns to adopt the mindset of setting aside 1–2 mg of pure compound for submission to CO-ADD for free antimicrobial testing as part of their routine workflow. The compound must be chemically stable and soluble in either water or DMSO, and there is no restriction on the number of compounds one can submit. A single contribution from one individual may seem insignificant, but with the collective efforts of the global chemistry community, the bigger picture becomes apparent as we strive to build the world's first antimicrobial-focused open access database of unique molecules tested for antimicrobial activity under standardized conditions. The endeavor will generate a powerful knowledge repository of structure–activity and structure–toxicity data that will be freely available to the worldwide research community. Thus, chemists

engaged in R&D not necessarily related to antibiotic discovery may be able to make a small but nonetheless significant contribution toward the discovery of new antibiotic molecules.

Whilst global compound collections and open framework drug discovery initiatives are not new, intellectual property, conflict of interest and licensing policies often stifle collaborative innovation and frustrate early adopters. CO-ADD differentiates itself from existing initiatives in several important ways: its core focus is to find novel starting points for antimicrobial discovery; CO-ADD offers a truly open-access approach to compound screening – compounds are screened free-of-charge, are not preselected on the basis of 'lead-like' filtering criteria, and are accepted from anywhere in the world; there is no encumbrance on intellectual property; the provider of the compound retains all rights to the compound, assay results and IP; for the first time, the collective screening results for both active and inactive compounds will be made publicly available in a central repository to assist researchers in understanding what physicochemical properties are important for antimicrobial development (CO-ADD participants have 18 months to patent and develop their compound before they are asked to make structures and results available to the open-access database). We emphasize that curation of existing literature datasets to obtain reliable standardized data for comparison is highly challenging due to the multitude of bacterial strains and assay conditions used across different studies.

So how does it work? Compounds submitted to CO-ADD undergo a primary screen at a single concentration (32 μg/ml) against a select panel of key ESKAPE bacterial pathogens (*Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* [MRSA]), and the two fungal pathogens *Cryptococcus neoformans* and *Candida albicans*. Membrane-deficient and efflux pump impaired *E. coli* mutants are also screened to provide additional information pertaining to bacterial cell penetration and efflux. Notably, screening is conducted using well-defined Clinical and Laboratory Standards Institute (CLSI)-compliant protocols with standard ATCC reference strains to enable the direct comparison of thousands of screening results independent of compound source. Any actives identified from the primary screen are then funneled into a hit confirmation cascade to rule out nuisance compounds by way of dose response antimicrobial assays, QC analysis and cytotoxicity/critical micelle concentration/hemolysis assays. Results from the initial screening and hit confirmation steps are then disclosed to the submitter for their evaluation. Promising results at this stage can trigger the hit validation cascade if sufficient material is available, which will enable further testing against a broader panel of multidrug-resistant clinical isolates, including assessment in the presence/absence of serum and lung surfactant. Finally, an initial investigation into drug-like properties, including microsomal and plasma stability, and drug–plasma protein binding will deliver a data package suitable to assess the suitability of a candidate for further chemistry optimization. To rule out singleton hits, promising compounds are resynthesized alongside several structural analogs.

Community response

Since its launch in February 2015, CO-ADD has devoted significant resources toward raising awareness to the plight of antibiotic resistance. The program is gaining traction and support through global participation at scientific conferences in Europe, Russia, Asia-Pacific and the USA, and CO-ADD team visits to chemistry departments at numerous academic institutions. Community engagement has been encouraging, with 104 groups from 34 countries participating in the scheme at the time of writing, each submitting anywhere from 10 to 150,000 compounds for screening. By reaching out to the chemical community through organizations such as the Royal Society of Chemistry, the American Chemical Society, the Royal Australian Chemical Institute, Gesellschaft Deutscher Chemiker and MedChemNet, we are looking to engage individuals, laboratories and institutions and build a network of collaborators that will collegially support the arduous process of antibiotic discovery.

It remains to be seen if the premise adopted by CO-ADD will aid in reinvigorating the antibiotic pipeline. With global strategies currently failing to provide us with a much needed arsenal of new antibiotic drug candidates, we hope that the open access approach offered by CO-ADD will encourage the chemical community to join us in a long-term internationally coordinated approach toward antibiotic discovery.

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Special Report
Special Focus Issue – Part I: New Frontiers in Antibacterial Drug Discovery

Future Medicinal Chemistry

Polyamine derivatives: a revival of an old neglected scaffold to fight resistant Gram-negative bacteria?

Emergence of multidrug-resistant pathogens was responsible for microbial infections and inefficacy of numerous antimicrobial therapies has induced a need for the research of new classes of antibiotics. In this review, we will focus our interest toward the biological properties of polyamino antimicrobial agents.

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Each year in the USA, at least 2 million people become infected with bacteria that are resistant to antibiotics and at least 23,000 people die annually as a direct result of these infections. The introduction in the Mid-20th century of efficient antibiotic therapies for infectious diseases has completely modified clinical practices in the development of life-threatening conditions leading to reduce the incidence of death resulting from bacterial infections. However, the rise of antibiotic resistance since few decades has resulted in a very pressing need for the discovery of novel antibiotics or treatment strategies [1]. In this context, numerous active avenues of research ongoing to develop the next generation of antibacterial drugs are under current investigations such as the combination of two active antibacterial agents into one hybrid compound or synthetic peptide mimics [2].

Some strains have become resistant to practically all of the commonly available agents. A notorious case is the methicillin-resistant *Staphylococcus aureus*, which is resistant not only to methicillin but usually also to aminoglycosides, macrolides and cyclines. Such strains are also resistant to disinfectants, and can act as a major source of hospital-acquired infections. An even more serious threat may be the emergence of multidrug-resistant (MDR) Gram-negative pathogens that are a global public health concern as therapeutic options for treating such infections are dwindling. Thus, multidrug resistance in bacteria occurs by the presence of plasmids or transposons, of genes, with each coding for resistance to a specific agent and/or by the action of multidrug efflux pumps, each of which can pump out more than one drug type.

Furthermore, the emergence of 'pan-resistant' Gram-negative strains, notably those belonging to *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, occurred more recently, after most major pharmaceutical companies stopped the development of new antibacterial agents. Hence, there are almost no agents that could be used against these strains, in which an outer membrane barrier of low permeability is combined with multitudes of specific resistance mechanisms.

On the basis of such observations, there is a need for the development of new strategies and a revival for the use of neglected polyamino derivatives has emerged as antimicrobial agents able to fight resis-

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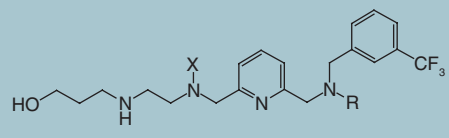
Table 1. Minimum inhibition concentrations of antibiotics to *P. aeruginosa* PAO1 in the presence of polyamines.

Antibiotic	Without compound	Spd	Spn	Put	Cad
Ampicilline	>1024	64	64	128	128
Aztreonam	4	0.5	0.5	1	0.5
Ceftazidine	2	0.5	0.5	1	0.5
Chloramphenicol	128	32	32	64	64
Nalidixic acid	128	64	64	64	64
Erythromycine	128	128	64	128	128

Compound concentrations were as follows: 20 mM Spd, Put and Cad; 1 mM Spn.
Cad: Cadaverine; MIC: Minimum inhibition concentration; Put: Putrescine; Spd: Spermidine; Spn: Spermine.
Data taken with permission from [15].

tant Gram-negative bacteria. Polyamines are small aliphatic hydrocarbon molecules with quaternary nitrogen groups that have a net positive charge at physiological pH. During the 60s and 70s polyaminated molecules were identified in all forms of life such as bacteria, fungi, plants and all types of eukaryotic cells. They were described to be critical for all types of cellular proliferation by determining the metabolic pathways of synthesis and degradation. It is well known that polyamines are ubiquitous compounds with different properties such as growth cell, cellular reparation, gene transcription, protein and nucleic acid synthesis [3]. They have been widely studied for their implication in cancer [4] since among all the envisioned approaches for preventing cancer development, a reduction of intracellular

Table 2. Structures and antimicrobial activities of meta-substituted benzyl pyridinopolyamine derivatives 1–12.



X = $CH_2C_6H_4CH_3$-m

1, R = R_1; **3**, R = R_3; **5**, R = R_5

2, R = R_2; **4**, R = R_4; **6**, R = R_6

X = $CH_2C_6H_4CF_3$-m

7, R = R_1; **9**, R = R_3; **11**, R = R_5

8, R = R_2; **10**, R = R_4; **12**, R = R_6

$R_1 = CH_2Ph$ $R_4 = CH_2C_6H_4NO_2$-m

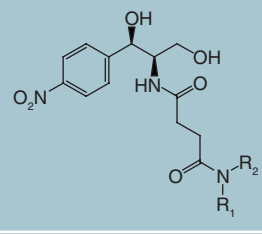
$R_2 = CH_2C_6H_4F$-m $R_5 = CH_2C_6H_4COOCH_3$-m

$R_3 = CH_2C_6H_4CH_3$-m $R_6 = CH_2C_6H_4CF_3$-m

Compound	*S. aureus*	*E. faecalis*	*E. coli*	*S. pyogenes*	*P. aeruginosa*	*P. vulgaris*	*K. pneumoniae*	*C. albicans*
1	3–6	3–6	12	1–5	>100	>100	6–12	<12.5
2	3–6	3–6	>100	1–5	>100	>100	12–25	<12.5
3	3–6	3–6	>100	1–5	>100	>100	6–12	<12.5
4	3–6	3–6	>100	1–5	>100	>100	>100	25
5	6–12	3–6	>100	1–5	>100	>100	>100	<12.5
6	6–12	3–6	>100	1–5	>100	>100	>100	>100
7	12–25	6–12	>100	1–5	>100	>100	25–50	>100
8	6–12	3–6	>100	3–6	>100	>100	>100	>100
9	6–12	3–6	>100	1–3	>100	>100	50–100	>100
10	3–6	3–6	>100	1–3	>100	>100	>100	>100
11	6–12	3–6	>100	1–3	>100	>100	>100	>100
12	3–6	6–12	>100	2.5–5	>100	>100	>100	>100

All values are minimum inhibitory concentrations (μg/ml).
Data taken with permission from [17].

Table 3. Determination of IC_{50} for chloramphenicol and its polyamino parent derivatives against wild-type and mutant *Staphylococcus aureus* and *Escherichia coli*.



13: R_1 = H, R_2 = $(CH_2)_4NH_2$

14: R_1 = H, R_2 = $(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$

15: R_1 = $(CH_2)_3NH_2$, R_2 = $(CH_2)_4NH_2$

16: R_1 = $(CH_2)_3NH_2$, R_2 = $(CH_2)_4N(CH_2Ph)_2$

17: R_1 = H, R_2 = $(CH_2)_3NH(CH_2)_4N(CH_2Ph)_2$

Compound	MRSA (GRE2272)	*S. aureus* (WT)	*E. coli* (A2058G)	*E. coli* (WT)	*E. coli* (ΔtolC)
CAM	8.0	3.1	15.5	6.2	2.0
13	>200	>200	>200	>200	>200
14	>200	45.3	>100	>100	>100
15	>100	12.7	>150	>150	>100
16	7	4.7	9.4	9.4	19.0
17	>100	13.7	32.3	35.5	42.5

All values are IC_{50} (μM).
WT: Wild-type.
Data taken with permission from [18].

polyamines synthesis has been suggested. Structurally, polyamines possess positively charged nitrogen atoms and can serve as electrostatic bridges between negative phosphate charges and others polyanions such as DNA and RNA [3] leading to chromatine and DNA modification occurring preferentially in polyamine-depleted cells. Finally, polyamines play a role in cell proliferation by interacting with phosphoprotein p53 involved in different gene regulation. It is reported that polyamine toxicity at high concentration may be the result of formation of high amounts of hydrogen peroxide during interconversion steps and consequently due to the oxidative stress [3]. Furthermore, polyamine cytotoxicity appears highly correlated with the concentration of formed acrolein [5] in the plasma of chronic renal failure patient. In this case, the level of spermine and spermidine decreased whereas the acrolein concentration increased [6]. Thus the apparent critical influence of polyamines on cell development and survival and their recognition by the polyamine transporters have both led to polyamines being increasingly considered for the design of a range of chemotherapeutic agents [7].

On the other hand, it has been widely demonstrated that polyamines can act as endogenous modulators of outer membrane permeability [8] of bacteria inducing resistance to cationic peptide, aminoglycoside or quinolone antibiotics [9].

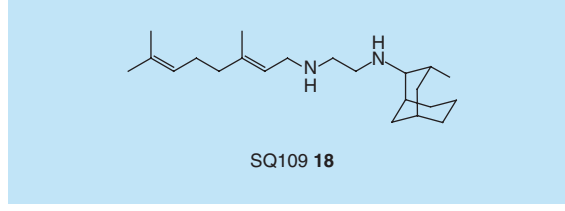
Polyamines are old polycationic molecules widely distributed in nature described for very first time in 1677 in seminal fluid [10]. It has been widely demonstrated that some of them such as cadaverine [11] or spermine [8] could decrease bacteria outer membrane permeability by being natural regulators of porin activity and subsequently reduce bacterial susceptibility to antibiotic treatments [12–14].

Surprisingly, it has been found that polyamines at millimolar levels can increase the susceptibility of *P. aeruginosa* to a variety of antibiotics (Table 1) [15], whereas Vaara *et al.* by using submillimolar concentrations have observed a discrepant outcome [16]. It has appeared that polyamines might be potentially useful in antipseudomonal therapies by increasing the effectiveness of numerous β-lactam antibiotics.

Thus, these results suggested that the development of a new approach involving polyamino derivatives as potent either antimicrobial agents or chemosensitizers of ineffective antibiotics against MDR bacteria.

Development of new polyamino antimicrobial agents

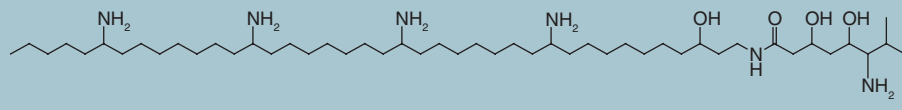
Different polyamino antimicrobial agents have been designed and we can classify these derivatives according two major classes belonging to a steroidal or a non-steroidal family.



SQ109 **18**

Figure 1. Structure of derivative SQ109 18.

Table 4. Structure and antimicrobial activities of zeamine 19.



Zeamine **19**

Strain	*S. aureus* ATCC25531	*B. cereus* XJ8	*E. coli* CFT073	*B. cepacia* H111	*P. aeruginosa* PAO1	*K. pneumoniae*
19	0.3	3	3	50	5	6

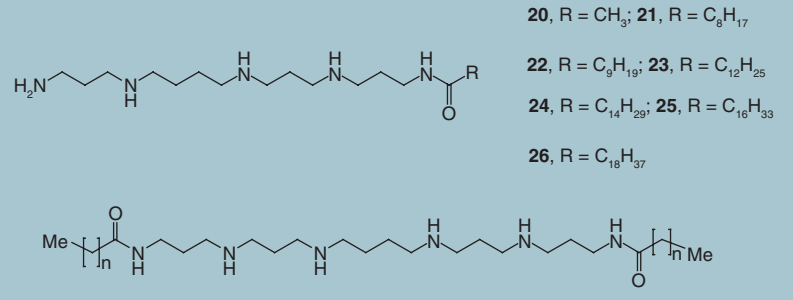
All values are minimum inhibitory concentrations (μg/ml).
Data taken with permission from [21].

Nonsteroidal derivatives

In 1998, Cook *et al.* have prepared a 1638-member meta-substituted benzyl pyridinopolyamine library by solution-phase chemistry. Twelve compounds **1–12** exhibit potent, highly selective activity against Gram-positive bacteria over Gram-negative bacteria and very high specificity for bacteria compared with the fungus *Candida albicans*. Thus, *Streptococcus pyogenes*, *S. aureus* and *Enterococcus faecalis* were inhibited at MICs of 1–12 μM, whereas MICs for *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris* and *P. aeruginosa* were >100 μM. It clearly appeared that functional groups in the meta-positions of the benzyl functionality set do indeed provide sufficient differentiating diversity to allow biological activities to be separated and identified from a library by iterative and positional scanning deconvolution processes (Table 2) [17].

On the other hand, a series of chloramphenicol (CAM) amides with polyamines **13–17** were recently synthesized either by direct attachment of the PA chain on the 2-aminopropane-1,3-diol backbone of CAM, previously oxidized selectively at its primary hydroxyl group, or from chloramphenicol base (CLB) through acylation with succinic or phthalic anhydride and finally coupling with a PA. In this context, conjugates **16** and **17** possessing a dibenzylated spermidine moiety through the succinate linker were the most potent antibacterial agents against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacterial strains (Table 3) [18].

Table 5. Structure and antimicrobial activities of mono- and bis-acyl polyamines 20–32.



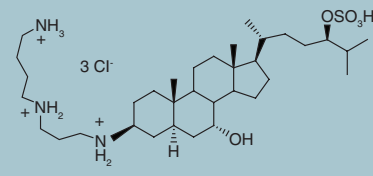
20, R = CH_3; **21**, R = C_8H_{17}
22, R = C_9H_{19}; **23**, R = $C_{12}H_{25}$
24, R = $C_{14}H_{29}$; **25**, R = $C_{16}H_{33}$
26, R = $C_{18}H_{37}$

27, n = 7; **28**, n = 8; **29**, n = 10;
30, n = 13; **31**, n = 15; **32**, n = 17

Compound	*S. aureus* (WT)	*E. coli* (A2058G)	Compound	*S. aureus* (WT)	*E. coli* (A2058G)
20	250	62.5	**27**	15.6	31.25
21	125	62.5	**28**	3.9	31.25
22	62.5	62.5	**29**	15.6	31.25
23	15.6	31.25	**30**	250	62.5
24	15.6	31.25	**31**	250	62.5
25	15.6	62.5	**32**	125	62.5
26	15.6	62.5			

All values are minimum inhibitory concentrations (μM).
WT: Wild-type.
Data taken with permission from [22].

Table 6. Antibacterial activities of squalamine 33.



Squalamine **33**

Compound	Gram-positive bacteria			Gram-negative bacteria			
	S. aureus	*S. pneumoniae*	*E. faecalis*	*E. coli*	*P. aeruginosa*	*K. pneumoniae*	*E. aerogenes*
33	2–8	32	–	8	2–8	8	32

Values are minimum inhibitory concentrations (mg/l).
Data taken with permission from [23].

Among infectious diseases, tuberculosis still remains one of the leading single agent killer in the world with around 2 million of deaths each year. Polyamino derivatives N-geranyl-N′-(2-adamantyl) ethane-1,2-diamine SQ109 **18** (Figure 1), a second-generation agent from the first-line drug ethambutol demonstrated interesting activities against both *M. tuberculosis* and *M. smegmatis* with MICs of 0.5 and 2 μg/ml, respectively [19].

Nevertheless, the bioavailability of this product remains low limiting its development and therapeutical use [20].

In 2010, Wu *et al.* isolated from the bacterial pathogen *Dickeya Zeae* strain DZ1 a new antibacterial compound namely zeamine **19**. Of peculiar note is that numerous MDR bacteria such as *P. aeruginosa* and *B. cenocepacia* are susceptible to zeamine with excellent to moderate MIC values varying from 0.3 to 50 μg/ml depending on the nature of the considered bacterial strain (Table 4) [21].

Finally, homologous series of mono- and bis-acyl polyamines **20–32** with varying acyl chain lengths were also designed and presented moderate MICs against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacterial strains (Table 5) [22].

Steroidal derivatives

On the other hand, numerous compounds possessing a sterol core were identified to possess interesting antibacterial activities. Among them, Squalamine **33**, a natural polyaminosterol derivative isolated from the shark *Squalus acanthias* was reported to be active against a large panel of micro-organisms with MICs ranging from 1 to 8 μg/ml against Gram-positive and Gram-negative bacteria (Table 6) [23].

On the basis of such results, the synthesis of numerous derivatives **34–37** from stigmasterol was achieved by Shu *et al.* (Figure 2) demonstrating that 3β analogs exhibit better activity than 3α ones [24]. From his part,

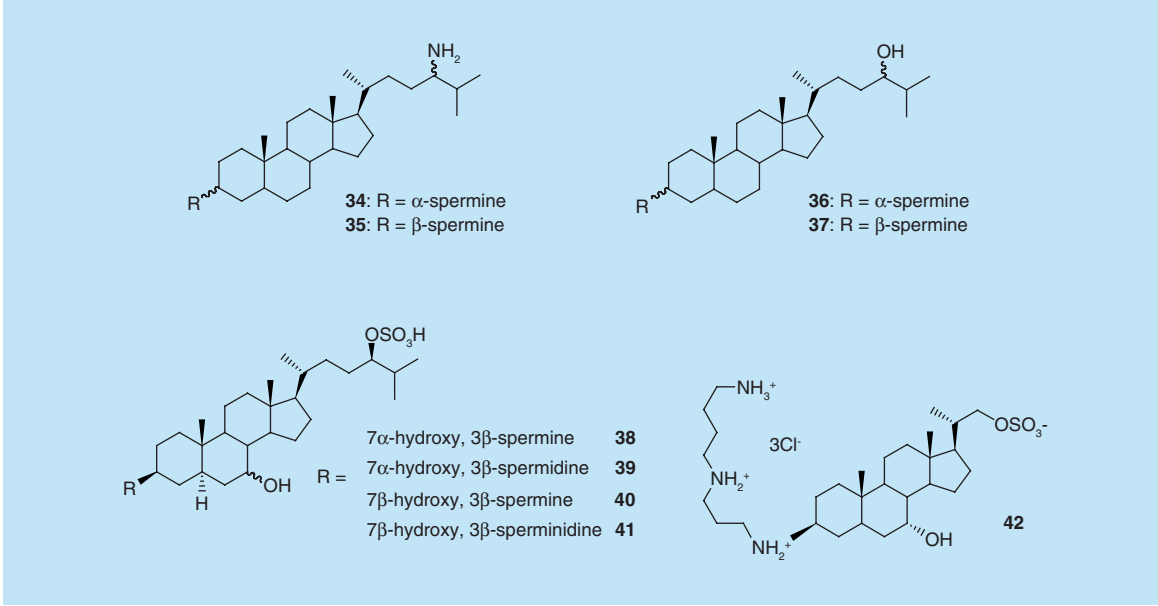


Figure 2. Structures of polyaminosterol derivatives 34–42.

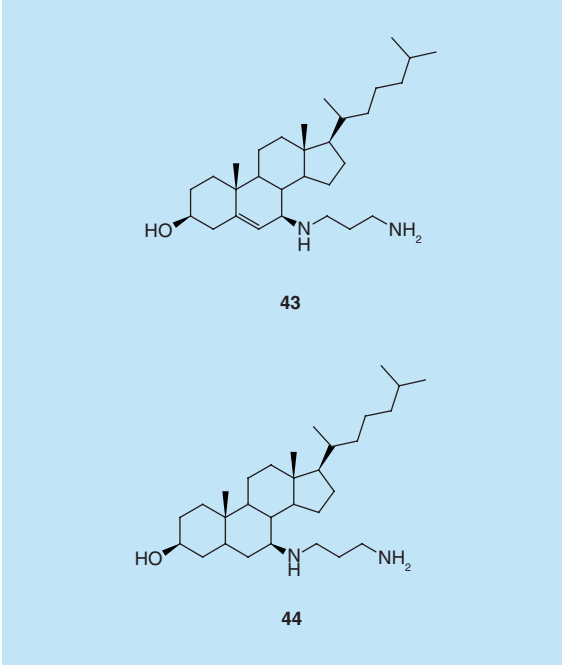


Figure 3. Structure of 7-polyaminosterol derivatives 43–44.

Selinsky *et al.* reported that squalamine analogs **38–41** differing in the identity of the polyamine attached at C3 of the sterol, and the stereochemistry of a hydroxyl substituent at C7 possessed different antimicrobial activities [25]. Thus, analogs with a tetra-ammonium spermine polyamine appear to be somewhat more active than analogs with a shorter trisammonium spermidine polyamine, and analogs with an axial (α) hydroxyl substituent at C7 are more active than analogs with the corresponding equatorial (β) hydroxyl one. Otherwise, 7β-OH spermine analog is the most active compound against *E. coli*, but the least effective against *P. aeruginosa* (MICs varying from 1 to 32 μg/ml, respectively).

On the other hand, Khan *et al.* reported the synthesis of a series of 3β-hydroxy-7α-amino 23,24-bisnor-5α-cholan-22-ol derivatives such as compound **42** which demonstrates a good activity against Gram-positive bacteria (MIC values 1.6–25 μg/ml) with respect to Gram negative ones (MIC value 6.3 to >100 μg/ml) [26,27]. Similar results were encountered by Kim *et al.* by using 3-polyamino-23,24-bisnorcholane derivatives [28].

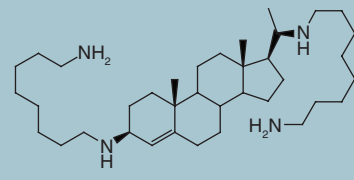
Amino deoxycholic acid derivatives were also reported to be active against numerous bacteria and even against vancomycin- and methicillin-resistant strains suggesting a high correlation between the cationic charge of the polyamine chain group and the biological activity [29]. Recently, numerous 3 and 7-polyaminosterol squalamine analogs such as **43–44** (Figure 3) were synthesized and demonstrated good activities against both Gram-positive and Gram-negative bacterial strains with MICs varying from 2.5 to 10 μg/ml [30,31] even against MDR strains recovered from cystic fibrosis patients (137 strains) [32].

In the case of Gram-positive bacteria, Alhanout *et al.* demonstrated that the activity of squalamine or its parent derivatives is due to a strong depolarization of the membrane with drained cytoplasmic content suggesting a 'detergent-like' mechanism [33]. On the opposite, in the case of Gram-negative bacteria these derivatives are able to disturb the membrane integrity of Gram-negative bacteria by interaction with negative charges of phosphate groups of the lipopolysaccharide (LPS) at the surface of the outer membrane [34].

On the basis of such a mechanism, 3,20-Amino- and polyaminosteroid analogs of squalamine such as derivative **45** were synthesized and evaluated for their *in vitro* antimicrobial properties against reference and clinical bacterial strains exhibiting MICs ranging from 2.5 to 40 μg/ml (Table 7) [35].

Antiseptics and disinfectants are widely used in hospitals for materials disinfection in order to avoid nosocomial infections [36]. Thus, medical devices with long residence times such as catheter were susceptible to bacterial biofilm colonization and may be a source of infection. In this context, soluble squalamine tablets have been developed for the rapid disin-

Table 7. Antibacterial activities of the 3,20-polyaminosterol derivative 45.

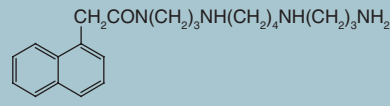


45

Compound	*S. aureus*	*E. coli*	*P. aeruginosa*	*I. limosus*	*B. cepacia*
45	2.5	5	2.5	10–20	>40

All values are minimum inhibitory concentrations (ml).
Data taken with permission from [35].

Table 8. Chemical structure of naphtylacetylspermine 46 and methoctramine 47 and their use as chemosensitizers against *Escherichia coli*.



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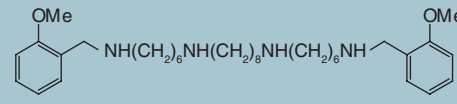


47

Antibiotic	Used concentrations (µg/ml) of polyamines 46 and 47												
	None	Naphtylacetylspermine 46								Méthoctramine 47			
None		1	2	4	8	16	32	64	128	1	2	4	8
Novobiocine	128	128	64	32	16	8	4	1	0.5	128	128	32	16
Erythromycine	64	64	64	64	31	16	8	4	4	64	64	32	16

All values are minimum inhibitory concentrations (µg/ml).
Data taken with permission from [37].

fection of home nebulizers of cystic fibrosis patients. Thus, 0.5 g/l squalamine reduced the levels of viable *S. aureus* and *P. aeruginosa* by 5 log10 and the level of viable *C. albicans* by 4 log10 after 20 min.

Development of polyamino chemosensitizers for antibiotic activity enhancement

One of the first studies was realized by Yasuda *et al.* in 2004 dealing with the synthesis and use of naphtylacetylspermine **46** and methoctramine **47** as chemosensitizer agents increasing *E. coli* membrane permeability. No intrinsic antimicrobial properties were encountered for these compounds but they are able to highly potentiate novobiocine or erythromycin antimicrobial activities (Table 8). It has been suggested that these compounds could alter membrane integrity by displacing divalent cations leading to an enhancement of noviobiocine and eryhtromycine entrance [37].

Otherwise, due to its previously described properties, an approach using squalamine as a chemosensitizer agent has been envisioned against resistant strains by using it at 1/5 and 1/10 of its MIC value to enhance significantly the activity of numerous antibiotics against isogenic multidrug *E. coli* AG100 and AG100Atet strains (Table 9) [38].

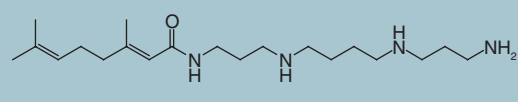
Such a result appears to be of great interest for the development of new drug combinations against MDR bacteria. Due to all of these considerations, LPS assembly became recently an interesting target due to the fact that its disruption could enhance the entrance of antibiotics through Gram-negative outer membranes. Thus, geraniol, a natural monoterpene encountered in many plant extracts, was demon-

Table 9. Effect of subinhibitory concentrations of squalamine 33 on the antibiotic susceptibility of various Gram-negative bacteria.

Strains	Addition	CHL	CIP	TET	FEP	ERY
	MIC (µg/ml)					
E. coli AG100	0	8	0.25	2	0.5	512
	+1/5 Sq	0.5	0.03	0.125	0.06	256
	+1/10 Sq	1	0.015	0.25	0.12	256
E. coli AG100A (acrAB-)	0	2	0.06	2	0.5	128
	+1/5 Sq	1	0.06	0.12	0.12	64
	+1/10 Sq	2	0.03	0.12	0.12	64
E. coli AG100tet (acrAB-, over-producing other pumps)	0	16	1	64	1	256
	+1/5 Sq	2	0.03	8	0.12	256
	+1/10 Sq	4	0.03	16	0.25	128

Sq 33 was used at 1/10 and 1/5 of its MIC determined for each strain.
CHL: Chloramphenicol; CIP: Ciprofloxacin; ERY: Erythromycin; FEP: Cefepime; MIC: Minimum inhibition concentration; Sq: Squalamine; TET: Tetracycline.
Data taken with permission from [38].

Table 10. Decrease of chloramphenicol and nalidixic acid resistance in the presence of compounds 48–50.



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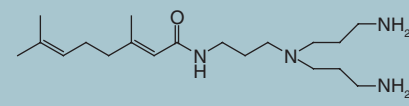

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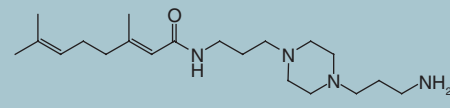


50

Compound	*Enterobacter*			*Salmonella*		
	Concentration (μM)	MIC ratio		Concentration (μM)	MIC ratio	
		CHL†	NAL†		CHL†	NAL†
PAβN	38	16	8	38	8	64
48	31	16	4	31	8	32
49	62.5	8	8–16	125	16	64
50	250	8	4	250	8	64

†MIC ratio is determined as the MIC of the antibiotic alone for each strain (i.e., chloramphenicol or nalidixic acid) to the MIC of the same antibiotic in the presence of compounds **48–50**. A ratio up to 1 indicates an improvement of the activity of the antibiotic in the presence of the considered compound.
MIC: Minimum inhibition concentration.

strated to act in a synergistic manner with several antibiotics against Gram-negative bacterial species. Numerous geraniol derivatives including geranylamine and polyaminogeranic acid molecules **48–50** were prepared and successfully investigated as chemosensitizers of chloramphenicol and nalidixic acid against *Enterobacter* and *Salmonella* strains (Table 10). It has been also demonstrated that they can alter the activity of a drug transporter and inhibit the major Enterobacteriaceae efflux pump, AcrAB-TolC [39].

In this context, another original chemical strategy was developed to prepare non cytotoxic ianthelliformisamine derivatives **51–56** which dramatically affected the antibiotic susceptibility of *E. aerogenes*, *P. aeruginosa* and *K. pneumoniae* MDR strains (Table 11) [40].

This efficiency was correlated with the inhibition of a dye transport, suggesting an action of these molecules on the activity of drug transporters. Due to their low cytotoxicity these kind of molecules could open the way for the development of new therapeutical strategies.

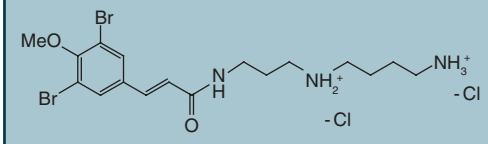
Conclusion

It clearly appears that even if polyamines are essential for life and are widely involved for growth cells their particular structure confers them the possibility to lead to the emergence of a new class of antimicrobial agents against MDR pathogens and to become a potent last-line therapeutic drug in the future.

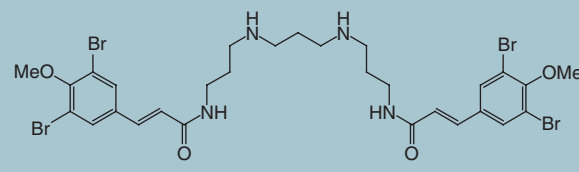
Future perspective

During the last decades, the search and commercial development of new antibiotics did not follow the rhythm of emergence of MDR bacteria. An alternative to this strategy is the finding of active molecules (that we will call antibiotic potentiators or chemosensitizers), preferably with a weak antibiotic activity and that in combination with antibiotics are able to enhance or synergize the antimicrobial activity of the latter. Antibiotic adjuvants can function either by reversing resistance mechanisms in naturally sensitive pathogens or by sensitizing intrinsic resistant strains. Thus, the use of antibiotic adjuvants has two beneficial outcomes: enhancement of the antimicrobial effect and reduction of the occurrence of mutations that results in resistance. In this context and as underlined in this review the search for molecules such as polyamine derivatives which act by membrane depolarization and/or integrity membrane disruption could constitute an efficient alternative since their mechanism of action may significantly reduce the development of resistance. Finally, we can envision that the continuous advances in the development of new and potent high-throughput technologies will definitively allow the discovery of new compounds with antibiotic adjuvant activity which is a less expensive alternative to the problem of multidrug resistance.

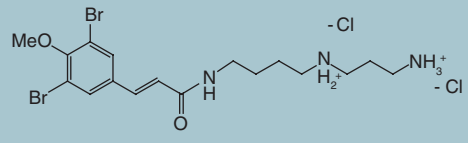
Table 11. Concentration of Ianthelliformisamine derivatives 51–56 necessary to restore doxycycline activity (2 μg/ml) against Ea289, PAO1 and KPC2 ST258 Gram-negative bacterial strains.



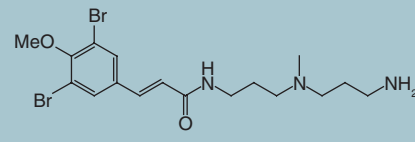
Ianthelliformisamine B 51



Ianthelliformisamine C 52



Tokaridine C 53



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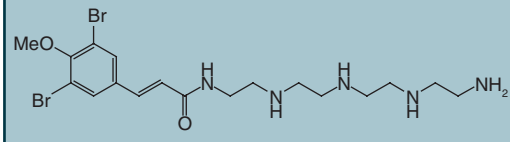

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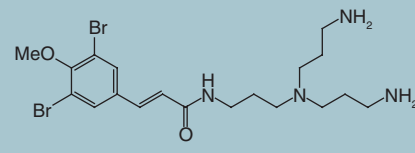

56

Compound	Concentration of Ianthelliformisamine derivative used (μM)		
	Ea289	PAO1	KPC2 ST258
51	25	12.5	3.12
52	12.5	3.12	12.5
53	6.25	3.12	3.12
54	12.5	6.25	3.12
55	12.5	6.25	3.12
56	6.25	3.12	3.12

Ea289: *Enterobacter aerogenes* 289; KPC2ST258: *Klebsiella pneumoniae* ST2558; PAO1: *Pseudomonas aeruginosa*.
Data taken with permission from [40].

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Executive summary

- Each year in the USA, 2 million people become infected with bacteria and at least 23,000 people die annually as a direct result of these infections. Thus, the rise of antibiotic resistance since few decades has resulted in a very pressing need for the discovery of novel antibiotics or treatment strategies.
- Efficacious small-molecule enhancers designed to efficiently target bacterial membranes could serve as lead compounds in the repositioning process of old neglected antibiotics.
- Search for polyamine derivatives acting by membrane depolarization and/or integrity membrane disruption could constitute an efficient alternative since their mechanism of action may significantly reduce the development of resistance.
- Enlarge the specificity of polyamine derivatives toward bacteria in order to minimize adverse effects expected when used to fight infections.

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Review
Special Focus Issue – Part I: New Frontiers in Antibacterial Drug Discovery

Future Medicinal Chemistry

New Delhi metallo-β-lactamase-1: structure, inhibitors and detection of producers

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Since its discovery in 2008, New Delhi metallo-β-lactamase-1 (NDM-1)-producing Enterobacteriaceae have disseminated globally, facilitated predominantly by gut colonization and the spread of plasmids carrying the bla_{NDM-1} gene. With few effective antibiotics against NDM-1 producers, and resistance developing to those which remain, there is an urgent need to develop new treatments. To date, most drug design in this area has been focused on developing an NDM-1 inhibitor and has been aided by the wealth of structural and mechanistic information available from high resolution x-ray crystallography and molecular modeling. This review aims to summarize current knowledge regarding the detection of NDM-1 producers, the mechanism of action of NDM-1 and to highlight recent attempts toward the development of clinically useful inhibitors.

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Keywords: detection • Enterobacteriaceae • inhibitors • mechanism • NDM-1 • New Delhi metallo-β-lactamase-1 • substrates

Multidrug-resistant Gram-negative Enterobacteriaceae present an ever increasing threat to current, highly antibiotic-dependent, healthcare systems. The increasing prevalence of multidrug-resistant organisms (MRO), including Enterobacteriaceae, could mark the beginning of an era characterized by treatment-resistant infections, prolonged illnesses, increased healthcare burdens and increased risk of mortalities. These bacteria have been found to be resistant to most β-lactam antibiotics, including carbapenems, the current mainstays for the treatment of Enterobacteriaceae-based infections [1]. The WHO report 'Antimicrobial resistance: global report on surveillance 2014' reflects the international dimensions of the current threat posed by antibiotic resistance and includes specific foci on the epidemiology of seven resistant bacterial species and approaches used to combat resistance [2].

The multidrug resistance exhibited by some Enterobacteriaceae can be attributed to the acquisition of a variety of resistance mechanisms, the most important of which is the production of β-lactamase enzymes, for example, oxacillinase-48 (OXA-48), Verona integron-encoded metallo-β-lactamase-1 (VIM-1), imipenemases (IMPs) and cefotaximases (CTX-Ms) [3]. NDM-1 [4] is one the most recent additions to the β-lactamase family, controversially named after its presumed place of origin, New Delhi in India [5]. A related metallo-β-lactamase (MBL), Florence imipenemase 1 (FIM-1) was isolated and cloned from a multidrug-resistant *Pseudomonas aeruginosa* clinical isolate in 2013 [6]. The first recorded case of an infection involving NDM-1, reported in 2009, involved a 59 year old Swedish patient (of Indian background) who had acquired a urinary tract infection (UTI) after traveling to New Delhi in 2007 [4]. The urine and

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fecal samples from this patient identified the presence of carbapenem-resistant *Klebsiella pneumoniae* and *Escherichia coli* strains. Genotyping and phenotyping of these strains revealed that resistance was due to a novel mechanism, NDM-1 production, which is encoded by the bla_{NDM-1} gene. Antibiotic susceptibility testing showed that NDM-1 production also conferred resistance to all other β-lactam antibiotics, except for the monobactam, aztreonam and the amidinopencillin, mecillinam [7]. Interestingly, a later publication, documenting the SENTRY antimicrobial surveillance program in 14 Indian hospitals in 2006–2007 reported that 15 of the 39 isolated strains of carbapenem-resistant Enterobacteriaceae carried the bla_{NDM-1} gene [8].

A wide range of antibiotic resistance genes have been found to be coharbored by the plasmids carrying bla_{NDM-1}, resulting in additional resistance mechanisms against β-lactams (e.g., due to the presence of extended-spectrum β-lactamases [ESBLs] and AmpC cephalosporinases), macrolides (due to erythromycin esterase), tetracyclines (due to increased efflux) and sulfonamides (due to altered dihydropteroate synthases) [9]. The presence of the ESBLs and cephalosporinases as a result of these coharbored resistance genes accounts for the resistance of many NDM-1 producers to aztreonam, which is not a substrate for the NDM-1 enzyme [10].

Most clinical NDM-1 producers are only susceptible to the last line antibacterial agents colistin, tigecycline or fosfomycin, all of which have limitations which restrict their use [11]. Colistin is a polymyxin antibiotic which NDM-1 producing Enterobacteriaceae remain highly susceptible to, with isolates from the UK, Chennai and Haryana showing 89, 94 and 100% susceptibility, respectively [12]. Colistin is, however, far from an ideal drug as a result of its associated nephrotoxicity and neurotoxicity [13]. Tigecycline and fosfomycin also have their limitations. Tigecycline is only approved for the treatment of complicated skin and skin structure infections (cSSTIs), complicated intra-abdominal infections and community-acquired pneumonia in adults; it has been deemed to be unsuitable for the treatment of UTIs due to its high degree of biliary excretion and low urinary concentrations [14]. There are issues of accessibility to fosfomycin in many countries, and its use as monotherapy for severe infections is not recommended, so it is only suitable for monotherapy against UTIs [10].

Due to the varied acquisition of resistance across NDM-1-producing Enterobacteriaceae, treatment is largely guided by antimicrobial susceptibility testing. Unfortunately, minimum inhibitory concentration (MIC) testing in 2011 of colistin, tigecycline and fosfomycin against 28 NDM-1 producing isolates of global origins found that 33% were resistant, or borderline susceptible, to at least one of these antibiotics [15].

NDM-1 producing bacteria have been isolated from both nosocomial and community-acquired infections and the emergence of these bacteria can be attributed to a number of factors, including increased access to international travel, an increase in travel to access cheaper medical care and the over-the-counter availability of broad spectrum antibiotics in some countries.

Since the initial report, other NDM-1-producing enterobacteria have been identified throughout the world, with most originating from the Indian subcontinent (which also accounts for 71.1% of the NDM-1-producing bacteria in autochthonous case reports in the period 1 December 2009–31 December 2012) [16]. Other suggested reservoirs of NDM-1 producers are the Balkans (possibly resulting from medical tourism) [17,18], and the Middle East (a common transit point for passengers travelling to the Indian subcontinent) [19]. In the period 1 December 2009–31 December 2012 there were 136 case reports of NDM-1-producing bacteria in the PubMed database, detailing 950 isolates, with the most common being *K. pneumoniae* (359, 37.8%) and *E. coli* (268, 28.2%). In 2009, NDM-1 producing multidrug resistant bacteria became the most prevalent carbapenem-resistant Enterobacteriaceae in the UK, consisting of 44% of the 73 identified carbapenemase producers [12].

Other NDM-1-producing organisms include *Enterobacter cloacae*, *Klebsiella oxytoca, Acinetobacter baumannii, Pseudomonas aeruginosa, Proteus* spp., *Citrobacter freundii*, *Morganella morganii* and *Providencia* spp. [16].

The pathogenesis of NDM-1-producing bacteria is largely dependent upon the typical infecting patterns of the particular host species. As a result, NDM-1 producing bacteria can contribute to a range of infections, including urinary tract and pulmonary infections, peritonitis, soft tissue infections, device-associated infections and septicemia. Studies of the gut colonization of patients in a hospital in Pakistan found a positive correlation between hospital 'length of stay' and the presence of fecal carriage of NDM-1-positive bacteria [20]. There has, however, been no evidence to support the greater virulence of bacteria producing NDM-1 compared with other strains [10].

A lack of correlation between the presence of NDM-1 producers and the presentation of clinical symptoms has also frequently been observed [3]. Furthermore, a systematic review of the literature between 2008 and 2011 reported that, in 65% of cases, colonization with NDM-1 producers occurred independently of clinical evidence of infection [21].

In addition to asymptomatic gut colonization, it is known that patients infected with NDM-1 producing bacteria can continue to be colonized for periods of up to 13 months [22]. Another report documented the presence of NDM-1 producers in urine or fecal samples at a 7-month follow-up [23]. Such prolonged periods of colonization represent a possible reservoir for NDM-1 dissemination [24]. Furthermore, a study of tap water and sewage samples from New Delhi detected the presence of bla_{NDM-1} in two out of 50 tap water samples and 51 out of 171 sewage samples, among bacteria of the *Aeromonas* and *Vibrio* genera [25]. This discovery indicated that the bla_{NDM-1} gene was not restricted to hospital-acquired bacterial species, but was also likely to be circulating among bacterial species highly prevalent in the community. The high prevalence of NDM-1-positive bacteria in such samples suggests the presence of an environmental reservoir, in which bacteria can reproduce and spread without necessarily leading to infection in patients [25].

β-Lactamase nomenclature

NDM-1 is a class B1 β-lactamase as it has two Zn2+ ions bound in its active site. By convention, β-lactamases are classified according to either their amino acid sequence or functional characteristics. Classification by amino acid sequence is the simplest, and currently most adopted, approach and involves dividing β-lactamases into classes A, B, C and D according to the conservation of their specific amino acid motifs [26,27]. Ambler class A, C and D β-lactamases have a serine-dependent mechanism of hydrolysis, while class B β-lactamases require the presence of at least one zinc ion in the active site for hydrolysis, and are commonly referred to as MBLs for this reason. Within the MBLs, further divisions are made according to metal binding site properties (Supplementary Table 1); NDM-1 is a class B1 β-lactamase (as is FIM-1), as x-ray crystallography has determined the presence of two Zn^{2+} ions bound in its active site [28,29].

Despite the presence of some conserved regions and tertiary structure fold similarities, NDM-1 shares little amino acid sequence identity with other MBLs; its closest relatives being FIM-1 (with which it shares 39–40% sequence identity) [6] and VIM-4 (37% sequence identity) [30].

As the MBLs belong to Ambler Class B, the suicide β-lactamase inhibitors, which inhibit the formation of a covalent enzyme–drug complex by the serine β-lactamases, are inactive. Suicide β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam (Ambler Class A inhibitors) and avibactam (Ambler Classes A, C and D inhibitor) are thus ineffective against NDM-1 and other MBLs.

Detecting NDM-1 producing bacteria

As a constituent of normal human intestinal flora, Enterobacteriaceae are easily spread and are difficult to eliminate; the rapid identification of NDM-1 producing Enterobacteriaceae in the clinical setting is a

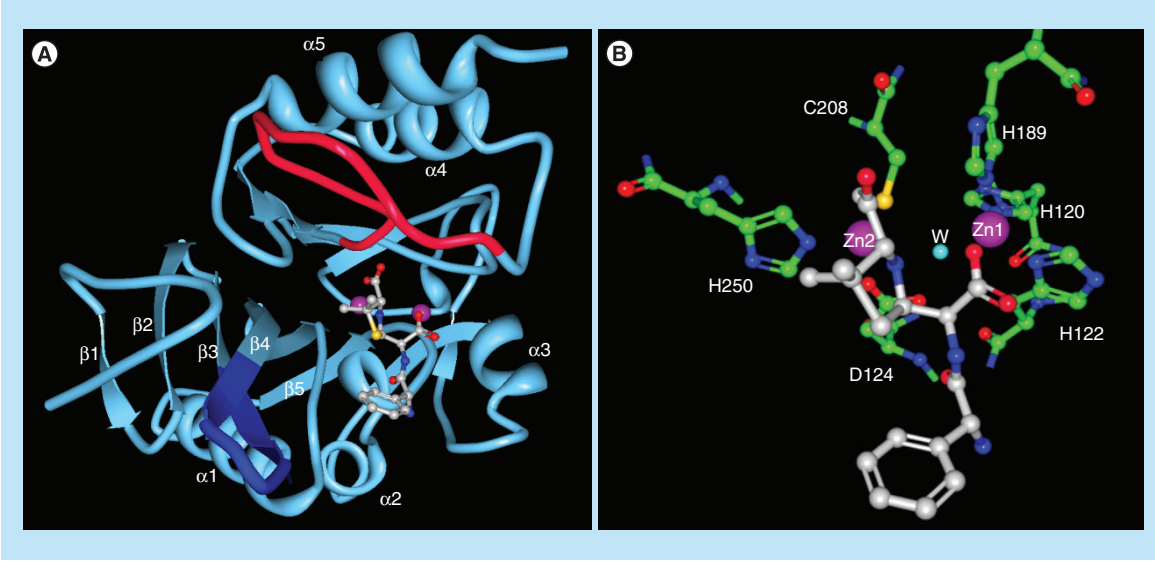


Figure 1. Overall structure of New Delhi metallo-β-lactamase-1 in complex with hydrolyzed ampicillin (PDB 3Q6X) [28]**. (A)** Protein backbone of New Delhi metallo-β-lactamase-1, shown as a pale blue cartoon with the helices and strands labeled. Loops **L3** (blue) and **L10** (red) are highlighted and the two zinc ions at the active site are shown as cerise spheres; **(B)** Enlargement of the active site with helices and strands hidden for clarity. The active site water/hydroxide (W) is shown in turquoise between the two zinc ions, and the residues coordinated to the zinc ions are labeled; Zn1 (H120, H122 and H189) and Zn2 (D124, C208 and H250). All PDB images were generated in RCSB Protein Workshop [43,44].

Table 1. Summary of important New Delhi metallo-β-lactamase-1 active site amino acids.

Amino acid	Role
Substrate interaction	
Leu65 and Met67 (Loop L3), Trp93 (Loop L6), Ile35, Val73	Formation of a large hydrophobic binding surface for hydrophobic interactions with β-lactam R groups [28,56,68]
Asn220	Involved in substrate recognition and hydrolysis [68]
	May stabilize the tetrahedral intermediate product via formation of an oxyanion hole in conjunction with Zn1 [56,66]
	Hydrogen bonds to carbonyl oxygen of substrate [28]
Gln123, Asp124	Hydrogen bonds with oxygen atoms adjacent to hydrophobic β-lactam R groups [28]
Lys211	Involved in substrate recognition and hydrolysis [68]
	Formation of a salt bridge to maintain correct substrate orientation for hydrolysis [28,30]
Trp93, Gln123, Asp 124 and His250	Formation of a hydrophilic hole for substrate binding [57]
Maintaining active site conformation	
Tyr229	Stabilizing loop L10 via hydrophobic and hydrophilic interactions [28,57]
Lys125	Stabilizing loop L6 via hydrogen bonding networks and restricting Zn1-coordinating residues [28]
His120, His122, His189	Zn1-coordinating residues [28]
Asp124, Cys20, His250	Zn2-coordinating residues [28]
Phe70	Contribute toward the flexibility and movement of loop L3 when interacting with the substrate [57]

vital step in the fight to slow the spread of antibiotic resistance through horizontal transfer.

Detection techniques enable patients to be screened for gut colonization, which is currently believed to be one of the major contributors to NDM-1 dissemination. Such testing needs to be as rapid and reliable as possible so that, along with the isolation of patients who are suspected to have been colonized/infected, it forms the basis of an effective infection control policy. Reliable and accurate screening tools are thus of immense importance and can even assist in the prevention of outbreaks of infections due to NDM-1-producing bacteria. Screening for carbapenemase-producing Enterobacteriaceae (CPE) initially involves antibiotic susceptibility testing using breakpoint values from the Clinical and Laboratory Standards Institute (CLSI) for carbapenems (Supplementary Figure 1) (1 mg/l for doripenem, imipenem and meropenem, 0.5 mg/l for ertapenem) [31]. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) has proposed meropenem as the indicator antibiotic for the detection of CPE (except OXA-48 producers), as it has the best balance between its sensitivity and specificity [32].

Follow-up testing of suspected MBL producers can be based upon phenotypic or genotypic methods, with the ideal method representing a balance between simplicity/cost–effectiveness and time to detection/reliability (Supplementary Table 2). Molecular methods generally result in a more rapid time to detection and greater sensitivity and specificity, but are usually more costly than phenotypic methods and require specialist interpretation. The cost per test and screening frequency are important factors when considering the numbers of patients who may need to be screened, while the availability of any necessary equipment is an important consideration for screening in any global location.

The modified Hodge test (MHT) detects carbapenemase activity by the production of a clover leaf shape only when the clinical isolate produces these enzymes (Supplementary Figure 2), thus enabling the growth of a carbapenem susceptible strain (*E. coli* ATCC 25922) toward a carbapenem (meropenem, imipenem and ertapenem) disk [32–34]. This screening method is generally quite sensitive, with the exception being the production of false negatives for NDM-1-producing *A. baumannii* species [8]. Major drawbacks, however, include its time consuming nature and poor specificity for NDM-1-producing Enterobacteriaceae, reflected by frequent false positives [35]. Enhanced differentiation of MBL producers is possible if zinc sulfate is added to an imipenem disk or to the Mueller–Hinton agar growth medium [36].

The EDTA double disk synergy test has also been reported to be a reliable means of detecting MBL producers, and is based on the principle that EDTA is an effective inhibitor of MBLs [37]. This method involves the comparison of zone diameters around meropenem-EDTA disks and meropenem-only disks. Strains are interpreted as MBLs if they produce a significantly wider zone diameter (>5 mm) around the meropenem-EDTA disk in comparison to the meropenem-only disk [38]. A similar test can also be performed with imipenem-EDTA disks [39].

Culture based screening methods using chromogenic media have gained much popularity because of their suitability for the testing of rectal swabs (screening for gut colonization) [40]. Such media contain substrates for specific enzymatic activity to aid species identification, as well as antibiotics to select for the growth of target species. The specific enzymatic activity is detected as a result of the cleavage of a covalent bond between an enzyme-targeting moiety and a chromogen (Supplementary Figure 3). Once liberated, the color of the chromogen is indicative of the presence of the specific enzyme (and thus the organism producing it). For example, *E. coli* can be detected as a result of its β-glucuronidase and/or β-galactosidase activity, while *Klebsiella-Enterobacter-Serratia* spp. exhibit β-glucosidase activity. A comparison between the chromogenic media, chromID® CARBA and Colorex™ KPC, found that chromID® CARBA had a much better recovery of NDM-1 producing isolates; detecting an additional 23 isolates (from a total of 64 isolates) [34]. chromID® CARBA was also found to have substantially higher sensitivity and specificity for carbapenemase-producing Enterobacteriaceae when compared with Brilliance™ CRE, detecting an additional 12 patients (from a sample size of 32) carrying NDM-1 producers [20].

chromID® ESBL, which is commonly used for the detection of ESBL producers, has also been found to be quite reliable for detecting NDM-1 producers [10], and screens for bacteria according to their resistance to the extended-spectrum cephalosporin, cefpodoxime. This method, however, will detect ESBL producers together with NDM-1 producers, thus increasing the workload associated with NDM-1 screening and potentially reducing sensitivity if large numbers of ESBL producers overgrow species with NDM-1 [40].

Although all of the above phenotypic methods are simple, cheap and relatively easy to perform and interpret, the major drawback is usually time to detection (often 18–24 h for chromogenic methods). One culture-based method which overcomes the lengthy delays to identification (during which the patient may have to remain isolated to prevent bacterial transmission) is Carba NP [41], which is capable of confirming the presence of carbapenemase (including NDM-1)

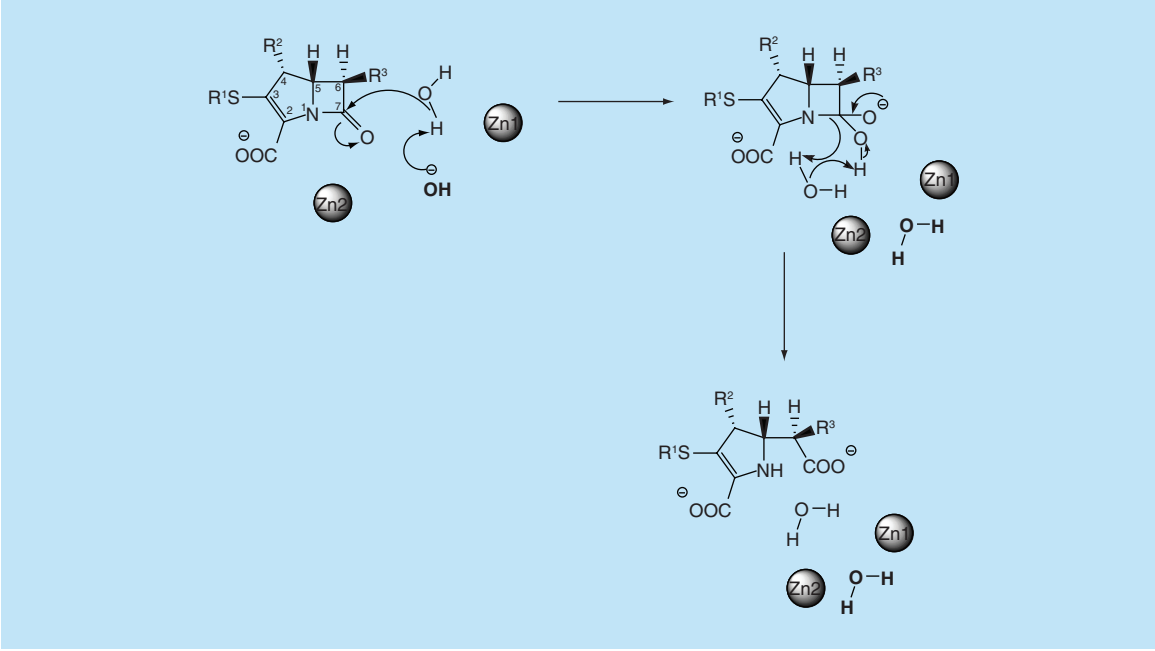


Figure 2. Proposed mechanism for the New Delhi metallo-β-lactamase-1-catalyzed hydrolysis of carbapenems.
The 'oriented' hydroxide ion (bold) serves as a general base to accept a proton from a bulk water molecule and the hydroxide ion thus formed undergoes nucleophilic attack on the lactam carbonyl group.
Adapted from Kim *et al.* [45].

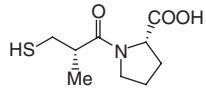
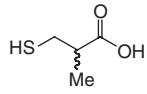
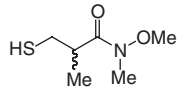
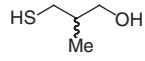
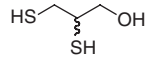
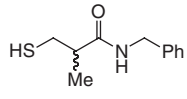
Table 2. Proposed nucleophiles and associated hydrolysis pathways.

Nucleophile identity	Hydrolysis pathway	Energetics
Bulk water molecule from the solvent environment (Figure 1)	The bulk water molecule co-ordinates to the zinc ion and donates a proton to the shared active site hydroxide (general base) [28]. This generates a new hydroxide which then attacks the carbon atom of the β-lactam carbonyl group [45]	Energetically favorable (energy barrier ~80 kJ/mol) [45]
Active site/oriented water molecule	The active site water donates a proton to either: – Asp124 (general base) [70]; – Bulk water molecule (general base) [45]. This generates an active hydroxide form which then attacks the carbon atom of the β-lactam carbonyl group to form a carboxylate intermediate [45,69,70]. *N*4 is directly protonated by the nucleophile [45]	Energetically unfavorable (energy barriers ≥200 kJ/mol) [45]

producers in 30–120 min, with high specificity and good sensitivity (which may be related to the level of carbapenemase expression) [42]. This test relies upon the fact that the product of carbapenem hydrolysis (in this case imipenem) has an increased number of carboxylic acid groups, resulting in the increased acidity of the medium. The decrease in the pH of the test sample upon carbapenem hydrolysis is detected by the phenol red in the medium turning from red to yellow (Supplementary Figure 4).

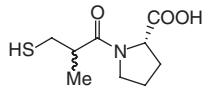
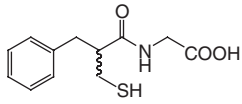
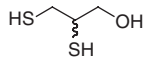
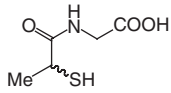
As it is a genotypic method which is capable of specifically identifying NDM-1 producers, PCR remains the gold standard for molecular-based identification of these bacteria; it can detect the presence of bla_{NDM-1} genes in less than 6 h, with excellent sensitivity and specificity (both can be 100%) [46,47]. However, as only a small fragment of the bla_{NDM-1} gene is amplified, these methods are only able to report the presence of this one variant. To identify other variants (as a result of mutations), PCR amplification of the full-length bla_{NDM} gene plus DNA sequencing would be required. This process is time consuming and cannot provide information on the carbapenemase activity of the coded NDM protein. Other associated limitations of PCR include its high running costs and the expertise required to operate the specialized equipment, meaning that not every global facility will have access to this technique. In order to determine the carbapenemase activity of NDM variants, a new technique, PCR-based *in vitro* protein expression (PCR-P), has been employed, integrating long-fragment quantitative PCR (to

Table 3. Structure and New Delhi metallo-β-lactamase-1 IC_{50}s of D-captopril analogs.

Compound	Structure	IC_{50} (μM)
1		7.9 ± 0.1
2		a (*R*) 53 ± 9 b (*S*) 81 ± 10
3		a (*R*) 48 ± 2 b (*S*) 20 ± 1
4		15 ± 2
5		10.4 ± 1
6		a (*R*) 1.5 ± 0.2 b (*S*) 5 ± 0.4

Data taken from [74].

Table 4. Structure and metallo-β-lactamase IC_{50}s of approved drugs containing thiol groups.

Compound	Structure	IC_{50} (μM)		
		NDM-1	IMP-7	VIM-1
1		6.4	2.9	6.8
7		1.8	5.3	5.8
5		1.3	3.5	0.6
8		84	5.9	8.6

IMP: Imipenemase; NDM-1: New Delhi metallo-β-lactamase-1; VIM-1: Verona integron-encoded metallo-β-lactamase-1.
Data taken from [77].

detect the full length *bla*$_{NDM}$) with a functional assay measuring the degradation of imipenem by the *in vitro* synthesized protein [48].

Another molecular-based technique with applications in the detection of pathogenic bacteria is MALDI-TOF MS, which has been proposed as a rapid and accurate alternative to the MHT. This technique detects the presence of the carbapenem and its degradation (hydrolysis) products after interaction with carbapenemase-producing bacteria [49–51]. Using this technique with meropenem, all carbapenemase producers were identified within 2.5 h, with no false positives or negatives [52]. MALDI-TOF is not routinely available in all microbiological diagnostic laboratories and, like PCR, is expensive and requires specialist operators/interpretation.

A UV-based assay has been developed which, like the MALDI-TOF method above, detects carbapenemases on the basis of their hydrolysis of imipenem, and the UV absorbance of the hydrolyzed product (at 297 nm). Although it has a sensitivity of 100% and specificity of 98.5% and is cheap, this method is relatively labor intensive and requires some specialist interpretation (the cut-off for a positive result being based upon the difference between the absorbance slope per minute for imipenem hydrolysis by a culture extract and that resulting from auto hydrolysis) [53].

Based upon the techniques currently available, Nordmann and coworkers have proposed a flowchart for the rapid detection and characterization of CPE, involving the Carba NP test (to differentiate between CPE and non-CPE), followed by PCR or DNA microarray (to determine the carbapenemase genes) and finally sequencing [53].

NDM-1 substrates

NDM-1 effectively hydrolyzes all β-lactam antibiotics except for the monobactam, aztreonam [4] and the amidino penicillin, mecillinam [54], but kinetic studies have found variations in the degree of affinity and catalytic efficiency for the hydrolysis of various substrates. For example, it has been shown that most penicillins and cephalosporins (e.g., cephalothin, cefotaxime and cefuroxime) bind tightly to NDM-1, as indicated by lower K_m values in comparison to IMP-1 and VIM-2 (Supplementary Table 3) [4]. Despite this, NDM-1 hydrolyzes these substrates less efficiently, as reflected by low k_{cat} values, with exceptions being the carbapenems (imipenem and meropenem), for which NDM-1 exhibits both tight binding (low K_m) and efficient catalysis rates (high k_{cat}).

Of greatest concern is the lack of novel and effective antimicrobial agents in the drug discovery pipeline for NDM-1 producing Enterobacteriaceae [55]. There is an urgent need for the development of novel metallo-β-lactamase inhibitors or new antibacterial agents. The challenges associated with the design of such agents, coupled with low financial returns due to short treatment durations, has contributed to the gradual exit of many major pharmaceutical companies from this area of research. Academic institutions and smaller commercial groups have, however, taken up the challenge and continue endeavors in this area.

NDM-1 variants & structure

NDM-1 consists of 270 amino acids in a single polypeptide chain, which exists in the typical αβ/βα sandwich fold characteristic of MBLs [28]. Two twisted β-sheets are found packed together in the centre and

surrounded on either side by two solvent-exposed α-helices [28,56]. One β-sheet is made up of seven antiparallel β-strands whilst the second is made up of four. The 5th α-helix acts as a bridge to hold the two β-sheets together (Figure 1).

In solution, the active form of NDM-1 has been reported to be a monomer, similar to other B1 MBLs [57], but some crystal structures have shown that NDM-1 can exist as a dimer due to hydrophobic and van der Waals interactions. The ability to exist as a dimer in both membrane-bound and purified states has been hypothesized to contribute toward the unique resistance mechanisms of NDM-1 [56].

At present, there are 24 crystal structures for NDM-1 deposited in the Protein Data Bank (PDB [58]) and these show a considerable degree of variation in the identity of the bound metal ion, cocrystallized substrate, length of NDM-1 *N*-terminus and conformation of substrate binding loops [59]. Areas of differentiation may present as structural sites important for substrate recognition or hydrolysis [60]. Substrates which have been cocrystallized include; ampicillin, benzylpenicillin, L-captopril, methicillin, meropenem and oxacillin. A comparison of root-mean-square deviation (RMSD) values between four NDM-1 structures (3Q6X, 3RKJ, 3RKK and 3S0Z) found that loop L3 (residues 63–73) contained the greatest variability, suggesting a high degree of flexibility in this region [29].

Since the first characterization of NDM-1, a further 14 variants of NDM have been identified [61], seven of which differ from NDM-1 by a single amino acid substitution and six by two amino acid substitutions, with only one (NDM-10) differing by three amino acids (Supplementary Figure 5) [62]. The most recently discovered variant, NDM-14, was identified in a clinical isolate of *Acinetobacter lwoffii* JN49-1 from an intensive care patient in Jinan, PR China, and differs from NDM-1 by a single substitution (Asp130Gly) [63]. None of the amino acid substitutions observed thus far (Supplementary Figure 6) are in the key catalytic residues, or those involved with substrate interaction or the maintenance of the active site conformation, yet some of these variants have been reported to exhibit enhanced carbapenemase activity, and greater affinity for the carbapenems. The most common substitution (Met154Leu) occurs in seven variants (NDMs 4, 5, 7, 8, 12, 13 and 15) and is reported to increase the hydrolytic activity of the enzyme [64,65], presumably as a result of a change in the interactions of this amino acid with the zinc coordinating residues in Loop 7 (Supplementary Figure 7). The NDM-11 variant is unique in that the substitution at this site is Met154Val.

The NDM variants are far less numerous and diversified than other MBLs, such as VIM and IMP, but

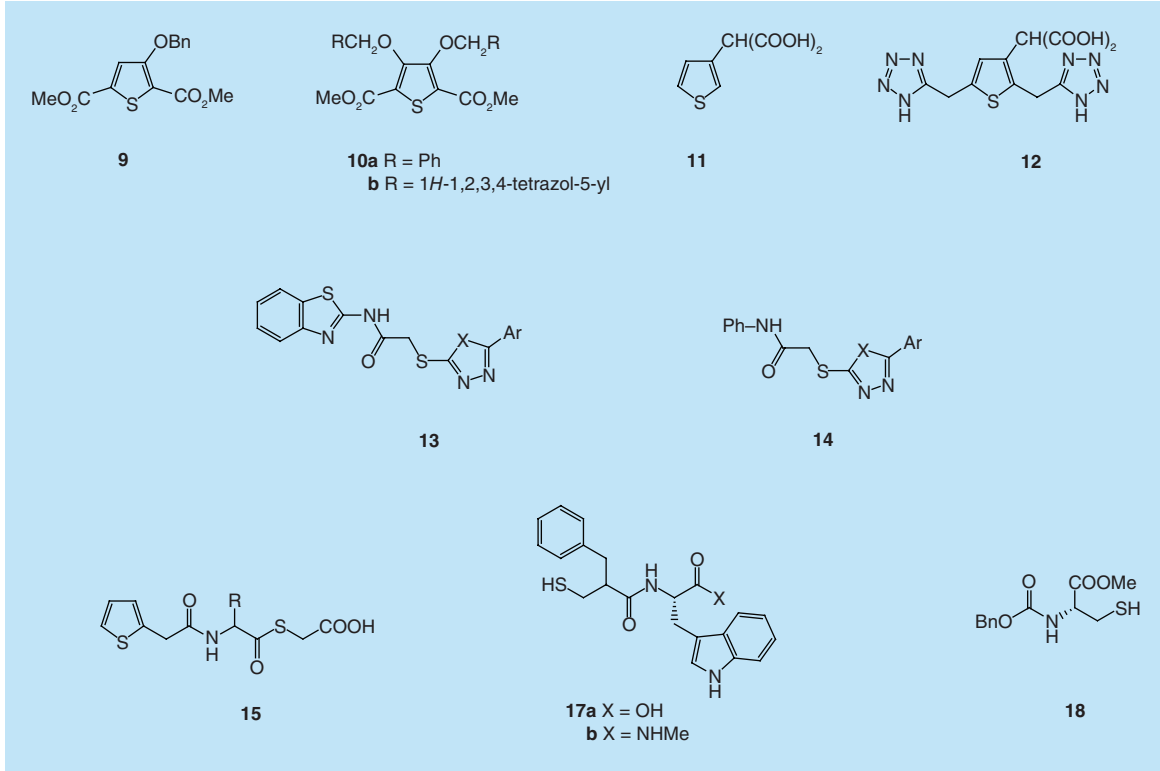


Figure 3. Sulfur-containing New Delhi metallo-β-lactamase-1 inhibitors.

Table 5. Structure and IC_{50}s of azolylthioacetamides 13 and 14 against representatives of the B1a, B1b, B2 and B3 β-lactamase sub-types.

Structure	K_i (μM)			
	CcrA (B1a)	NDM-1 (B1b)	ImiS (B2)	L1 (B3)
13a X = NH, Ar = 2-HOC_6H_4	6.3 ± 0.1	0.35 ± 0.01	>300	0.25 ± 0.03
13b X = O, Ar = 2-HOC_6H_4	7 ± 1	6.1 ± 0.2	15 ± 2	0.65 ± 0.09
13c X = S, Ar = 2-HOC_6H_4	–	0.81 ± 0.07	–	1.8 ± 0.01
13d X = NH, Ar = 4-pyridyl	8.5 ± 0.2	5.6 ± 0.1	0.75 ± 0.02	0.34 ± 0.05
13e X = O, Ar = 4-pyridyl	–	45 ± 6	1.10 ± 0.03	0.82 ± 0.08
13f X = S, Ar = 4-pyridyl	–	6.8 ± 0.4	> 300	0.33 ± 0.02
14a X = NH, Ar = 2-HOC_6H_4	0.30 ± 0.09	0.43 ± 0.03	2.2 ± 0.2	0.073 ± 0.007
14b X = O, Ar = 2-HOC_6H_4	52 ± 5	0.60 ± 0.07	3.3 ± 0.2	0.34 ± 0.02
14c X = S, Ar = 2-HOC_6H_4	–	0.64 ± 0.03	> 300	0.51 ± 0.03
14d X = NH, Ar = 4-pyridyl	–	0.42 ± 0.02	1.3 ± 0.4	0.25 ± 0.04
14e X = O, Ar = 4-pyridyl	–	0.23 ± 0.01	> 300	0.43 ± 0.09
14f X = S, Ar = 4-pyridyl	–	1.2 ± 0.1	–	0.38 ± 0.02

Data taken from [79].

this may simply be a function of the time since the discovery of the various genes [61].

Active site

The NDM-1 active site is located in a broad, shallow groove near the edge of the twisted β-sheets, flanked by loops L3 (residues 63–73) and L10 (residues 208–221) (Figure 1). The active site consists of two zinc ions; with tetrahedral coordination of Zn1 by three histidine residues (His120, His122 and His189) and the water molecule/hydroxide ion which complexes to both zinc atoms, with Zn2 also coordinated by His250, Cys208 and Asp124 (Table 1) [28,29,66]. These zinc ions function as the primary substrate recognition complex of NDM-1, coordinating to oxygen atoms found in the carboxyl and carbonyl groups of β-lactam substrates [45]. Zn1 is important in maintaining the correct orientation (for nucleophilic attack) of the substrate's carbonyl group, while Zn2 interacts with the substrate's amide nitrogen [67]. In B1 MBLs, Zn1 has generally been found to display higher occupancy than Zn2 [56]. The distance between the two zinc ions is found to vary across different crystal structures (3.2–4.6 Å).

Despite the similarity of NDM-1 to other B1 MBLs (especially VIM-2 and IMP-1) in terms of overall fold, areas of structural differentiation from these MBLs have been identified by superimposing NDM-1 with VIM-2 and IMP-1 crystal structures. One of the main differences exists at the *N*-terminus, where NDM-1 has an extra β-strand extension which binds to loop L3 via an Ile35 residue. This is believed to increase the hydrophobicity of the loop and to contribute toward the larger hydrophobic substrate binding surface of NDM-1 in comparison to VIM-2 and IMP-1 [28].

The active site groove of NDM-1 has also been found to have a larger surface area than that of other MBLs. This is due to the loops L3 and L10 being oriented further away from the zinc centre of the

Table 6. Structure and metallo-β-lactamase IC_{50}s of amino acid thioesters 15.

R	IC_{50} (μM)		
	NDM-1	ImiS	L-1
H	28.7	61.5	0.84
Me	95.7	46.3	0.91
^{i}Pr	12.4	18.9	0.45
^{i}Bu	28.5	3.6	0.069
Bn	36.3	65.4	2.9

Data taken from [80].

Table 7. New Delhi metallo-β-lactamase-1 inhibitors identified by meropenem hydrolysis ^{1}H NMR assay.

Compound	IC_{50} (μM)
L-1	175.0
16	1.6
17a	0.51
17b	44

Data taken from [82].

active site [56]. This groove is also large enough, on either side of the zinc ions, to accommodate for the binding of larger substrates [29]. Therefore, penicillin or carbapenem antibiotics with a variety of substituents at carbon 2 or 6 can be sterically accommodated (Supplementary Figure 1). Together with the flexibility of the loops and their lack of extensive interactions with the substrates other than the β-lactam moiety (a characteristic of a promiscuous enzymes) [45], these factors are thought to contribute toward the broad substrate binding profile of NDM-1.

Electron density data also suggest the presence of a water molecule or hydroxide ion positioned between the two Zn ions (Figure 2). As would be expected, the exact identity of the molecule occupying this space has been suggested to depend upon the pH conditions under which the crystals were grown; with a water molecule predominating at low pH and a hydroxide ion at higher pH [45].

Structural analyses of the NDM-1 active site have suggested that other amino acid residues may play important roles in substrate recognition and hydrolysis (Table 1). The roles of some of these residues have been investigated through the introduction of point mutations into the NDM-1 active site and subsequent screening for their effect upon substrate hydrolysis.

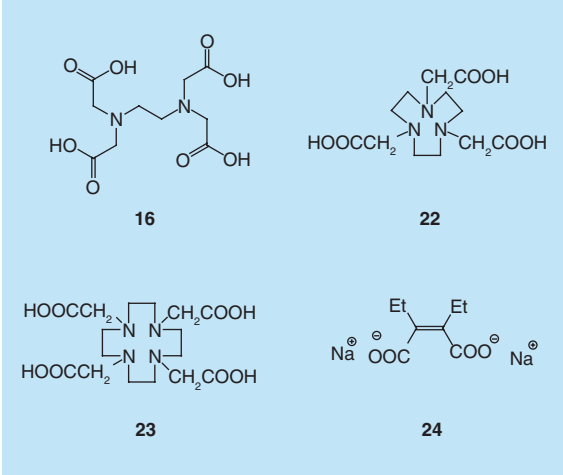


Figure 4. Metal chelating New Delhi metallo-β-lactamase-1 inhibitors.

One such study found that substituting alanine for leucine 65 (Loop 3) resulted in a reduction in the MICs of ampicillin and some cephalosporins, while substituting asparagine 220 (Loop 10) by alanine resulted in a dramatic loss in hydrolytic activity (decrease in MIC) toward ampicillin, cefepime, meropenem and imipenem [68]. Mutations at these residues thus resulted in a loss of NDM-1 hydrolytic activity. The coupling of information gained from such mutational studies with crystallographic studies may shed further light on the functional properties of NDM-1.

Hydrolysis mechanism

NDM-1 has been shown to hydrolyze substrates according to the general MBL hydrolysis mechanism, involving the coordination of the β-lactam carbonyl oxygen to Zn1, nucleophilic attack (addition/elimination) by the shared active site hydroxide, protonation of the nitrogen atom and C-N bond cleavage (Figure 2) [45].

The specific details of the hydrolysis pathway have not yet, however, been fully elucidated. Studies using molecular dynamics modeling, and quantum and molecular mechanics (QM/MM) analyses have proposed the presence of one or two transition states [28,69]. Due to the fast catalytic rate of NDM-1, however, structural verification of these transition states has so far been unsuccessful. Controversy has also surrounded the identity of the nucleophile and general bases involved in the NDM-1 hydrolysis mechanism (Table 2) [28,45]. Further studies at the molecular level are necessary in order to gain a clearer understanding of this mechanism, which may in turn provide important information supporting the rational design of clinically useful inhibitors.

NDM-1 inhibitors in development

In recent years there have been increasing global incentives to encourage and financially support the development of new antibiotics [55]. The design of drugs targeting NDM-1 has focused on two areas; compounds which act in synergy with carbapenems (thus restoring the efficacy of these against NDM-

1), or novel antibiotics which are not susceptible to NDM-1-catalyzed hydrolysis [56]. Most effort has currently been focused on the first area, as it offers the possibility of protection of previously effective β-lactam antibiotics from hydrolysis when administered in combination with the inhibitor [71]. Such inhibitors are hypothesized to act broadly across MBL subtypes because of the high degree of structural similarity found between MBLs [72].

Sulfur-containing inhibitors

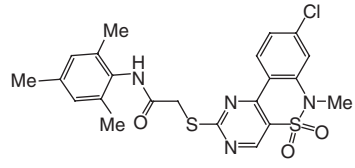
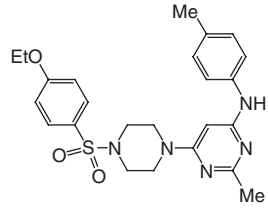
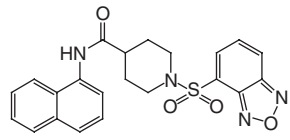
As we have seen, like angiotensin converting enzyme (ACE), MBLs contain a key zinc atom at the active site, so it is no surprise that both enantiomers of captopril (the first clinically approved ACE inhibitor) have been investigated as potential NDM-1 inhibitors [73]. D-Captopril **1** was shown to bind NDM-1 with high affinity and inhibit its activity with an IC_{50} (half maximal inhibitory concentration) of 7.9 μM, while L-captopril exhibited greatly reduced inhibition (IC_{50} 202.0 μM) [57]. A crystal structure of NDM-1 bound to L-captopril has been obtained, which validates this binding interaction, and shows L-captopril bound between Zn1 and Zn2 of the active site, interacting with both zinc ions via coordination bonds [66].

A recent study aimed to characterize the NDM-1 inhibitor pharmacophore by synthesizing and testing various D-captopril **1** analogs resulting from the replacement of the proline residue or modification of the 3-mercapto-2-methylpropanoyl moiety (Table 3) [74]. The moderate activity exhibited by the corresponding carboxylic acids **2** and, in particular, the Weinreb-Nahm amides **3** could be due to the well known ability of both carboxylates [75] and hydroxamates [76] to interact with zinc ions. The 3-mercapto-2-methylpropanoyl moiety was also simplified to produce mercaptoalcohols **4** and **5**, which had IC_{50}s of 15 and 10 μM, respectively. 2,3-Dimercaptopropanol (dimercaprol, British anti-Lewisite [BAL]) **5** is used clinically as an antidote for metal poisoning and may have potential as a lead compound for the preparation of NDM-1 inhibitors. The (*R*)-isomer of the benzyl amide analog **6a** was found to be the most potent NDM-1 inhibitor, with an IC_{50} of 1.5 μM (and more potent than its [*S*]-enantiomer **6b**).

Using a sensitive assay, based upon the hydrolytic cleavage of the fluorogenic dicephalosporinodifluorofluorescein releasing fluorescent difluorofluorescein (Supplementary Figure 8), Proschak and coworkers investigated the MBL (NDM-1, VIM-1 and IMP-7) inhibitory activity of a range of approved drugs containing a thiol group, with D/L-thiorphan **7**, the active metabolite of racecadotril, the peripherally-acting enkephalinase inhibitor, approved in most countries (other than the USA) as an antidiarrheal agent and 2,3-dimercaprol **5** being the most potent (Table 4). Surprisingly, tiopronin **8**, was significantly less active against NDM-1 than against VIM-1 and IMP-7 MBLs [77].

Thiophenecarboxylic acid derivatives **9–12** have been identified as potential inhibitors of NDM-1 by QM/MM modeling (Figure 3) [78]. Their weak inhibitory effect (all IC_{50}s > 100 μM) was confirmed

Table 8. New Delhi metallo-β-lactamase-1 IC_{50}s of sulphonamides 19–21.

Compound	Structure	IC_{50} (μM)
19		37.6 ± 0.9
20		31.4 ± 1.2
21		29.6 ± 1.3

Data taken from [84].

Table 9. Minimum inhibitory concentrations (mg/l) of carbapenems in combination with with 1,4,7-triazacyclononane-1,4,7-triacetic acid 22 (4 mg/l) against *Escherichia coli* expressing different metallo-β-lactamase.

MBL	MIC	
	Meropenem	Imipenem
NDM-1	0.125	1
NDM-4	0.06	0.25
VIM-1	0.06	1
IMP-8	0.06	0.125

MBL: Metallo-β-lactamase; MIC: Minimum inhibitory concentration.
Data taken from [88].

using an *in vitro* enzyme inhibition assay. It has been proposed that the sulfur atom of the thiophene ring interacts with both zinc ions and the oxygen atom of the bridging water in the NDM-1 active site, and that modifying the thiophene side chains to improve interactions with the six zinc coordinating amino acids (His189, His120, His122, Asp124, Cys208 and His250) could lead to more effective NDM-1 inhibitors.

Building upon their earlier discovery of the inhibition of MBLs by thioazoles, Yang and coworkers synthesized a series of diaryl-substituted azolylthioacetamides **13**, **14** and showed that some exhibited inhibition of all four MBLs tested, while others were mixed inhibitors of NDM-1 (Table 5) [79]. Based upon docking studies with the NDM-1 structure from the complex with hydrolyzed benzylpenicillin (PDB 4EYF) [66], these workers propose that the triazole group of **14a** coordinates to both zinc atoms, while the *N*-phenylthioacetamide group and 2-phenolic hydroxyl groups mimic the amido side-chain and 3-carboxylate group of hydrolyzed benzylpenicillin, respectively. The phenolic hydroxyl group is proposed to interact with Lys 211 [79].

Yang and coworkers have also prepared a series of thiophene-containing amino acid thioesters **15** and tested their activity as MBL inhibitors [80]. These compounds are potent inhibitors of the Ambler class B3 L1, but also exhibited activity against CcrA (class B1a), ImiS (B2) and NDM-1 (B1b) (Figure 3 & Table 6).

Breeze, Hu and coworkers designed a ^{1}H NMR spectroscopy assay to monitor meropenem hydrolysis in whole *E. coli* cells producing NDM-1; the conversion of meropenem to its hydrolyzed form can be monitored by changes in the chemical shifts of the C-4 or side-chain methyl groups (MeCHOH- or dimethylamino-). Using this assay, this group confirmed the inhibition of NDM-1 (Table 7) by captopril **1** and identified the chelator EDTA **16** (Figure 4) as a potent inhibitor [81]. Using this assay, this group also discovered thiol-containing amides which are potent NDM-1 inhibitors, with the most potent compound being the tryptophan-containing compound **17a**, which has greater activity than the corresponding *N*-methyl amide **17b** (Figure 3 & Table 7) [82].

Using an MS-based assay, involving either a 'direct' (nano ESI of an incubated mixture of NDM-1 and an inhibitor, detecting the protein–inhibitor complexes) or 'indirect' (protein–ligand complexes were purified, then the ligand dissociated and characterized by HRMS) approach, Chen *et al.* identified some thiol-containing NDM-1 inhibitors, with the most potent being the thiol-containing Cbz-protected amino acid **18** (Figure 3), which has a binding constant (K_d) of 1–2 μM and an IC_{50} of 1.81 ± 0.13 μM [83].

Virtual screening of a collection of drug-like molecules from the ZINC database (2.8 M compounds), after removal of ampicillin and nonconserved water molecules from the structure of NDM-1 in complex with hydrolyzed ampicillin (PDB 3Q6X), identified three sulfonamides **19–21** (Table 8) which are selective NDM-1 inhibitors [84]. These inhibitors exhibited no significant inhibition of SIM-1; pyrimidine **20** also did not inhibit VIM-2, while inhibitors **19**, **21** show greatly reduced inhibition. Molecular dynamics studies of the interaction between the most potent inhibitor **21** and NDM-1 suggest that it interacts extensively with the active site, with the benzoxadiazole group involved in a π–π-stacking interaction with the zinc-coordinating residue His122, and the sulfonamide group interacting with Zn2, Asn220, His120, His 122 and His189, as well as the key bridging water/hydroxide molecule. It is proposed that this interaction with the bridging water molecule may prevent the proton transfer which generates the nucleophile responsible for the attack on the β-lactam ring [84].

Metal complexing agents

As mentioned above, EDTA **16** (Figure 4) is an established MBL inhibitor which functions by chelating the critical Zn^{2+} ions in the active site. Studies have shown

that EDTA is more effective at inhibiting NDM-1 enzymes in comparison to other MBLs, having an IC_{50} of 1.6 μM [81], which is suggestive of a stronger ability to bind NDM-1 Zn^{2+} ions. Despite these promising findings, the toxicity associated with EDTA makes it unsuitable for therapeutic use, but its analogs and complexes have been employed as leads for the design of new NDM-1 inhibitors [85].

EDTA complexed with a calcium ion (calcium disodium EDTA) has been shown to have NDM-1 inhibitory activity – this complex has greatly reduced toxicity and is currently approved as an injection for the treatment of lead poisoning [86,87]. This complex has been shown to considerably reduce the MICs of carbapenems for NDM-1 producing bacteria. In addition, combination therapy with imipenem (IPM) and cilastatin sodium (CS), in a mouse sepsis model, resulted in a greater reduction in bacterial burden in comparison to IPM or CS treatments alone [87].

The restoration of the activity of carbapenems by the metal chelators 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) **22** and 1,4,7,10-tetra-azacyclononane-1,4,7,10-tetra-acetic acid (DOTA) **23** (Figure 4) was investigated by Essack and coworkers (Table 9). NOTA was more effective than DOTA in restoring the activity of meropenem and imipenem (breakpoint values 1 mg/l) and, as is to be expected, this activity was restricted to class B lactamases [88].

ME1071 (disodium 2,3-diethylmaleate) **24** (Figure 4) is a recently patented maleic acid derivative found to inhibit MBLs [89]. The synergism exhibited by ME1071 and any carbapenem was weaker for NDM-1 than for other carbapenemases (IMP, VIM). For example, the combination of ME1071 (32 mg/l) with biapenem caused the mean MIC for Enterobacteriaceae NDM-1 producers to fall (from 7.7 to 3 mg/l). Although this lowering of the MIC was much smaller than that seen for other carbapenemase producers it does lend some support to the potential for combining ME1071 with biapenem to provide synergistic effects in therapeutic applications.

Aspergillomarasmine A

Aspergillomarasmine A (AMA) **25** (Figure 5) is a natural product isolated from an extract of a strain of *Aspergillus versicolor* fungus, which has been found to be a nontoxic and potent, NDM-1 inhibitor [90,91].

AMA **25** was previously investigated as an inhibitor of angiotensin-converting enzyme (ACE, IC_{50} 1.2 μM) [92] and endothelin converting enzyme (IC_{50} 3.4 μM) [93,94]. An inductively coupled MS study confirmed that the mechanism for the AMA inhibition of NDM-1 involves depletion of Zn^{2+} ions in the NDM-1 active site. Furthermore, it has been found that, although inhibition with AMA is irreversible, the addition of excess $ZnSO_4$ to inhibited NDM-1 results in a full restoration of the NDM-1 enzymatic activity [90].

AMA inhibits NDM-1 with a K_i of 11 nM and an IC_{50} of 4.0 ± 1.0 μM; and has been shown to display a synergistic effect with meropenem, fully restoring its activity against NDM-1 or VIM-2 expressing carbape-

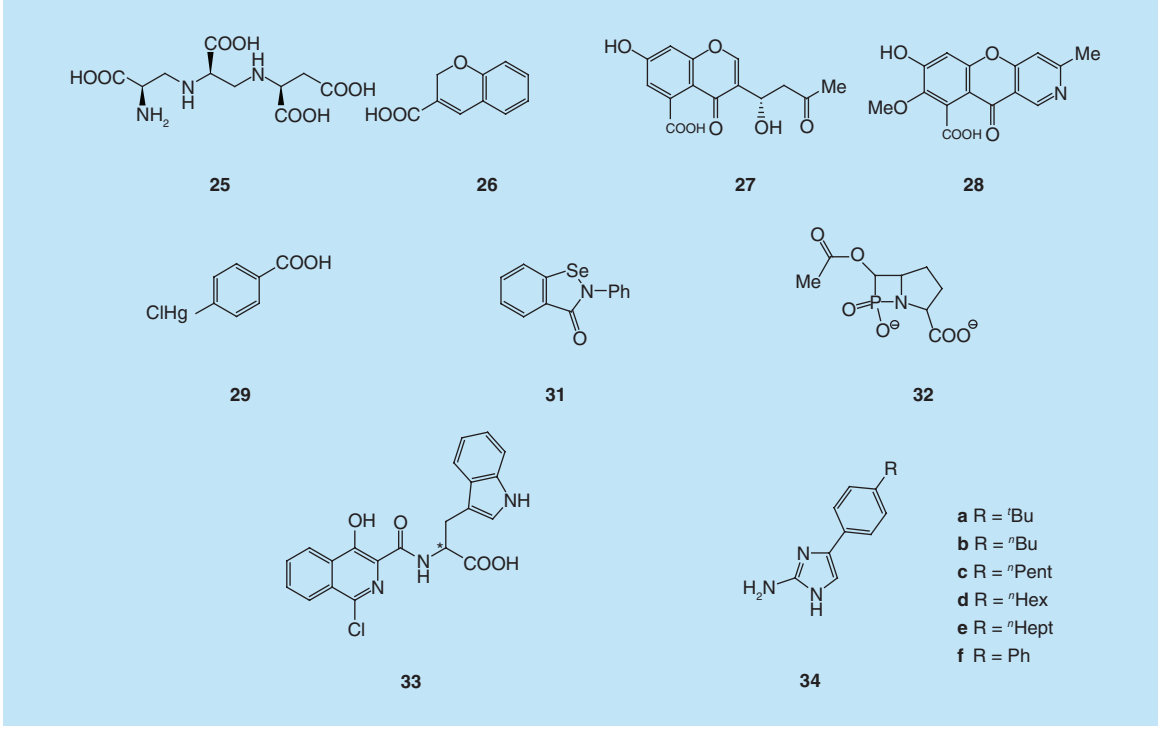


Figure 5. Miscellaneous New Delhi metallo-β-lactamase-1 inhibitors.

Table 10. Structure and IC_{50}s of isoqunolinols.

Isomer	IC_{50} (μM)				
	SPM-1	IMP-1	NDM-1	VIM-2	Bc II
33-(*S*)	23.2 ± 1.1	75.6 ± 1.5	61.4 ± 1.3	54.7 ± 1.4	61.3 ± 1.3
33-(*R*)	46.6 ± 1.1	74.1 ± 1.6	47.1 ± 1.1	55.3 ± 1.3	132.4 ± 1.3

IMP: Imipenemase; NDM-1: New Delhi metallo-β-lactamase-1; VIM-1: Verona integron encoded metallo-β-lactamase-1.
Data taken from [102]. .

nem-resistant Enterobacteriaceae, with little effect on SPM-1, IMP and AIM-expressing MBLs (fractional inhibitory concentration [FIC] indices for clinical CRE isolates ranged from 0.05 to 0.09) [90].

Individually, neither AMA nor meropenem successfully prevent lethal *K. pneumoniae* N11-2218 infection in CD1 mice but, when delivered in combination (both 10 mg/kg), survival rates increased to >95% at 5 days post infection.

Studies of the toxicity of AMA in mice have shown that AMA exhibits low toxicity, with an LD_{50} (lethal dose to 50% of animals) of 159.8 mg/kg; compared with 28.5 mg/kg for EDTA. Furthermore, AMA demonstrated good selectivity for NDM and VIM MBLs, causing only a 35% reduction in rabbit lung ACE activity, and no effect on mean atrial blood pressure in mice [90].

AMA **25** represents a good, nontoxic lead compound, with the potential to be administered as an NDM-1 inhibitor in combination with β-lactam antibiotics such as meropenem, to fully restore antibiotic activity, but a limitation may be its pharmacokinetics – it is highly hydrophilic, with a cLogP of -5.

Chromenes

Few rationally designed NDM-1 inhibitors have demonstrated efficacy at submicromolar levels and this may be due to the fact that the design of successful inhibitors has been impeded by a lack of knowledge regarding the structure, hydrolysis mechanism and conformational changes of NDM-1 [95]. In addition, the flexibility of the positions of the zinc ions in the NDM-1 active site, and the presence of mobile loops makes structure-based drug design more difficult. In an example of the use of *in silico* screening to design MBL inhibitors, Proschak and coworkers utilized a consensus docking protocol, in which the SPECS database was docked into the crystal structures of NDM-1, IMP-1, VIM-2 and VIM-4 using three different software suites (MOE, GOLD and PLANTS). [95] Only those fragments with a RMSD ≤2Å between the predicted binding pose for all three packages were subjected to consensus filtering, in which the predicted binding modes were compared and only the fragments that were placed within RMSD ≤2Å to each other in all four MBLs were retained. As the predicted binding modes in VIM-4 were distinctly different to the other three MBLs, it was omitted from the consensus filtering and the 27 compounds which met the criteria were assessed using the commercially available Fluorocillin assay. To confirm the reversible binding of the most active compound, the chromene **26** (NDM-1 IC_{50} 50 ± 9 μM, binding efficiency index [BEI] = 24) (Figure 5), to NDM-1, saturation transfer difference (STD)-NMR was used, yielding a K_D of 1.7 mM [96].

Two polyketides isolated from *Penicillium* sp. *109F 484* obtained from the rhizosphere soil of the plant *Picea asperata* exhibit weak NDM-1 inhibition (Figure 5). The chromenone **27** and the chromeno[3,2-*c*]pyridine **28** inhibited NDM-1, with IC_{50}s of 94.9 and 87.9 μM, respectively, but neither had any inherent activity (at 256 mg/l) against *K. pneumoniae*-producing NDM-1 nor had any effect on the MIC of meropenem when give in combination (at a concentration of 128 mg/l) [97].

Thiol modifying agents

Approaches that target conserved regions of MBL active sites have also met with drawbacks. For example, a single amino acid substitution of Cys208 by Asp208 resulted in an NDM-1 variant which retained almost full enzymatic activity when treated with Cys208 targeting NDM-1 inhibitors such as the thiol modifying agents *p*-chloromercuribenzoic acid **29** (NDM-1 IC_{50} 2.3 μM; TEM-1 IC_{50} >30 μM) (Figure 5) and sodium nitroprusside ($Na_2Fe[CN]_5NO{\cdot}2H_2O$) **30**, which is indicated for the treatment of severe hypertension (NDM-1 IC_{50} 9.0 μM; TEM-1 IC_{50} >30 μM) [98]. This substitution can arise through the accumulation of two-point mutations, reflecting the ease with which bacteria may develop resistance to such inhibitors.

Ebselen

Ebselen **31** (Figure 5), a selenium-containing molecule which is in clinical trials for the treatment of ischemia and stroke, has also been proposed to target the Cys208 zinc-complexing residue [99]. Co-adminstration of ebselen (which alone has no cytotoxic effect) with ampicillin or meropenem (ebselen:β-lactam

ratio 1.3–1.4:1) resulted in fourfold and 35-fold reductions in the MICs of these β-lactams, respectively. The combination of ebeselen and meropenem in the ratio 14:1 led to a 140-fold reduction in the meropenem MIC. Incubation of NDM-1 with ebselen **31** resulted in an ESI-MS peak corresponding to the addition of ebselen and loss of one zinc atom, confirming the formation of a covalent S–Se bond (ESI, Supplementary Figure 9).

Covalent irreversible inhibition by β-lactams

As we have seen, suicide inhibitors such as clavulanate are ineffective against MBLs, but the irreversible inactivation by supratherapeutic doses of the β-lactams, cefalotin and moxalactam is mediated by covalent bond formation to Cys208, while cefaclor inactivation is mediated by attack by Lys 211 (Supplementary Figure 10) [100], which is a key residue for interaction with the substrate (Table 1). The proposed mechanism involves the hydrolysis of cefaclor by NDM-1, followed by the attack of the amino group of Lys211 on an episulfonium ion and a series of proton transfers to give the covalently linked NDM-1-cefaclor adduct (Supplementary Figure 10).

β-Phospholactam

Mechanistic studies have suggested that the breakdown of the ring-opened β-lactam (nitrocefin) is rate-limiting for NDM-1, so Crowder and coworkers synthesized a β-phospholactam **32** as a tetrahedral transition state analog (Figure 5). After 30 min incubation with MBLs, this phospholactam **32** (100 μM) exhibited weak inhibition of IMP-1, CcrA and L1 (all 70%), Bla2 (94%) and NDM-1 (53%) [101].

4-Chloroisoquinolinols

Using a new fluorescence-based assay for the detection of MBL inhibition, which involves an increase (or decrease) in fluorescence upon hydrolysis of a fluorogenic (fluorescent) cephalosporin-linked coumarin to liberate the 7-hydroxycoumarin anion (ESI, Supplementary Figure 11), Schofield and coworkers identified weak pan-MBL inhibiting 4-chloroisoquinolinols **33** (Table 10) [102].

Aminoimidazoles

Building upon their previous work, Melander and coworkers have identified a series of aminoimidazoles **34** (Figure 5) which have the ability to suppress carbapenem resistance in NDM-1-producing *K. pneumoniae*, by acting synergistically to lower the MICs of both imipenem and meropenem. Treatment with a combination of the lead compound **34d** (30 μM) and these carbapenems resulted in a 16-fold reduction in the MICs, with the aminoimidazole exhibiting little bactericidal activity. This effect was not restricted to MBLs, as combination with imipenem or cefotaxime resulted in reduced MICs for the Class A KPC-2 and ESBL producing *K. pneumoniae* strains, respectively. This synergistic effect may, in part be due to an enhancement in cell permeability upon exposure to the aminoimidazoles [103].

Future perspective

The rapid spread of broad spectrum antibiotic resistance conferred by NDM-1 makes it a very real threat to current antimicrobial chemotherapy and calls for the prioritization of drug design attempts to discover effective antibiotics. The development of increasingly sophisticated virtual compound library screening techniques in recent years may hold great potential in the search for NDM-1 inhibitors [104], and, when coupled with high-resolution NDM-1 crystal structures, may lead to approaches which are able to rapidly and accurately assess binding interactions between NDM-1 and extensive libraries of chemical compounds; thus enabling the rapid identification of better inhibitors [105,106].

At present, molecules with zinc chelating properties have demonstrated the greatest promise [86], as, in addition to acting as NDM-1 inhibitors, such compounds can chelate metal ions present in bacterial cell membranes, thus helping to degrade bacterial biofilms [107]. Many studies also support the potential NDM-1 inhibiting capabilities of molecules which can chelate with zinc via sulfhydryl bonds [56,72,74], although, given the importance of zinc in other enzymes (approximately a third of human proteins are metalloproteins), such as the ACE and matrix metalloproteinase (MMP) endopeptidases, selectivity and nonspecific chelation, together with its associated biological effects, remain significant challenges [108].

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/full/10.4155/fmc-2016-0033

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Executive summary

Introduction

- New Delhi metallo-β-lactamase-1 (NDM-1) is one the most recent additions to the β-lactamases, key contributors to resistance in Enterobacteriaceae.
- NDM-1 was first reported in 2009 in a patient with carbapenem-resistant *Klebsiella pneumoniae* and *Escherichia coli* strains.
- Most clinical NDM-1 producers are only susceptible to the last line antibacterial agents colistin, tigecycline or fosfomycin.

Detecting NDM-1 producing bacteria

- Initial screening for carbapenemase-producing Enterobacteriaceae (CPE) involves carbapenem susceptibility testing
- Follow-up testing of suspected metallo-β-lactamase (MBL) producers can be based upon phenotypic or genotypic methods
- Carba NP can confirm the presence of carbapenemase producers in 30–120 minutes, with high specificity and good sensitivity
- Molecular-based identification of NDM-1 producers uses PCR, matrix-assisted laser desorption/ionization-time-of-flight MS, or UV-based assays
- A proposed flowchart for the detection and characterization of CPE involves the Carba NP test, followed by PCR or DNA microarray, and finally sequencing

NDM-1 variants and structure

- NDM-1 is a class B1 β-lactamase as it has two Zn2+ ions bound in its active site
- NDM-1 consists of 270 amino acids in a single polypeptide chain a typical αβ/βα MBL sandwich fold
- The NDM-1 active site is located in a broad, shallow groove near the edge of two twisted β-sheets
- The active site consists of two zinc ions; and the water molecule/hydroxide ion which complexes to both zinc atoms
- The zinc ions function as the primary substrate recognition complex of NDM-1, coordinating to oxygen atoms found in the carboxyl and carbonyl groups of β-lactam substrate
- NDM-1 has been shown to hydrolyze substrates via the coordination of the β-lactam carbonyl oxygen to Zn1, nucleophilic attack (addition/elimination) by the shared active site hydroxide, protonation of the nitrogen atom, and C-N bond cleavage

NDM-1 inhibitors in development

- Sulfur-containing inhibitors were initially based upon the strong interaction of thiol-containing compounds (e.g. captopril) with zinc
- Metal-chelating inhibitors, such as EDTA, also interact strongly with the active site zinc atom(s)
- Other known inhibitors include the natural product, Aspergillomarasmine A

Future directions

- Virtual library screening techniques may hold great potential in the search for NDM-1 inhibitors
- Zinc chelators have demonstrated the greatest promise as NDM-1 inhibitors
- A significant challenge will be selectivity, given the importance of zinc in other enzymes

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