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REVIEW ARTICLE

Speculative strategies for new antibacterials: all roads should not lead to Rome

Stuart Shapiro

In concert with improvements in personal hygiene and public sanitation, the discovery and development of antibiotics during the latter half of the last century has reduced substantially the morbidity and mortality associated with bacterial diseases. However, the past decade has witnessed a sharp reduction in interest in antibacterial drug development by 'big pharma', compounded by a decline in the breadth of chemical space for new antibacterial molecules and a failure to exploit the plethora of cellular processes potentially targetable by novel classes of antibacterial molecules. This review focuses on some strategies relating to antibacterial chemotherapy, paths less trodden, which the author considers worthy of further exploration.
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The need for new antibacterials to counter the rising tide of pathogen resistance, and the lack of progress to meet this crucial medical need, have been bewailed and discussed at length in the biomedical literature and will not be reiterated here; interested readers are referred to recent reports from the IDSA,[1] ECDC/EMEA,[2] Center for Global Development[3] and WHO.[4] Resistance is an inevitable outcome of the evolutionary dynamic set in motion by exposure of microorganisms to antibiotics; while drug makers may stand athwart their prey declaring 'Resistance is futile!', in fact antibiotic introductions constitute a classic case of *nascentes morimur*, where the best we can do is keep our current antimicrobial armamentarium a step or two ahead of the pathogens until successor antibiotics reach the market. Antibiotic stewardship and prudent prescribing strategies,[5] antibiotic cycling and rational combination therapy can retard the rate at which resistance emerges and disseminates but, given their genetic plasticity and physiologic resourcefulness, microorganisms will always outwit us; to the microbe's credit but our misfortune, reentrenchment of resistance usually occurs sooner rather than later. Thus, continuous identification and development of new, more efficacious products with acceptable safety profiles to combat agents of infection are required if we are to avoid sliding into a post-antibiotic era[6,7] with its ensuant toll in morbidity and mortality.

The author's professional interests in microbiology extend back some forty years. They focused initially on comparative bacterial physiology and enzymology though, during the 1980s, he was privileged to work in the laboratory of Professor Dr Leo C Vining (Biology Department, Dalhousie University, Halifax, Nova Scotia, Canada), where he studied the metabolic regulation of streptomycete antibiotic biosynthesis. Thereafter, his interests migrated from physiological control of actinomycete antibiotogenesis[8] to antibiotic discovery and preclinical development, pursued in both academic and industrial milieux. Over the past two decades he had the good fortune to work on a broad array of antimicrobial types, ranging from botanical and synthetic orochemoprophylactica[9] to 'classic' antibiotic families such as β-lactams, topoisomerase inhibitors, macrolides and dihydrofolate reductase inhibitors. During these years of 'active service' in the anti-infectives industry, he witnessed the transfer of responsibility for antibiotic discovery and development from big pharma to small-to-medium-size pharmaceutical and biotechnology firms often lacking the confluence of experience, creativity and financing to initiate, or to sustain, programs aimed at designing, identifying and commercializing innovative products for the antimicrobial market.

In light of the above, the author seeks to share some personal thoughts about avenues being explored, or remaining to be explored, *vis-à-vis* antibacterial chemotherapy.

HYBRID ANTIBIOTICS: ANTIBACTERIAL OPPORTUNITIES OR ANTIBACTERIAL TOURISM?

The term 'hybrid antibiotic' can refer either to unnatural antibiotics, usually of polyketide origin, produced by microorganisms as a consequence of mixing or altering biosynthetic genes;[10,11] or to synthetic molecules where two (structurally diverse) antibacterial pharmacophores are coupled to form a single molecular entity.[12] The following focuses exclusively on the latter.

Hybrid antibiotics represent a type of combination therapy in which the drug pair, rather than being independent entities, are

Harry Lime Institute for Penicillin Research, Schlettstadterstrasse 21, Basel, Switzerland
Correspondence: S Shapiro, Harry Lime Institute for Penicillin Research, Schlettstadterstrasse 21, CH-4055 Basel, Switzerland.
E-mail: stuart.shapiro@sunrise.ch
Dedicated to the memory of my beloved wife, Corine Michèle Shapiro-Bloch (1956–2013).
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covalently and stably linked. An antibiotic hybrid should at least mimic, if not improve upon, the synergism obtainable when the two different pharmacophores are administered simultaneously as separate drugs. Additionally, it is projected that independent mutations affecting each of the hybrid molecule's targets will be required to compromise completely its clinical value. A further point for consideration is the pharmacodynamics of the hybrid molecule when the poles of the hybrid consist of moieties with dissimilar pharmacodynamic drivers (*vide infra*).

A hybrid antibiotic ought to have the advantage of relatively straightforward pharmacokinetic and safety profiles, obviating the need to 'mix-and-match' pharmacological and toxicological properties that can complicate conventional combination therapy. Nonetheless, 'mix-and-match' problems often can be resolved on the basis of a good understanding of the properties of antibiotic pairs, supplemented by knowledge of the relevant physiological status of the patient (renal sufficiency, hepatic sufficiency, etc.) and prior experience with the same or similar drug combinations as reported in the biomedical literature.[13]

In conventional combination therapy, two independently administered drugs are free to seek their preferred targets. Where the poles of a hybrid antibiotic have to interact with spatially disparate sites, the amount of drug available to reach both targets is reduced, even in a working volume as small as that of a bacterial pathogen, when binding of the hybrid antibiotic to one target precludes simultaneous binding to its alternative target. This 'bilocation dilemma' can be addressed either by administering the hybrid at higher concentrations to facilitate partitioning of the drug between competing targets; or by designing a hybrid whose poles target independent but nearby drug-binding sites (*vide infra*).

One of the earliest attempts to create a hybrid antibiotic was the synthesis by Chu and Bardos[14] of 2(4)-imino-4(2)-amino-2,4-dideoxyriboflavin (Figure 1), a Januskopf structure (lacking a spacer moiety), containing structural features of 2,4-diaminopyrimidine antifolates and riboflavin antagonists. The *in vitro* antibacterial activity of this compound was reversed by exogenous folinic acid or riboflavin. Most experimental hybrid antibiotics consist of a pair of mostly intact antibiotic molecules, tethered by a linker or spacer, in which each antibiotic's binding site for its respective target is preserved.[12,15,16] At least four such products have entered clinical trials:

(i) MCB-3837 (Figure 2), the phosphate ester prodrug of a fluoroquinolone–oxazolidinone hybrid, MCB-3681, invented by the now-defunct Morphochem AG (Basel, Switzerland);
(ii) cadazolid (ACT-179811; Actelion Pharmaceuticals, Ltd., Allschwil (BL), Switzerland) (Figure 2), a fluoroquinolone–oxazolidinone hybrid structurally very similar to MCB-3681;
(iii) CBR-2092 (Figure 3), a fluoroquinolone–rifamycin hybrid invented by the now-defunct Cumbre Pharmaceuticals, Inc. (Dallas, TX, USA); and
(iv) TD-1792 (Figure 4), a cephalosporin–glycopeptide hybrid (Theravance, Inc., South San Francisco, CA, USA).

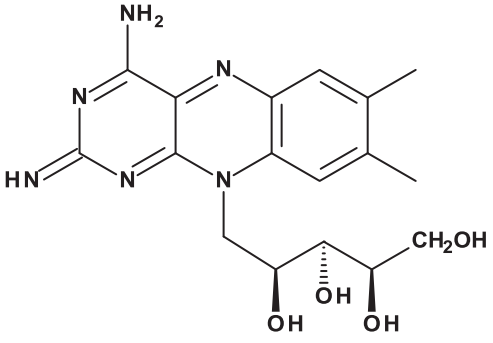

Figure 1 2(4)-Imino-4(2)-amino2,4-dideoxyriboflavin.

As discussed by Pokrovskaya and Baasov,[12] most hybrid antibiotics synthesized to date have a fluoroquinolone at one pole. Fluoroquinolones, exemplified by ciprofloxacin, are bactericidal drugs that target bacterial topoisomerases II and IV, and typically have a broad spectrum of activity that includes pseudomonads and other recalcitrant Gram-negative pathogens. When combined with oxazolidinones or rifamycins, however, the anti-Gram-negative activity of the fluoroquinolone component is compromised, rendering the hybrid predominantly anti-Gram-positive.[12] Loss of activity against Gram-negative pathogens likely is due, in large measure, to the bulk of the hybrids (mw MCB-3681, 627; mw

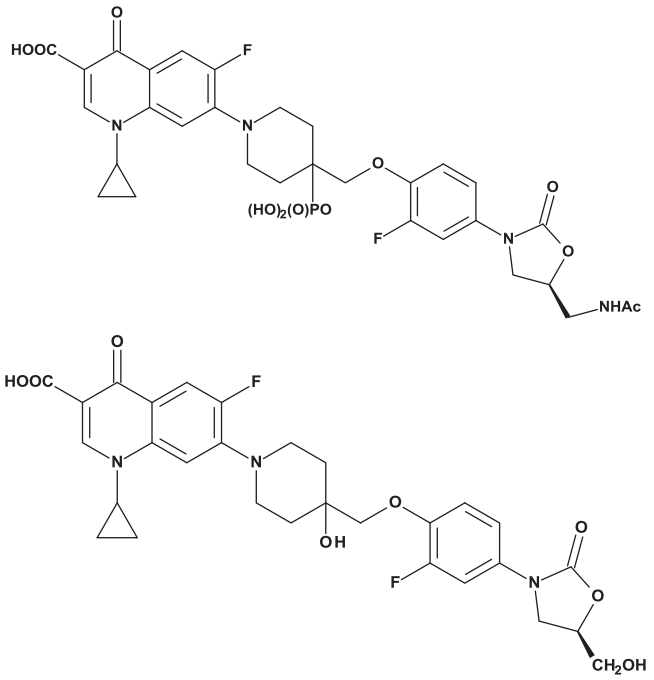

Figure 2 MCB-3837 (upper structure) and cadazolid (ACT-179811) (lower structure).

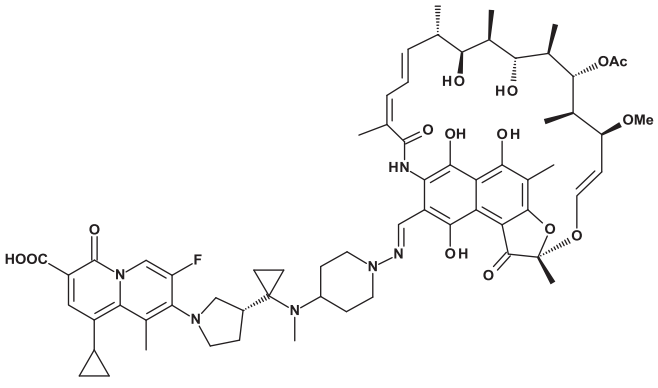

Figure 3 CBR-2092.

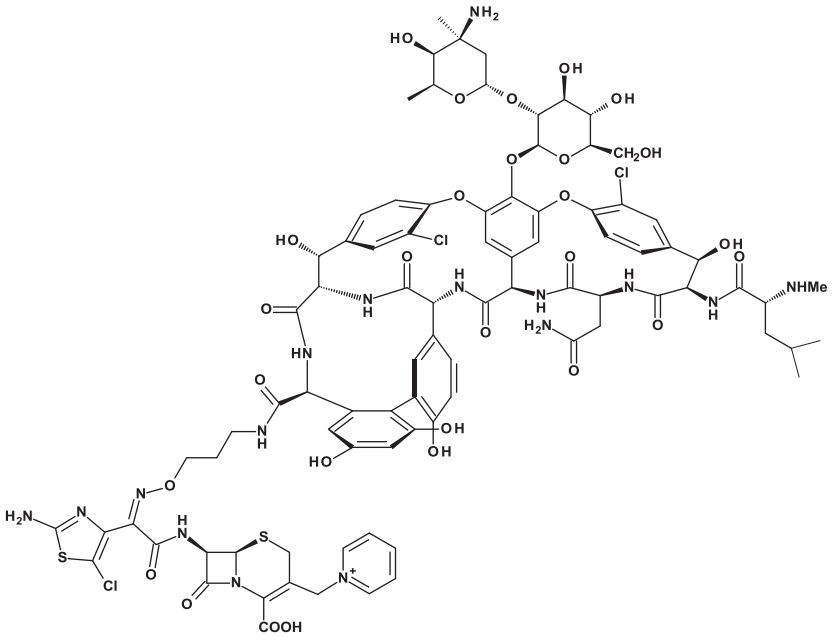

Figure 4 TD-1792.

cadazolid, 586; mw CBR-2092, 1205), which impedes passage through the outer membrane of Gram-negative pathogens, though efflux may have some role, as linezolid,[17] fluoroquinolones[18] and rifampicin[19,20] are substrates for efflux pumps in Gram-negative bacteria. Antibiotics belonging to the fluoroquinolone and rifamycin families have a proclivity for rapid development of endogenous resistance during serial passage in the presence of subinhibitory concentrations of either of these antibiotics, and it was noted by Robertson *et al.*[21] that passage of the CBR-2092-susceptible strain *Staphylococcus aureus* ATCC 29213 (broth MIC, 0.015 μg ml^{-1}) in the presence of CBR-2092 led to elevated MICs towards the hybrid through accumulation of mutations in RNA polymerase (Rpo) and topoisomerases II (Gyr) and IV (Par): 0.125 μg ml^{-1} by passage 2 (*rpoB*^{R484H}), 0.5 μg ml^{-1} by passage 5 (*rpoB*^{R484H}*gyrA*$^{\Delta L520}$), culminating at 64 μg ml^{-1} by passage 26 (*rpoB*^{R484H}*gyrA*$^{\Delta L520,\ S84L}$*parC*$^{R236(duplication),H103Y}$).

Cadazolid was supposed to have been introduced at the 22nd European Congress of Clinical Microbiology and Infectious Diseases in April 2012,[22] but actually debuted some months later during the 52nd Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC). The compound presumably is devoid of clinically useful anti-Gram-negative activity (except perhaps towards some *Bacteroides* spp., for which an MIC_{90} of 4 μg ml^{-1} was reported[23]), and it is in development for treatment of infections caused by *Clostridium difficile*, for which an MIC_{90} of 0.25 μg ml^{-1} was obtained.[23,24] Oxazolidinones (for example, linezolid) and fluoroquinolones generally have good oral bioavailability, but that of cadazolid is negligible.[25] Cadazolid is active against both linezolid- and moxifloxacin-resistant strains of *C. difficile*, and population analyses suggest that the hybrid molecule has a low frequency of resistance towards this pathogen. Serial passage on subinhibitory concentrations of cadazolid produced strains with MICs 1 $\log_2$ dilution step higher towards this compound than those of the parents,[23] though with <10 serial passages having been performed it is premature to reach firm conclusions about the lack of proclivity for endogenous resistance emergence of *C. difficile* towards cadazolid. The principal mode of action of this oxazolidinone–fluoroquinolone hybrid is the inhibition of protein synthesis rather than of DNA synthesis,[26] though different oxazolidinone–fluoroquinolone hybrids reportedly can target preferentially either topoisomerase or the ribosome.[27] Cadazolid has MIC_{90}s towards methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci of 0.25 μg ml^{-1} and 2 μg ml^{-1}, respectively,[23] making it of prospective clinical interest for treatment of systemic infections caused by these pathogens. However, the product seems to be in development exclusively for *C. difficile* infections, inviting questions about the systemic stability or toxicity of the drug, especially in the light of oxazolidinones being well-known mitotoxicants,[28] and fluoroquinolones being associated with a broad array of adverse effects[29–32] and increased risk of *C. difficile* infection.[33] Unfortunately, issues relating to safety pharmacology and toxicology of parenteral cadazolid have not been reported. Insofar as cadazolid, scheduled to commence a Phase III trial this year (http://www1.actelion.com/en/our-company/news-and-events/index.page?newsId=1666815), is being positioned narrowly as an oral treatment for *C. difficile* infection, it may be expected to encounter fierce competition, not only from the current generic standards of care, metronidazole and vancomycin, but also from a recently approved drug, fidaxomicin (lipiarmycin, a macrocycle), and numerous products in development addressing the same niche, including ramoplanin (a lipoglycodepsipeptide), surotomycin (formerly CB-183,315, a lipopeptide), rifaximin (an ansamycin), NVB302 (a lantibiotic) and LFF571 (a thiopeptide). Moreover, antibiotic treatment of *C. difficile* infection eventually may be supplanted by fecal transplant therapy.[34]

In 2009, Long and Marquess[15] published a table of hybrid antibiotics gleaned from the patent literature, to which may be added the aminoglycoside–fluoroquinolone hybrids of Pokrovskaya *et al.*,[35] the mutilin–fluoroquinolone hybrid of Asahina *et al.*,[36] and the cephalosporin–triclosan hybrid of Li *et al.*[37] This plethora of preclinical structures conveys the impression that efforts to create hybrid antibiotics often amount to little more than random pairing, where compounds with known antibacterial activity are combined arbitrarily in the hope of obtaining hits that will lead to clinical

candidates. Without denigrating the ingenuity and resourcefulness of the medicinal chemists tasked with synthesizing hybrid antibiotics, one is left with a nagging feeling that this effort could be directed both more rationally and more successfully.

One strategy is to circumvent the 'bilocation dilemma' by selecting pairs of antibiotics binding to independent targets in close proximity to one another. Such an approach was pursued by Theravance, Inc., whose scientists reasoned that tethering cephalosporins, which target the D,D-transpeptidase activity of penicillin-binding proteins in the bacterial periplasm (or 'inner wall zone'), to glycopeptides, which bind the terminal D-alanyl-D-alanine moiety of a lipid-bound periplasmic peptide involved in cell wall crosslinking, would yield hybrids where both poles can bind simultaneously to their targets. Synergism has been documented for combinations of β-lactam antibiotics with glycopeptides towards glycopeptide-resistant enterococci,[38,39] and when vancomycin and cephalosporins were attached at different positions, all of the hybrids exhibited synergistic antibacterial activity. That the specifics of attachment of vancomycin to cephalosporin had little impact on activity implies that the two poles of a single dimer do not bind simultaneously to their different targets,[40] the observed synergism arising from inhibition at successive steps in the same pathway, as occurs when sulfamethoxazole, a dihydropteroate synthetase inhibitor, is combined with trimethoprim, a dihydrofolate reductase inhibitor[41] (also see Osborne *et al.*[42]). The compound which Theravance progressed into clinical development, TD-1792, is a heterodimer of vancomycin and a ceftazidime-like β-lactam, bactericidal towards MRSA and heterogeneous vancomycin-intermediate *S. aureus* (hVISA), with an MIC towards these staphylococci of 0.03 μg ml^{-1},[43] as well as an MIC towards *C. difficile* of 1 μg ml^{-1}.[44] The pharmacodynamic driver for efficacy of vancomycin is AUC/MIC, whereas that of cephalsoporins is t > MIC.[45] In a murine neutropenic thigh model of MRSA infection, Hegde *et al.*[46] found that AUC/MIC was a better predictor of TD-1792 efficacy than t > MIC. Of course, the spectrum of a molecule of this size (mw TD-1792, 1983) is restricted to Gram-positive pathogens. Results of a Phase II trial of TD-1792 in complicated skin and soft tissue infections have been published.[47]

Besides periplasmic constituents, another target of potential interest for rationally designed hybrid antibiotics is the ribosome. High-resolution X-ray structures of antibiotics bound to the 50S ribosomal subunit of bacteria[48–52] showed overlap between the binding sites of erythromycin, clindamycin and chloramphenicol, and it is likely that, for most eubacteria, conservation of ribosome structure across species and genera translates into similarities in modes of binding of these antibiotics to the peptidyl transferase center or exit tunnel of the ribosome.

Even before high-quality crystallographic data for antibiotics bound to bacterial ribosomes were available, biochemical studies[53,54] had indicated that erythromycin, clindamycin and chloramphenicol shared nearby, probably overlapping, binding sites, which led some groups, such as that of Jiří Zemlička at the Wayne State University School of Medicine (Detroit, MI, USA) to rationally design hybrid antibiotics with improved binding affinities for ribosomes. In the early 1980s, Zemlička and Bhuta[55] reported synthesis of sparsophenicol (Figure 5), derived from chloramphenicol and sparsomycin (a product of *Streptomyces sparsogenes*), which strongly inhibited protein synthesis *in vitro*, but was devoid of antibacterial activity. A decade later, Zemlička *et al.*[56] reported synthesis of chloramlincomycin (Figure 6), in which the aliphatic arm of chloramphenicol is linked to the pyranoside of clindamycin; and lincophenicol (Figure 6), in which most of the chloramphenicol

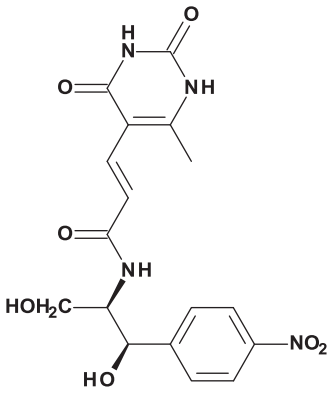

Figure 5 Sparsophenicol.

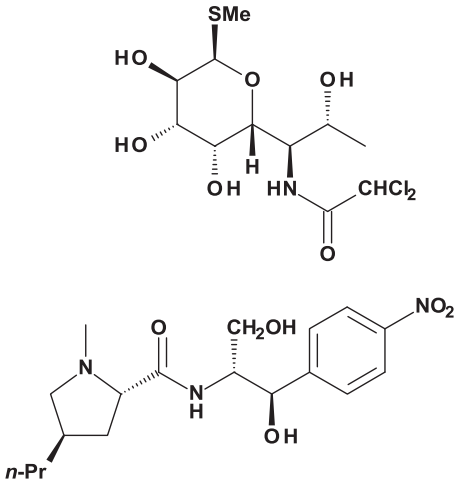

Figure 6 Chloramlincomycin (upper structure) and lincophenicol (lower structure).

molecule is linked to the pyrrolidine of clindamycin. Neither hybrid molecule inhibited polyphenylalanine biosynthesis in an *in vitro* system prepared from *Escherichia coli* though, surprisingly, chloramlincomycin had an MIC of 6.25 μg ml^{-1} vs a strain of *Streptococcus pyogenes* towards which chloramphenicol had an MIC of 3.13 μg ml^{-1}.

Guided by precise information about antibiotic binding to ribosomes afforded by X-ray analysis, scientists at Rib-X Pharmaceuticals, Inc. (New Haven, CT, USA) synthesized Rx-2102 (Figure 7), a hybrid of florfenicol and azithromycin connected by an 4-(*n*-propyl)-1,2,3-triazole spacer, with low MICs ($\leqslant$1 μg ml^{-1}) towards not only a wild-type pneumococcus, but also towards pneumococcal strains resistant to azithromycin due to an A2058G (*E. coli* numbering) transition and to A2058 monomethylation + an L4 mutation.[52]

While hybrid antibiotics are, by definition, heterodimers, some effort also has been directed towards the rational design of homodimers. Vancomycin, for example, forms a dimer in aqueous solution, and dimerization of this glycopeptide is enhanced in the presence of the cell wall analog di-*N*-acetyl-L-Lys-D-Ala-D-Ala. On the presumption that vancomycin dimerization has an important role in its antibiotic activity,[57] medicinal chemists have synthesized vancomycin dimers as prospective antibiotics. Griffin *et al.*[58] systematically probed the effect of linkage orientation and linker length on antibacterial potency towards Gram-positive cocci, and observed that both

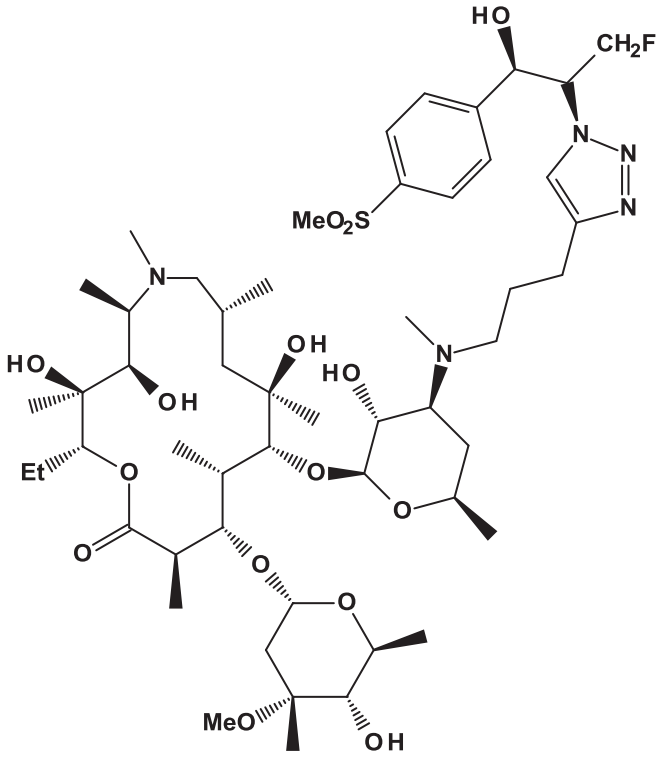

Figure 7 Rx-2102.

parameters influence the *in vitro* activity of vancomycin dimers, though no single dimer proved uniformly more potent than all other dimers (or vancomycin, for that matter) against pneumococci, enterococci and *S. aureus* displaying various resistotypes.

The considerable investment of intellectual and material resources to date has generated few hybrid antibiotics that can be considered 'clinical trial-worthy'. It is likely that the success rate could be improved substantially if laboratories would forego the temptation to pressgang into hybrids whichever antibiotics pass their way, and instead focus on innovative products that try to address the 'bilocation dilemma', as Zemlička, Theravance and Rib-X have sought to do. Moreover, given the particularly urgent need for new drugs targeting Gram-negative pathogens, medicinal chemists would do well to apply principles of rational drug design to tether pharmacophores whose combined size (and/or membranotropic properties) enable the hybrids to surmount the barrier posed by the outer membrane.

POLYMYXINS AND NEOPOLYMYXINS: BACK TO THE FUTURE?

The alarming increase in incidence of infections attributable to multidrug-resistant Gram-negative pathogens has led to a resurgence in the use of an old antibiotic class, the polymyxins. These cationic lipocyclopeptides, products of *Bacillus* spp., were discovered during the 1940s and introduced into the clinic during the 1950s, but superseded by other drugs with anti-Gram-negative activity due to toxicity issues, including allergenicity, neurotoxicity, and most notably nephrotoxicity.[59] Two generic polymyxin products are available, polymyxin B and colistin (polymyxin E). While generally thought to be interchangeable, there are some differences between these products. Polymyxin B is administered intravenously as the active principle, whereas colistin is administered as a sulfomethylated prodrug to reduce its toxicity.[60] The two antibiotics often are considered to be equivalent, though Elemam *et al.*[61] identified KPC-producing *Klebsiella pneumoniae* strains more susceptible (up to 4 $\log_2$ dilution steps) to colistin than to polymyxin B. Moreover, polymyxin B and colistin may have different clearance mechanisms.[62]

During the past decade, efforts have been made to modulate the renotoxicity associated with polymyxins without compromising their pharmacodynamic properties by using 'rational' dosing regimens.[63–66] However, internationally accepted guidelines for use of polymyxins to treat serious Gram-negative infections have yet to emerge from these studies.

An alternative to improved dosing is the discovery and development of new polymyxins unencumbered by the toxicities associated with colistin and polymyxin B. At the 50th ICAAC in 2010, Cubist (Lexington, MA, USA) and BioSource Pharm, Inc. (Spring Valley, NY, USA) presented a dozen posters about a novel polymyxin, CB-182,804 (Figure 8), which has been followed up by a single article devoted to the product.[67] Towards a collection of over 5400 clinical isolates of Enterobacteriaceae (*E. coli*, *K. pneumoniae*, *Enterobacter* spp.) and non-fermentative bacilli (*Acinetobacter baumannii*, *Pseudomonas aeruginosa*), MIC_{90}s for CB-182,804 were higher than those for polymyxin B by 2 $\log_2$ dilution steps;[67] moreover, the data presented at ICAAC failed to demonstrate convincingly that the nephrotoxic potential of CB-182,804 was diminished significantly compared with that of polymyxin B. Since 2010 there has been no update on the status of CB-182,804 on Cubist's website and, absent evidence to the contrary, it is likely that the development program for this product has been shelved.

Others have pursued a more rational and systematic approach to the discovery of safer polymyxin derivatives, predicated on the assumption that cationicity is an important determinant of aminoglycoside nephrotoxicity,[68,69] though Bosmans and De Broe[70] remark that aminoglycoside nephrotoxic potential does not coincide (strictly) with cationic load. Northern Antibiotics Oy (Helsinki, Finland) has created synthetic polymyxins, or 'neopolymyxins', modeled after polymyxin B and colistin, bearing net charges over the range $+2$ to $+5$. When a subset of these compounds was assayed for binding affinity to rat renal cortex brush border membranes,[71] an approximately linear relationship between cationic load and membrane affinity was obtained (Figure 9) (M Vaara and T Vaara, personal communication).[72]

Most of Northern Antibiotic's efforts have focused on NAB739 (Figure 8), a neopolymyxin modeled after polymyxin B but with a net charge of $+3$. The relatively modest affinity of this synthetic lipocyclopeptide for rat renal brush border membranes suggests a reduced potential for inducing acute kidney injury, consistent with pharmacokinetic results, where bolus administration in Sprague-Dawley rats led to a much higher renal clearance ($0.53 \pm 0.30\,ml\,min^{-1}\,kg^{-1}$ vs $0.010 \pm 0.008\,ml\,min^{-1}\,kg^{-1}$) and urinary recovery ($19.40 \pm 7.38\%$ of dose vs $0.18 \pm 0.14\%$ of dose) for NAB739 than for colistin.[73]

No cytotoxicty towards Chinese hamster lung fibroblasts was observed for NAB739 at concentrations up to $128\,\mu g\,ml^{-1}$.[72] Studies with porcine renal proximal tubule epithelial cells showed that the concentration of NAB739 required to induce necrosis in this cell line was nearly an order of magnitude higher than that required for polymyxin B.[74] More recently, Vaara and Vaara[75] found that the IC_{50} of NAB739 towards HK-2, an immortalized human proximal tubule cell line that does not require electroporation for susceptibility to polymyxins, was $337\,\mu g\,ml^{-1}$, whereas IC_{50}s for polymyxin B and colistin were $13\,\mu g\,ml^{-1}$ and $45\,\mu g\,ml^{-1}$, respectively.

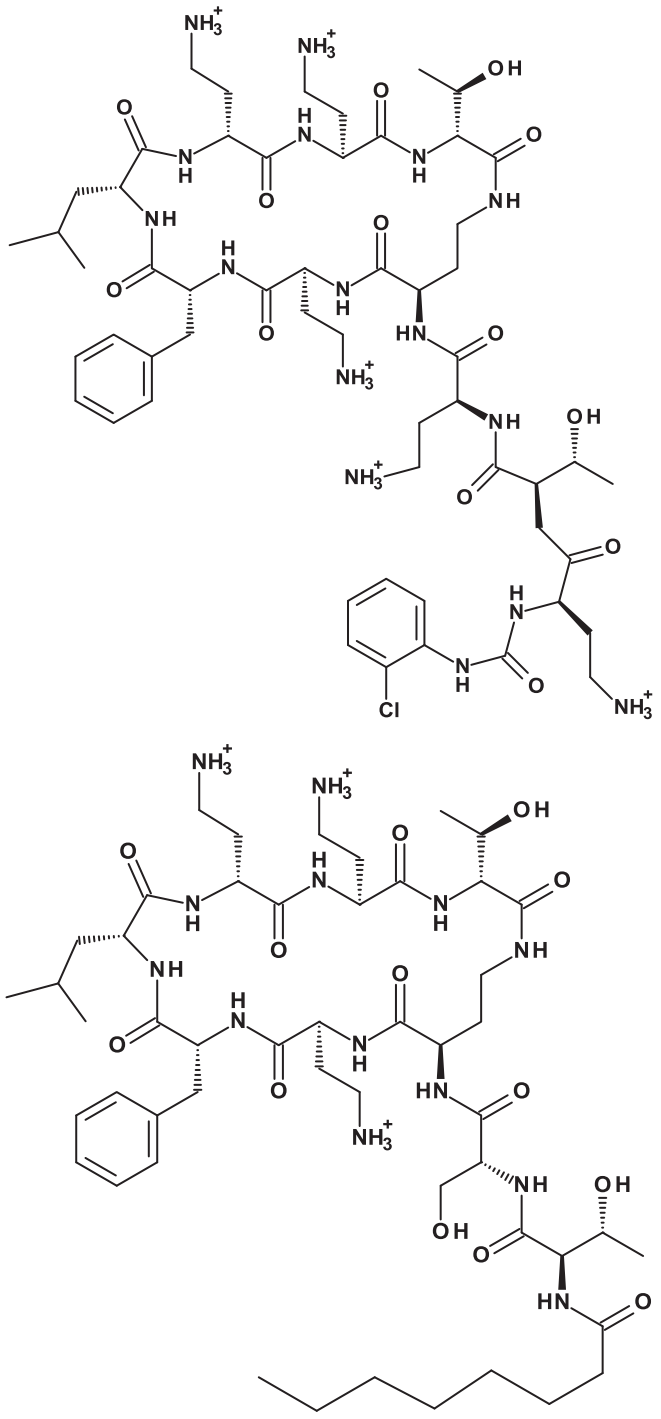

Figure 8 CB-182,804 (upper structure) and NAB739 (lower structure).

MIC_{90}s ($\mu g\,ml^{-1}$) for NAB739 were 2, 2, 8 and 16 towards *E. coli*, *K. pneumoniae*, *Acinetobacter* spp., and *P. aeruginosa*, respectively, compared with 2, 1, 2 and 2 for polymyxin B.[76] In a mouse model of intra-abdominal infection with *E. coli* IH3080 (O18:K1:H7),[77] NAB739 dosed at $4\,mg\,kg^{-1}$ proved approximately as effective, in terms of change in log_{10} CFU per peritoneum 6 h after initial treatment, as polymyxin B dosed at $2\,mg\,kg^{-1}$.

The neopolymyxin congeners invented by Northern Antibiotics remain at an early stage of preclinical development. While the antibacterial activity of NAB739 towards *E. coli* and *K. pneumoniae* approximates that of polymyxin B, its activity towards *A. baumannii* and *P. aeruginosa* is noticeably less. However, if the safety profile of NAB739 proves disproportionately better than that of colistin or polymyxin B, then the therapeutic window for NAB739 may be wide enough to enable its use for treatment of serious infections attributable to non-fermentative Gram-negative bacilli, especially in renally compromised patients unable to tolerate an additional nephrotoxic burden. The outcome of a thorough preclinical toxicology study of NAB739 (and its 'colistin analog', which may be even less nephrotoxic than NAB739) in rodent and non-rodent species, encompassing the latest information about renal biomarkers, is awaited eagerly.

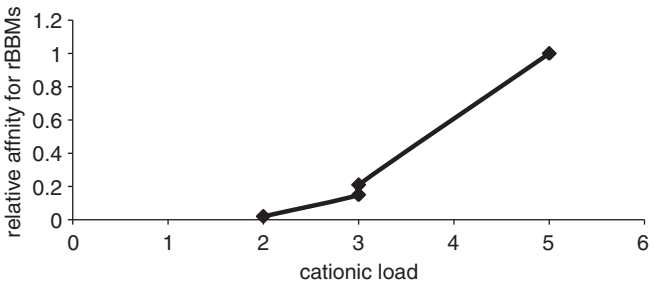

Figure 9 Cationic load vs (neo)polymyxin affinity for renal brush border membranes (rBBMs), normalized to polymyxin B (affinity = 1). A full color version of this figure is available at *The Journal of Antibiotics* journal online.

ANTIMETABOLITES: TIME FOR A RENAISSANCE?

An antimetabolite is a compound whose structural resemblance to an essential metabolite (biosynthetic intermediate or end product) enables it to interfere with one or more essential physiological processes, leading to bacteriostasis or bactericidality. The first synthetic antibiotic, Prontosil, was an antimetabolite prodrug whose active principle, sulfanilamide, resembles *p*-aminobenzoic acid and is a competitive inhibitor of dihydropteroate synthetase.[78] While nucleotide analogs have been introduced as antivirals (for example, azidothymidine) and a single antifungal antimetabolite (5-fluorocytosine) has been approved for therapeutic use, the only antimetabolites used to treat bacterial infections are sulfa drugs and trimethoprim. As these interfere with different steps in the pathway leading to folic acid ('vitamin B_9'), sulfa drugs, especially sulfamethoxazole, and trimethoprim often are combined into a single orally available medication.

During the past 70 years, hundreds of antimetabolites (analogs of nucleosides, amino acids, carbohydrates, lipids, vitamins and so forth) have been synthesized or isolated from natural sources, but few of these have achieved marketing approval by health regulatory agencies, sometimes for reasons of toxicity, and sometimes due to lack of efficacy during clinical trials. However, an urgent need for new antibiotics to treat refractory multidrug-resistant infections warrants reconsideration of the antimetabolite approach as a source of new antibacterials. The success of sulfonamides and trimethoprim as inhibitors of the biosynthesis of folic acid raises the question of whether this strategy can be applied successfully to other vitamins.

Consider, for example, biotin ('vitamin H', 'vitamin B_7'), a small sulfur-containing molecule that acts as a carboxyl shuttle in physiological carboxylations, biosynthesized by microorganisms and plants but not mammals.[79] During the early 1950s, Grundy *et al.*[80] isolated from the spent medium of *Streptomyces virginiae* a small molecular

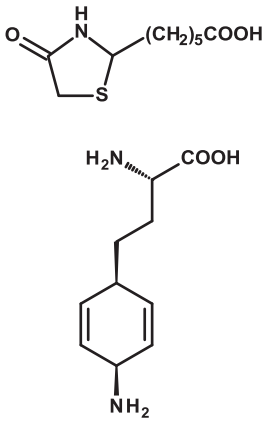

Figure 10 Actithiazic acid (acidomycin) (upper structure) and amiclenomycin (lower structure).

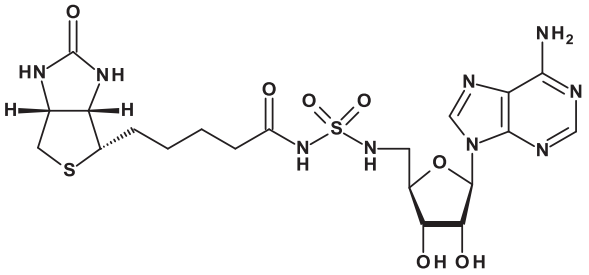

Figure 11 5′-Amino-5′-*N*-(biotinyl)sulfamoyl-5′-deoxyadenosine.

weight compound, actithiazic acid (acidomycin) (Figure 10), with *in vitro* antimycobacterial activity. Twenty years later, scientists at the Institute of Microbial Chemistry in Tokyo[81,82] isolated from culture filtrates of *Streptomyces lavendulae* subsp. *amiclenomycini* an L-amino acid, amiclenomycin (Figure 10), whose *in vitro* inhibitory activity, restricted to mycobacteria, was reversible by exogenous biotin (whereas amiclenomycin originally was proposed to have a *trans* structure, Mann *et al.*[83] showed that it has a *cis* structure). Eisenberg and Hsuing[84] attributed the action of actithiazic acid to inhibition of biotin formation from dethiobiotin by biotin synthase (BioB), though just how actithiazic acid blocks the formation of the tetrathiophene ring of biotin remains obscure. In 2005, Mann *et al.*[83] described how amiclenomycin reacts with pyridoxal-5′-phosphate in the active site of BioA (7,8-diaminopelargonic acid aminotransferase), an enzyme involved in an early step of biotin biosynthesis, to form an aromatic adduct that remains tightly bound to the active site, thereby aborting biotin synthesis. A study by Shi and Aldrich,[85] using synthetic analogs of amiclenomycin, indicated that inhibitory activity is dependent upon the ring moiety being planar rather than puckered.

Thus, already 40 years ago, there was evidence that some microbial natural products affecting biotin biosynthesis selectively inhibited mycobacteria. The past decade has witnessed a renewal of interest in antibiotins as a potential means of treating multidrug-resistant and extremely drug-resistant tuberculosis. Park *et al.*[86] reported that deletion of the gene encoding BioA makes *M. tuberculosis* dependent on exogenous biotin, with little or no growth at $<0.006\,\mu g\,ml^{-1}$ of biotin and wild-type growth at $>0.06\,\mu g\,ml^{-1}$. As the biotin titer in human serum is $\sim 0.0005\,\mu g\,ml^{-1}$, *M. tuberculosis* cannot acquire sufficient biotin from a human host to establish and maintain an active infection, so the disease state is dependent upon *de novo* synthesis of biotin by the pathogen. The authors remark that the conclusion of Kitahara *et al.*[82] that the ability of *M. tuberculosis* to establish and maintain an infection is independent of *de novo* biotin synthesis is an artifact of amiclenomycin's poor pharmacokinetic behavior, including spontaneous aromatization.

Rather than targeting biotin biosynthesis *per se* (for example, Soares da Costa *et al.*[87]), some research groups have focused on biotin protein ligase, which catalyzes attachment of biotin to the ε-amino group of a conserved lysyl residue in biotin-dependent enzymes. Towards this end, Duckworth *et al.*[88] synthesized a bisubstrate, 5′-amino-5′-*N*-(biotinyl)sulfamoyl-5′-deoxyadenosine (Figure 11), modeled after biotinyl-AMP formed by biotin protein ligase. The bisubstrate has an MIC $\leqslant 0.5\,\mu g\,ml^{-1}$ towards *M. tuberculosis* strains, with very low toxicities towards Vero (African green monkey kidney epithelial) cells and DU145 (human prostate cancer) cells, but was inactive (MICs $>256\,\mu g\,ml^{-1}$) towards Gram-positive cocci, Gram-negative bacilli and yeasts, possibly due to restricted uptake and/or efflux.

In contrast to biotin, mammalian cells possess the genetic machinery (localized to their mitochondria) for synthesis of *R*-(+)-α-lipoic acid, a coenzyme involved in decarboxylation reactions. Lipoic acid biosynthesis proceeds through attachment of octanoate to the lipoyl domain (the E2 subunit of α-ketoacid dehydrogenases or the H subunit of glycine decarboxylase) of a lipoate-dependent enzyme, followed by sulfurylation to generate a dithiolane ring. Endogenous lipoate synthesis is essential for mouse embryonic survival,[89] though the extent to which mammalian cells generally can satisfy their need for lipoate through *de novo* synthesis is unclear. Bacteria and mammalian cells possess lipoate protein ligases, that recycle lipoate released during protein degradation by attaching it to empty lipoyl domains, either directly or in concert with an amidotransferase.[90] Analogous to the reaction catalyzed by biotin protein ligase, lipoate attachment through the action of lipoate protein ligase proceeds through a lipoyl-AMP intermediate.[91]

Certain bacterial pathogens, such as *S. aureus*, *Enterococcus faecalis*, *S. pyogenes*, *Chlamydia trachomatis* and *Listeria monocytogenes*, are lipoate auxotrophs,[92–94] whereas *Burkholderia pseudomallei*, the causative agent of melioidosis, seems to have a complete pathway for lipoate biosynthesis but requires exogenous lipoate for virulence in mice and for optimal intercellular spreading.[95] Inhibition of lipoate protein ligase by a bisubstrate analog of lipoyl-AMP might afford a potent, possibly narrow-spectrum chemotherapeutic agent for treating infections attributable to certain pathogens. As of this writing, no such inhibitors have been described.

In contrast to biotin and α-lipoic acid, which in their catalytically active form always are covalently bound to their apoenzymes, the riboflavin (vitamin B_2, vitamin G)-derived coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) usually are not covalently attached to their apoenzymes.[96] Where flavinylation of an amino acid residue occurs, it will have proceeded without the intercession of a dedicated flavin protein ligase.[97,98] Free riboflavin is devoid of bioactivity; it must be 5′-phosphorylated by flavokinase to FMN or further adenylylated by FAD synthetase to generate enzymically-active species, which participate in diverse biochemical processes, mostly redox reactions.

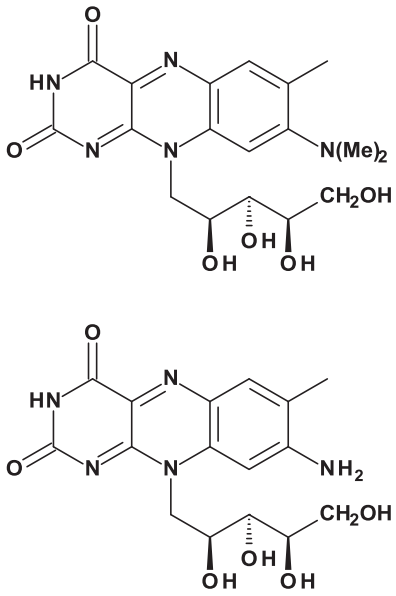

Figure 12 Roseoflavin (upper structure) and 8-demethyl-8-aminoriboflavin (lower structure).

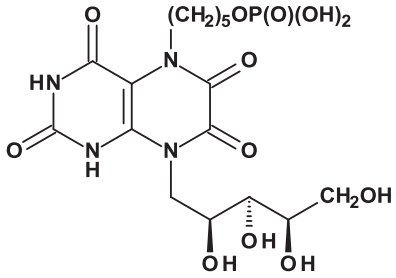

Figure 13 5-(1,5,6,7-Tetrahydro-6,7-dioxo-8-D-ribityllumazin-5-yl-)pentane 1-phosphate.

The riboflavin biosynthetic pathways in microorganisms and plants are well established.[99] Riboflavin auxotrophy is rare among bacteria, but riboflavin is an essential growth factor for some Gram-positive pathogens, including *E. faecalis*, *S. pyogenes* and *L. monocytogenes*;[100] whereas some Gram-negative species, such as *E. coli* and *Salmonella* spp., lack flavin uptake systems and are dependent on endogenous riboflavin synthesis.[101] Morgunova *et al.*[102] have argued that *de novo* biosynthesis of riboflavin probably is essential for survival of pathogenic mycobacteria.

Streptomyces davawensis produces roseoflavin (Figure 12),[103] the only known natural riboflavin analog with antibiotic activity, which targets the FMN riboswitch in some bacteria.[104,105] Roseoflavin is a substrate for human flavokinase and human FAD synthetase, whereas its biosynthetic precursor, 8-demethyl-8-aminoriboflavin (Figure 12),[106] is a substrate only for human flavokinase. As most flavoproteins use FAD rather than FMN as their coenzyme,[96] Pedrolli *et al.*[107] suggested that 8-demethyl-8-aminoriboflavin might be a better lead structure for development of novel flavin antibacterials, though targeting the biosynthetic pathway for riboflavin might prove a better strategy, as this pathway is lacking in mammals.

Most work on antiriboflavins has focused on the last two enzymes of the riboflavin pathway: lumazine synthase, which condenses 3,4-dihydroxy-2-butanone-4-phosphate with 6-(1-D-ribitylamino)-5-aminouracil to form 6,7-dimethyl-8-ribityllumazine; and riboflavin synthase, which combines two molecules of 6,7-dimethyl-8-ribityllumazine to yield one molecule of riboflavin and one molecule of 6-(1-D-ribitylamino)-5-aminouracil. In the course of a longstanding research effort aimed at deciphering the mechanisms of these enzymes, Cushman *et al.*[108] obtained a bienzyme competitive inhibitor, 5-(1,5,6,7-tetrahydro-6,7-dioxo-8-D-ribityllumazin-5-yl-) pentane 1-phosphate (Figure 13), with K_i values of 13 μg ml^{-1} for *Bacillus subtilis* lumazine synthase, 0.006 μg ml^{-1} for *M. tuberculosis* lumazine synthase, and 0.07 μg ml^{-1} for *E. coli* riboflavin synthase. A lumazine synthase/riboflavin synthase dual inhibitor should confer a therapeutic advantage over a monoenzyme inhibitor, with vitamin synthesis constricted at two bottlenecks, requiring mutations in two enzymes to achieve full resistance. Results of whole-cell assays with this compound were not presented, and such compounds might encounter difficulties gaining access to bacterial cytoplasm. In a follow-up study, Cushman *et al.*[109] used high-throughput screening of a commercial chemical library to identify [5-(4-chlorophenyl)-5-hydroxy-3-(trifluoromethyl)-4,5-dihydro-1*H*-pyrazol-1-yl](*m*-tolyl) methanone (Figure 14), a noncompetitive inhibitor of *M. tuberculosis* riboflavin synthase (K_i, 8.4 μg ml^{-1}), with an MIC (microplate Alamar Blue assay) towards this pathogen (strain H_{37}Rv, replicating phenotype) of 14 μg ml^{-1}.

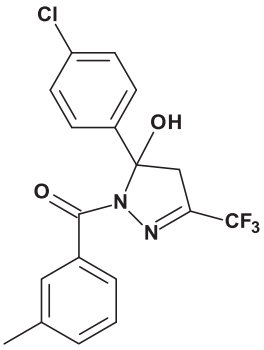

Figure 14 5-(4-Chlorophenyl)-5-hydroxy-3-(trifluoromethyl)-4,5-dihydro-1*H*-pyrazol-1-yl](*m*-tolyl)methanone.

Besides the examples given here, other coenzymes, such as thiamine,[110–113] CoA[114–116] and menaquinone,[117,118] have been examined as potential targets for antibacterial therapy, and it remains to be seen which, if any, of these avenues will attract adequate interest, manpower, and financing to support a sustained research effort.

RIBOSOMAL METHYLTRANSFERASE INHIBITORS: EMBRACING EPIGENICITY?

Target modification by methylation of rRNA constitutes an efficient means by which bacteria achieve resistance to ribosomotropic antibiotics. Gram-positive and Gram-negative species can harbor different families of enzymes catalyzing transfer of methyl moieties to strategic sites on bacterial ribosomes, resulting in high levels of resistance towards a broad spectrum of antibiotics.

Aminoglycoside-modifying enzymes remain the predominant mechanism of resistance to this class of antibiotics, though the past decade has seen the emergence in Asia and global dissemination of 16S ribosomal methyltransferases (16S-rMTases), principally among Enterobacteriaceae, but also among *P. aeruginosa* and *A. baumannii*, which pose serious problems for aminoglycoside therapy.

Zhou *et al.*[119] reported that 26% of 741 consecutive strains of Gram-negative bacilli collected at a Shanghai hospital contained a 16S-rMTase; and >90% of nearly 200 multidrug-resistant *E. coli* and *K. pneumoniae* isolates collected in Peking by Yang *et al.*[120] contained a 16S-rMTase (also see Li *et al.*[121]). SENTRY data for 2007–2008 indicated prevalence rates for 16S-rMTases among Enterobacteriaceae of 10.5% in India, 6.9% in China, 6.1% in Korea, 5% in Taiwan and 3.1% in Hong Kong.[122] While the prevalence of 16S-rMTases outside Asia remains low, it is just a matter of time before this resistance mechanism migrates westward and becomes established in Europe and North America.

Most clinically relevant 16S-rMTases, belonging to the Rmt or Arm families, methylate N^7-G1405 and confer resistance to 4,6-disubstituted 2-deoxystreptamine aminoglycosides (foe example, gentamicin, tobramycin, amikacin and plazomicin (ACHN-490)[122]) but not 4,5-disubstituted 2-deoxystreptamine aminoglycosides (for example, neomycin), 4-monosubstituted 2-deoxystreptamine aminoglycosides (for example, apramycin), or those lacking a 2-deoxystreptamine moiety (for example, streptomycin). Much less commonly encountered is NpmA, whose methylation of N^1-A1408 confers a broad range of resistance, encompassing neomycin and apramycin as well as 4,6-disubstituted 2-deoxystreptamine aminoglycosides, but not streptomycin.[123,124] Distribution of these 16S-rMTases remains confined to Gram-negative species, though their ability to function in Gram-positive pathogens such as *S. aureus* is worrisome.[125] Moreover, 16S-rMTases often are harbored on the same plasmid encoding metallo-β-lactamase NDM-1 and other resistance mechanisms,[121,126] contributing to the threat of panresistant Gram-negative pathogens.

KsgA is another 16S-rMTase, responsible for dimethylations of N^6-A1518 and N^6-A1519, whose distribution includes Gram-positive species and mycobacteria as well as Gram-negative microorganisms. Contrary to the situation with Rmt or Arm methyltransferases, high-level resistance towards kasugamycin is manifested in the absence of KsgA activity, when A1518 and A1519 are not methylated.[127] Of greater relevance than its effect on kasugamycin resistance is the influence on ribosome biosynthesis of KsgA, where it appears to function as a late-stage ribosome biogenesis factor, in which context KsgA has been proposed as an attractive antibacterial drug target.[128]

The Erm ('erythromycin ribosomal methylase') enzymes comprise another family of structurally homologous methyltransferases, which methylate N^6-A2058 in the peptidyl transferase center of 23S rRNA. Overlap of binding sites for antibiotics to the bacterial ribosome around A2058 leads to multidrug resistance towards macrolides, lincosamides and class B streptogramins,[129,130] hence the term 'MLS_B' in connection with resistance due to Erms. Far more Erms than 16S-rMTases have been described so far (http://faculty.washington.edu/marilynr/ermweb1.pdf).

Erm-type 23S-rMTases occur in Enterobacteriaceae, *Haemophilus* spp., Gram-positive and Gram-negative anaerobes and mycobacteria,[131,132] but the clinical impact of MLS_B resistance is probably greatest among Gram-positive cocci. ErmA is commonly found among methicillin-resistant *S. aureus* and tends to be produced constitutively, whereas ErmC is encountered more often among methicillin-susceptible *S. aureus* and tends to be inducible, though there may be regional differences; *ca.* 10% of clinical isolates of *S. aureus* harbor both ErmA and ErmC[133–135] (also see Spiliopoulou *et al.*[136]). ErmB is found mostly in streptococci and enterococci; some 95% of clinical isolates of *Enterococcus faecium* are resistant to erythromycin, primarily due to constitutive production of ErmB[133] (*cf.* Portillo *et al.*[137]). Constitutive producers of Erm tend to be more resistant that inducible producers,[138] though this can be species-specific[139] and may depend upon whether A2058 is mono- or dimethylated.[140]

During the past decade, still another 23S-rMTase of prospective clinical importance has been described. Cfr ('chloramphenicol-florfenicol resistance') methylates C^8-A2503 of bacterial 23S rRNA, conferring resistance to phenicols, lincosamides, oxazolidionones, pleuromutilins, A-type streptogramins and 16-member macrolides.[141,142] Though this enzyme is encountered infrequently, it has been responsible for several outbreaks of linezolid resistance among staphylococci,[143–145] (also see Pournaras *et al.* [146]) and the *cfr* gene has been detected in a strain of *Proteus vulgaris* of porcine origin.[147]

By restricting propagation of ribosomes with low avidities for antibiotics, administration of a suitable methyltransferase inhibitor to Arm or Rmt producers ought to enhance susceptibility to aminoglycosides in a pathogen population where these enzymes are significant determinants of antibiotic resistance. Similarly, administration of an Erm or Cfr inhibitor ought to promote a shift in MIC towards lower values among pathogens where one or the other methyltransferase is the main determinant of drug resistance. To date, discovery of selective methyltransferase inhibitors to overcome bacterial resistance has focused largely on Erms, though there is no reason why analogous programs to identify Cfr or 16S-rMTase inhibitors could not be pursued.

Most methyltransferases use *S*-adenosyl-L-methionine (SAM) as a methyl donor, and the catalytic domain of SAM-dependent methylases is well conserved.[148] Therefore, competitive inhibitors of SAM or its demethylation product *S*-adenosyl-L-homocysteine, such as those described by Hadjuk *et al.*[149] or Hanessian and Sgarbi,[150] could prove unduly toxic towards the human Erm orthologues DIM1 and mt-TFB,[151] as well as other essential mammalian SAM-dependent enzymes[152,153] (*cf.* Lu *et al.*[154]). The streptomycete product sinefungin (Figure 15), a structural analog of SAM, is too toxic to be of either veterinary[155] or human clinical use.

Nearly two decades ago, Clancy *et al.*[156] screened 160 000 substances in the Pfizer chemical library and identified several compounds belonging to different structural types that enhanced the activity of azithromycin *in vitro* towards *S. aureus*, *E. faecalis* and group B streptococci with an MLS_B resistotype and that were believed not to be competitive with SAM. When several hits were tested in mice with lethal systemic infections caused by an MLS_B-resistant *S. aureus* or a macrolide-susceptible *S. pyogenes*, none of them were able to rescue staphylococcal-infected animals, and only one of them, containing a penem moiety, was able to protect some streptococcal-infected animals. However, no animal experiments involving coadministration of a macrolide or clindamycin with any of the hits were described explicitly, and the project appears to have been discontinued. (It stands to reason that Clancy *et al.*[156] would have

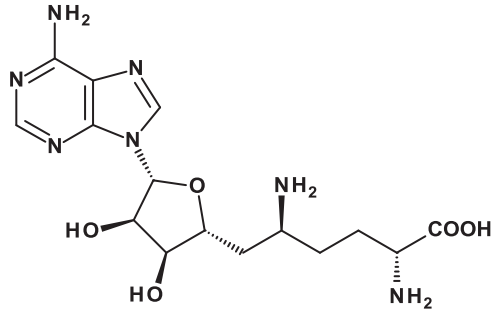

Figure 15 Sinefungin.

performed experiments in which mice given lethal doses of MLS_B-resistant Gram-positive cocci were adminstered a purported Erm inhibitor to assess its impact on rescuing animals cotreated with a macrolide or clindamycin, and Feder *et al.*[157] assume that this was indeed the case. However, Clancy *et al.*[156] do not state explicitly that such experiments were done.) Though there is no indication of binding exosites for Erms,[158] Giannattasio and Weisblum[159] reported several peptide inhibitors of ErmC′ (a soluble form of ErmC produced by *B. subtilis*), which they proposed acted at sites other than the active site of the enzyme.

The specificity of 16S- and 23S-rMTases for only one or two adjacent ribosomal nucleotides, and the inherent toxicological risk of blocking the SAM binding site, clearly point towards the RNA binding site as the preferred target for design of ribosomal methyltransferase inhibitors. The three-dimensional structures of ErmC′ and ErmAM (a B-type Erm from *Streptococcus pneumoniae*) have been solved. Although no Erm-RNA cocrystals have been obtained to date, the RNA binding pocket of ErmC′ has been characterized,[148] which can serve as a starting point for *in silico* docking of combinatorial chemistry scaffolds. Moreover, Schluckebier *et al.*[160] obtained the structure of M.*Taq*I, a restriction-modification enzyme from the thermophile *Thermus aquaticus*, cocrystalized with a 10-base pair duplex oligodeoxynucleotide and the nonreactive SAM analog 5′-[2-(amino)ethylthio]-5′-deoxyadenosine. The catalytic domain of M.*Taq*I, an N^6-adenine-specific DNA monomethylase, aligns with those of Erms and KsgA.[161]

Using an *in silico* approach, Alvesalo *et al.*[162] identified a pair of 2,1,3-benzoxadiazole derivatives, 'MB12' and 'MB13' (Figure 16), inhibitory towards *Chlamydia pneumoniae*, a cause of atypical community-acquired pneumonia, to the extent of 56.1% and 85.5%, respectively, at 15–17 μg ml^{-1}. At that concentration, the compounds had no cytotoxicity or viability effects on human lung cells. Feder *et al.*[157] remark that these compounds bound in the SAM pocket of ErmC′ rather than in the rRNA pocket. Using their own docking approach, Feder *et al.*[157] identified a compound, 4-methyl-2,6-di[(4-methylphenyl)thio]nicotinonitrile ('RF00667') (Figure 17), predicted to bind exclusively in the rRNA pocket of ErmC′, which was a noncompetitive inhibitor of ErmC', with an IC_{50} of 65 μg ml^{-1}. However, macrolide or clindamycin MICs, as a function of inhibitor concentration in pathogens expressing the MLS_B resistotype, were not reported. The authors suggested that a superior selective inhibitor of Erms might be obtained by linking SAM or an SAM analog with an rRNA analog, as the two substrates bind in adjacent clefts separated by a peptide ridge.

Recently Baker *et al.*[163] described a scintillation proximity assay, adaptable to high-throughput format, for quantifying transfer of [^{3}H-CH_3]SAM into 16S rRNA by RmtA or KsgA, and into 23S rRNA by ErmC′. Baker and Rife[161] used this assay to screen compounds for their ability to inhibit reactions catalyzed by ErmC′ and KsgA, supplemented by cocrystallization studies with ErmC′ to determine in which substrate pocket hits were binding. Following on the report of Clancy *et al.*[156] that the IC_{50} for ErmC′ of N^6-cyclopentyladenosine ('UK-80882') (Figure 18) was not sensitive to increasing concentrations of SAM, Baker and Rife[161] prepared and tested this compound as a reference inhibitor, plus several new N^6-substituted adenosine derivatives as prospective ErmC' or KsgA inhibitors. In their first library, they found N^6-octylamine adenosine to be the most active compound towards ErmC′ (51% inhibition, compared with 32% for an equimolar concentration of N^6-cyclopentyladenosine), whereas the most active compound towards KsgA was N^6-octyladenosine (39% inhibition, compared with 16% for an equimolar concentration of N^6-cyclopentyladenosine). Cocrystallization with ErmC′ showed that N^6-octylamine adenosine bound to the SAM pocket rather than the rRNA pocket, but careful analysis of this costructure suggested additional strategies for obtaining inhibitors that would bind preferentially to the rRNA site. When such compounds were prepared during a second round of synthesis, improved inhibition towards ErmC′ was observed for several of them; the most active compound in this second library, 5′-deoxy-5′-butylphenylamine-N^6-cyclopentyladenosine, inhibited ErmC′ (93%) nearly as much as an equimolar concentration of sinefungin (98%). Kinetic studies of ErmC′ suggested that these inhibitors were binding in the rRNA pocket rather than in the SAM pocket, though confirmation by X-ray analysis of cocrystals with 'second-generation' inhibitors was not successful at showing a bound ligand in any site. Most 'second-generation' inhibitors were poorly active towards KsgA, indicating that, despite their overall structural and biochemical similarities, the two enzymes can behave differently towards inhibitors, and that optimizing the inhibitory power of a compound towards one enzyme may compromise its inhibitory

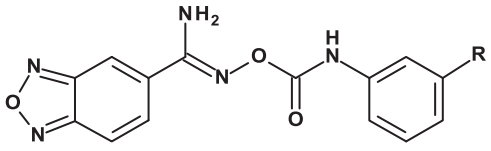

Figure 16 MB12 (R = H) and MB13 (R = Cl).

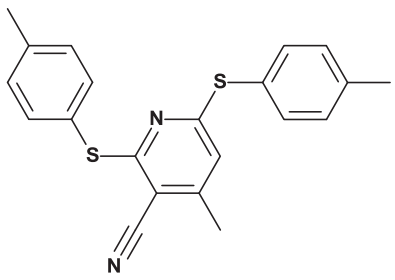

Figure 17 4-Methyl-2,6-di[(4-methylphenyl)thio]nicotinonitrile ('RF00667').

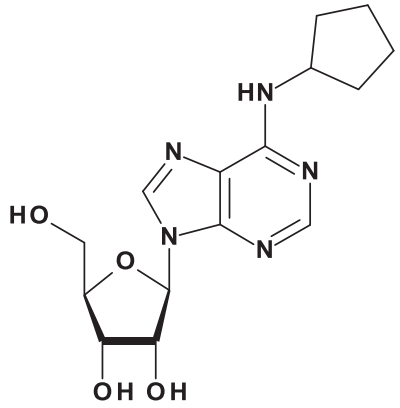

Figure 18 N^6-Cyclopentyladenosine ('UK-80882').

power towards the other. Whether this reflects a specific difference between ErmC′ and KsgA, or a general difference between 16S-rMTases and 23S-rMTases, remains to be clarified.

Clearly, much more effort needs to be invested in this approach to determine whether it is likely to lead to therapeutically useful products.

EXPLOITING BACTERIAL TOXIN–ANTITOXIN SYSTEMS: PRETTY POISON?

The existence of bacterial toxin–antitoxin systems was uncovered during the early 1980s in the context of post-segregational killing[164] and, during the past decade, analysis of hundreds of bacterial chromosomes has uncovered thousands of presumptive open reading frames for toxins and their cognate antitoxins.[165–167] A single bacterial strain can harbor genes for a surprisingly large ensemble of toxin–antitoxin systems: the *E. coli* K-12 chromosome contains at least eight confirmed toxin–antitoxin systems and 28 putative toxin–antitoxin systems,[168] whereas at least 88 different toxin–antitoxin systems may be encoded by *M. tuberculosis*.[169] The toxin component is invariably a protein, whereas its corresponding antitoxin can be a protein or an RNA molecule. The antitoxin is more unstable than its cognate toxin and needs to be synthesized continuously to keep the toxin neutralized. If the intracellular titer of antitoxin falls below a critical concentration in the presence of cognate toxin, the free toxin will exert its deleterious effects.

Five types of toxin–antitoxin systems have been described, according to the structure and mode of action of the antitoxin. In Type I systems, the antitoxin is an siRNA that binds toxin mRNA and precludes its translation; whereas in Type II and Type III systems, the antitoxin is a protein and RNA molecule, respectively, that binds the toxin to form a biologically inactive complex.[170] In Type IV systems, the antitoxin is a protein that interacts not with the toxin or its mRNA but with the toxin's target, protecting it from deactivation.[171] In Type V systems, the antitoxin is a protein with RNAse activity that selectively degrades toxin mRNA before it can be translated.[172] Type I and Type II systems are commonest, and most toxin–antitoxin systems in *E. coli* are of these types.

Toxins exert their physiological effects by uniquely targeting an intracellular component of transcription (DNA gyrase), translation (mRNA, tRNA, ribosomes and elongation factors), the bacterial cytoskeletal system (FtsZ and MreB), phospholipid synthesis (inhibition of phosphatidylethanolamine synthesis), the cell membrane,[173] or peptidoglycan synthesis (production of UDP-GlcNAc-3′-phosphate, a competitive inhibitor of MurA) required for replicative growth and/or survival.[174] Type I toxins perturb membrane structure, collapsing ion gradients and preventing ATP synthesis, whereas the vast majority of Type II toxins are mRNA-specific endonucleases that arrest translation by mRNA cleavage.[170,175] Given the abundance of toxins and antitoxins that may be present at any given time, a bacterium needs to harmonize these components in relation to environmental cues to ensure that, under favorable conditions, replicative growth proceeds in an unencumbered fashion while cessation of growth, or even suicide,[176,177] ensues when conditions become unfavorable. So far as is known, bacterial-like toxin–antitoxin systems do not occur among eukaryotes. Some homologous eukaryotic ribonucleases, associated with nonsense-mediated decay of RNA and processing of pre-18S ribosomal RNA, belong to the PIN-domain family (Pfam: PF01850) and share the same overall folding pattern with some bacterial toxins possessing endonuclease activity, but these homologous eukaryotic and prokaryotic proteins have very low sequence identity.[178]

There appears to be a relationship between antibiotic action and bacterial toxin–antitoxin systems, not surprising when one considers that antibiotics impose conditions hostile to bacterial propagation or survival. Pioneering work on the relationship between antibiotics and bacterial toxin–antitoxin systems, performed in the laboratory of Hanna Engelberg-Kulka (Hebrew University-Hadassah Medical School, Jerusalem, Israel), showed that thymine starvation in *E. coli*, induced by sulfamethoxazole or trimethoprim, reduced transcription of the operon encoding the MazEF (Type II) toxin–antitoxin system. This was followed by preferential decay of the MazE antitoxin and accumulation of free MazF,[179] an endoribonuclease that preferentially cleaves single-stranded mRNAs at ACA sequences, leading to inhibition of translation and cell death.[180] Death of *E. coli* consequent to exposure to other antibiotics, such as rifampicin, spectinomycin, chloramphenicol and nalidixic acid, likewise appear to be mediated by the MazEF system.[181]

These observations beg the question: can bacterial toxin–antitoxin systems be exploited for clinical ends? Can drugs that interfere selectively with the toxin–antitoxin balance in bacterial pathogens form the basis of a chemotherapeutic approach? Given the diverse modes of action of toxins and their interactions with cognate antitoxins, it is not surprising that strong 'cross-talk' between non-cognate toxins and antitoxins, though not unknown,[182,183] is uncommon. Therefore, a chemotherapeutic strategy predicated on toxin–antitoxin interference likely would be a narrow-spectrum therapy for serious infections associated with a particular species or genus, such as *S. aureus* or *P. aeruginosa* or *M. tuberculosis*, where the targeted toxin–antitoxin system is widely preserved and expressed. The feasibility of such an approach is supported by the finding of Williams *et al.*[184] that all 78 methicillin-resistant *S. aureus* clinical isolates examined harbored transcriptionally functional *mazEF* genes on their chromosome, and that all 42 *P. aeruginosa* clinical isolates examined harbored transcriptionally functional *relBE* and *higBA* (both Type II) genes on their chromosome. Towards this end, possible strategies would include:[185]

- molecules that bind to an antitoxin, precluding formation of a complex with its cognate toxin;
- molecules that promote dissociation of existing toxin–antitoxin complexes;
- molecules that enhance proteolysis or nucleolysis of free or complexed antitoxins; and
- molecules that inhibit transcription of the toxin–antitoxin operon.

To date, there have been only two reports on the discovery of molecules capable of disrupting toxin–antitoxin interactions. Lioy *et al.*[186] devised a cell-based high-throughput protocol to search for disruptors of the plasmid-borne Type II ε–ζ antitoxin–toxin system found predominantly among Gram-positive species.[187] Assisted by molecular dynamic simulations, they identified sets of oligopeptides that interfered with ε–ζ interactions with high binding affinity (*ca.* 0.6 μM), though purified single oligopeptides proved to be much weaker binders. The following year, Chopra *et al.*[188] focused on the Type II MoxXT antitoxin–toxin module from *Bacillus anthracis* and, using a rational drug design approach, obtained an octapeptide predicted to mimic binding of MoxX antitoxin to MoxT toxin. When this octapeptide was synthesized and tested, it was found to have an IC_{50} for interference of MoxX–MoxT binding approaching 2 μM.

While the targeted utility of inhibitors of bacterial antitoxins is clear, it is also important to consider the association of toxins with

persistence, in which small subpopulations of bacteria (for *E. coli ca.* 0.001% of logarithimically growing cells, up to 0.1% of stationary cells[189]) enter into what appears to be a state of dormancy, when the cells become highly tolerant to antibiotics, if not actually resistant to them. When antibiotic concentrations fall to subinhibitory levels, outgrowth by persisters reestablishes the population, a phenomenon believed to underlie the occurrence of chronic infections.[190,191] Transcriptome analysis has indicated involvement of chromosomally encoded toxin–antitoxin modules in bacterial persistence; and by demonstrating that successive deletion of the 10 mRNase-encoding toxin–antitoxin operons of *E. coli* progressively reduced the level of persisters, Maisonneuve *et al.*[192] showed that persistence is a phenotype common to toxin–antitoxin modules (*cf.* Kwan *et al.*[193]). TisB, a Type I toxin that interferes with energy metabolism,[194] appears to be responsible for the majority of *E. coli* persisters under conditions of SOS induction.[195] If, indeed, persistence is predicated largely upon the action of bacterial toxins, then agents that disharmonize the toxin–antitoxin equilibrium need to ensure that enough of the appropriate free toxin is made available intracellularly to compromise survival to the point of achieving bacteriological cure, while minimizing risk of persister formation.

LEGACIES TO ENCOURAGE AND LEGACIES TO AVOID

Rome holds a special place in the collective mind of Western civilization. The national languages of most countries in Western Europe, as well as more refined standards of literature, art, and monumental architecture, concepts of civil engineering and urban design, and the fundamentals of Western politics and jurisprudence, derive from ancient Rome. Roman demography was highly stratified, with a small population of wealthy elites living alongside a very large population of the poor and downtrodden who endeavored to eke out a subsistent existence without recourse to the social and charitable safety nets to which contemporary Western societies are accustomed. Unlike present cities, especially in the developing world, which continue to grow at increasingly unsustainable rates due to influx from neighboring rural regions, the population of Rome appears to have gone into decline after about 100 BCE.[196] Social, military, climatic and fecundity issues certainly had a bearing on the demographics of ancient Rome, but the poor hygienic conditions that most of the population probably experienced, compounded by a lack of knowledge about disease etiology and control, makes it extremely likely that infectious diseases contributed in no small way to the population reduction and, more generally, to the misery of the population.[197,198]

The fight against infectious diseases is approaching an epidemiological watershed. While big pharma continues to pursue research programs on antivirals and, to a lesser extent (buoyed by the generosity of foundations supporting public health) antiparasitics, their activity in the antibacterial (and antimycotic) arena remains sadly lagging, despite such well-intentioned campaigns as the IDSA's '10 × '20' initiative,[199] the GAIN Act (http://www.pewtrusts.org/uploadedFiles/wwwpewtrustsorg/Fact_Sheets/Antibiotics_and_Innovation/Antibiotics_GAIN_FactSheet.pdf), and the European Commission's Action Plan against the Rising Threats from Antimicrobial Resistance (http://ec.europa.eu/dgs/health_consumer/docs/communication_amr_2011_748_en.pdf). If we are to avoid the nightmare scenario of a pre-Domagk era, when routine surgery or even simple cuts and sore throats bore a serious risk of life-threatening sequelae, pharmaceutical companies, big and small, need to rally around the public good and consolidate their efforts to promote the discovery and development of innovative therapeutic modalities for combating bacterial infections.

EPILOGUE

During spring 1981, as I was completing my doctorate in Biomedical Sciences at the Worcester Foundation for Experimental Biology, Inc. (Shrewsbury, MA, USA) (see Shapiro and Caspi[200]), I arranged for a post-doctoral position with Professor Dr Leo C Vining at Dalhousie University. Later that year, I moved to Halifax, where Leo graciously welcomed me. As my interests lay more in physiology and biochemistry than genetics, Leo suggested that I focus on mechanisms of nitrogen regulation of chloramphenicol biosynthesis in *Streptomyces venezuelae*.

Chloramphenicol, a broad-spectrum ribosomotropic antibiotic discovered at Parke, Davis & Co. in 1947, had been a subject of intense scrutiny by Leo and his collaborators for many years and, shortly before my arrival, Leo had completed some studies on carbon regulation of chloramphenicol biosynthesis. Nitrogen regulation of antibiotogenesis was virgin territory, and during this initial period, I focused on the effects of nitrogen sources on the phasing and output of chloramphenicol, with just a slight detour into carbon catabolite control to test a hypothesis proposed by Leo. This was followed by a second period when, as a research fellow and adjunct associate professor of biology, I worked on metabolic control of cephamycin C biosynthesis in *Streptomyces clavuligerus*.

In 1987, I was recruited to head the nascent Industrial Microbiology Laboratory at Sigma-Tau Industrie Farmaceutiche Riunite SpA. With feelings of excited anticipation about embarking upon a new career tempered by sadness about leaving such a wonderful working environment, I assumed a new position in Pomezia, an industrial suburb of Rome. We now resided on different continents, but Leo and I remained in contact, airmail being the preferred mode of communication. Arrival of one of Leo's missives was always a joyful occasion, not only for the information it conveyed but also because he was a superb wordsmith.

I met Leo twice more after leaving Halifax in late December 1987. The first occasion was in Česke Budějovice during the summer 1988, when we were both invited by Academician Dr Zdenko Vaněk to chair sessions at the Second International Symposium on Overproduction of Microbial Products, hosted by the Institute of Microbiology of the Czechoslovak Academy of Sciences. I saw Leo again in 1999, during a brief business trip to Halifax. Leo and his charming wife Pat still resided in their beautiful home on Regina Terrace, and they invited me to take high tea with them. We spent several hours reminiscing and bringing one another up to date, fond memories flowing fast and furious.

All good things come to an end. Leo's health gradually declined, our correspondence became less frequent, and I came to accept the fact that, sooner or later, I would receive some very distressing news. That news reached me on 12 April 2012, when Dr Francis Arhin and Dr Ashish Paradkar, two of his former graduate students, informed me that Leo passed away, peacefully, at home, surrounded by his loving family, on Sunday, 08 April 2012.

Leo, you will be sorely missed by those of us who had the pleasure and honor of knowing and working with you. God bless you, Leo.

CONFLICT OF INTEREST

The author declares no conflict of interest.

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