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Antibiotic adjuvants: multicomponent anti-infective strategies

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The unremitting emergence of multidrug-resistant bacterial pathogens highlights a matching need for new therapeutic options. For example, new carbapenemases such as KPC (class A *Klebsiella pneumoniae*) and NDM-1 (New Delhi metallo-β-lactamase 1) are surfacing, resulting in almost total resistance to β-lactam antibiotics. Furthermore, resistance is quickly disseminated, not only in the healthcare sector, but also within the community at large, because many resistance determinants are carried on mobile genetic elements readily shared among pathogens. The absence of new antibiotics has led to a growing reliance on older, more toxic drugs such as colistin, but resistance to these is already arising. One approach to combat this growing problem is the use of combination drug antibiotic adjuvant therapy, which potentiates the activity of antibiotics. Here, we review the current situation and discuss potential drug combinations that may increase the potency of antibiotics in the future. Adjuvant therapies include antibiotic combinations, synergy between antibiotics and nonantibiotics, inhibition of resistance and molecules that alter the physiology of antibiotic-insensitive cells, such as those in biofilms. We provide a rationale for these multicomponent strategies, highlighting current research and important considerations for their clinical use and pharmacological properties.

There is an urgent need for new antibiotics (Refs 1, 2, 3, 4). This is due to the emergence of newer pathogens with multidrug-resistance profiles such as *Acinetobacter baumannii* (Ref. 5) and the re-emergence of 'old' pathogens such as *Mycobacterium tuberculosis* (Mtb) (Ref. 6) and *Neisseria gonorrhoeae* (Ref. 7) in forms now resistant to frontline antibiotics. In addition, new resistance genes are evolving, which target important classes of drugs such as the KPC (class A *Klebsiella pneumoniae*) (Ref. 8) and NDM-1 (New Delhi metallo-β-lactamase 1) (Ref. 9) carbapenemases. Furthermore, horizontal transfer of antibiotic-resistance elements freely occurs among Gram-positive and Gram-negative pathogens and they are widely distributed in healthcare settings and in the community (Ref. 2). All of these challenges have combined to exert tremendous pressure on the infectious disease community to develop

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new drugs and establish the means to preserve the efficacy of existing antibiotics. Presently, there is an acute need for new agents that target Gram-negative pathogens (Refs 10, 11); however, multidrug-resistant Gram-positive pathogens such as vancomycin-resistant enterococci and methicillin-resistant *Staphylococcus aureus* (MRSA) also remain chronic concerns.

The challenge facing clinicians and drug discoverers alike in infectious disease medicine is antibiotic resistance. Resistance has emerged to all classes of antibiotics, resulting in a continuous need for new drugs. Following the discovery of penicillin in the 1940s, the emergence of resistance was countered by the discovery and rapid implementation of many new antibiotics. This period, termed the Golden Age of antibiotics, ended in the early 1960s when almost all the antibiotic chemical scaffolds in current clinical use were discovered (Ref. 12). Nevertheless, in answer to the emergence of resistance, medicinal chemical modification of known antibiotic scaffolds proved highly effective in generating multiple 'generations' of antibiotics that retained antimicrobial activity even in the face of emergent resistance. However, there is reason to believe, as a result of the continuing evolution of known resistance genes and the selection of new ones, that further retooling of many of the older antibiotic scaffolds will result in diminishing returns (Ref. 12).

An additional challenge of antibiotic resistance is that it can take on several distinct mechanisms, and many pathogens harbour several mechanisms simultaneously (Fig. 1). The dominant resistance mechanisms are enzyme-catalysed antibiotic modification and destruction, active efflux of compounds from the cell and alteration of antibiotic targets. Many pathogens circulating in healthcare facilities carry mobile genetic elements, such as plasmids, which incorporate several resistance genes conferring protection to more than one class of antibiotic. Furthermore, collections of resistance elements can be readily incorporated into bacterial chromosomes through transposons and integrons. The genome of a multidrug-resistant strain of *A. baumannii*, for example, harbours an 86 kb DNA insertion containing 45 antibiotic-resistance genes (Ref. 13)! The origin of many of these genes is likely to be the innumerable nonpathogenic bacteria found in many environments that also contain resistance genes (Refs 14, 15). This resistome is massive and includes elements that confer resistance to natural product antibiotics, their semisynthetic derivatives and completely synthetic compounds. Resistance is therefore inevitable.

Antibiotic resistance is a formidable challenge that will require several strategies to address. Chief among these is a need for new drugs. Both natural products and synthetic compounds have proved useful in this regard. However, very few new classes of antibiotics have been described over the past 40 years. There are several reasons for this decline in innovation, including the challenge of identifying new chemical matter that is effective and nontoxic (Ref. 16), an ever more complex regulatory environment and the movement of the pharmaceutical industry in favour of blockbuster drugs, which, more often than not, are used to treat chronic conditions requiring long-term drug therapy. As a result, the antibiotic pipeline is growing ever more dry (Ref. 17).

The focus of most new antibiotic discovery is on the well-established and clinically proven 'one-compound–one-drug' paradigm. This approach has resulted in the discovery of most of our current collection of antimicrobial agents. However, given the challenges of resistance and the difficulty of new drug discovery, the time is ripe for the consideration of new strategies.

The combination of anti-infective compounds to improve therapeutic outcome and even to diminish resistance is a well-established strategy in infectious disease medicine. For example, the triple-drug therapy for human immunodeficiency virus (HIV) infection HAART (highly active antiretroviral therapy) has proved highly successful in lowering viral levels to below the detection limit and reducing the frequency of mutations conferring resistance (Ref. 18). Historically, the treatment of bacterial, fungal and parasitic infections with drug combinations has been a clinical mainstay (Ref. 19).

An exclusive focus on combinations of antimicrobial agents is too narrow to address the need for new antibacterial drugs. Instead, a more comprehensive approach is the development of antibiotic adjuvants that include not only antibiotics but also other bioactive molecules. For the purposes of this review, we

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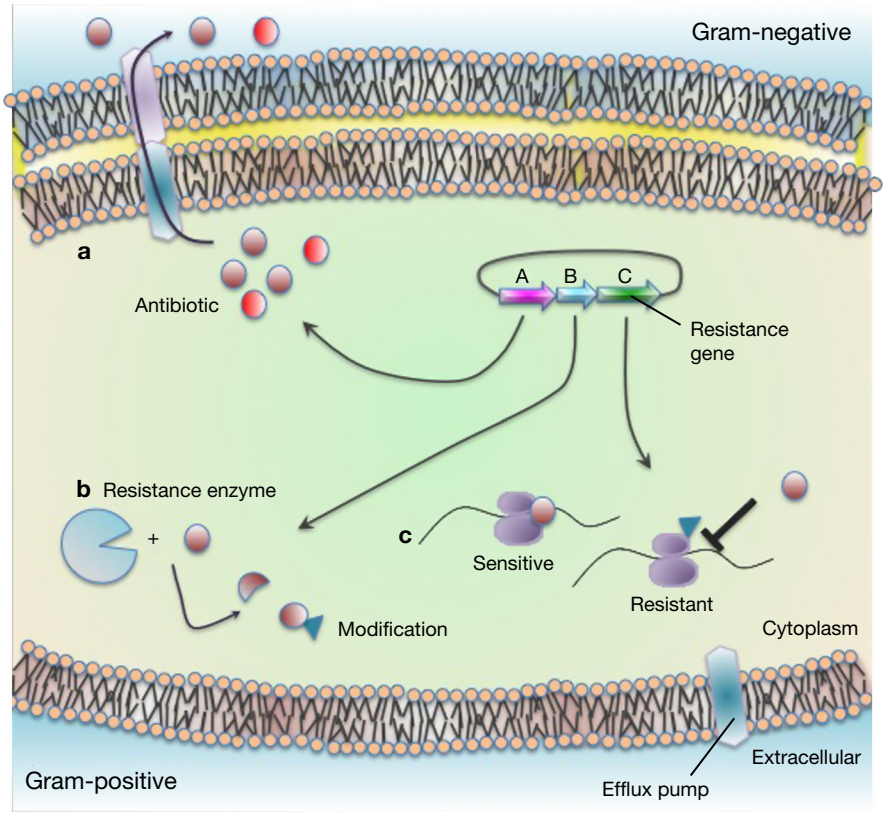

Mechanisms of antibiotic resistance

Figure 1. Mechanisms of antibiotic resistance. Major routes of antibiotic resistance: (a) efflux of antibiotics occurs by multidrug efflux pumps that effectively pump several types of antibiotics out of the cell; (b) enzymatic modification or degradation of antibiotic molecules can occur, rendering them inactive; and (c) alteration of the antibiotic target, for example the ribosome, prevents binding of the antibiotic and loss of activity. The mechanisms are similar in Gram-positive and Gram-negative organisms except for efflux, in which pumps can span both inner and outer membranes of Gram-negative organisms. Often, more than one antibiotic-resistance gene is clustered on mobile genetic elements or integrated into the chromosome.

define the concept of antibiotic adjuvants as combinations of antibiotics with other strategies that enhance antimicrobial activity against the pathogen (Fig. 2). Combinations of compounds that impact the host, such as steroids, will not be discussed here, but are also important antibiotic adjuvant strategies.

The mechanistic logic underlying the activities of antibiotic adjuvant combinations is diverse. Combinations of bioactive molecules can result in the sequential or orthogonal inhibition of steps necessary for essential physiological pathways. One of the best-documented examples of this is the sulfamethoxazole and trimethoprim antibiotic combination that inhibits folic acid metabolism in bacteria. Molecules that block antibiotic resistance are another example of adjuvants that can have significant clinical impact. The archetypal example is the β-lactamase inhibitor clavulanic acid, which is administered in conjunction with amoxicillin, resulting in the highly successful drug Augmentin®. Another strategy is the enhancement of antibiotic uptake to overcome drug efflux or physiological barriers such as the outer membrane of Gram-negative bacteria. Antibiotics are usually most effective with actively growing cells; molecules that enhance

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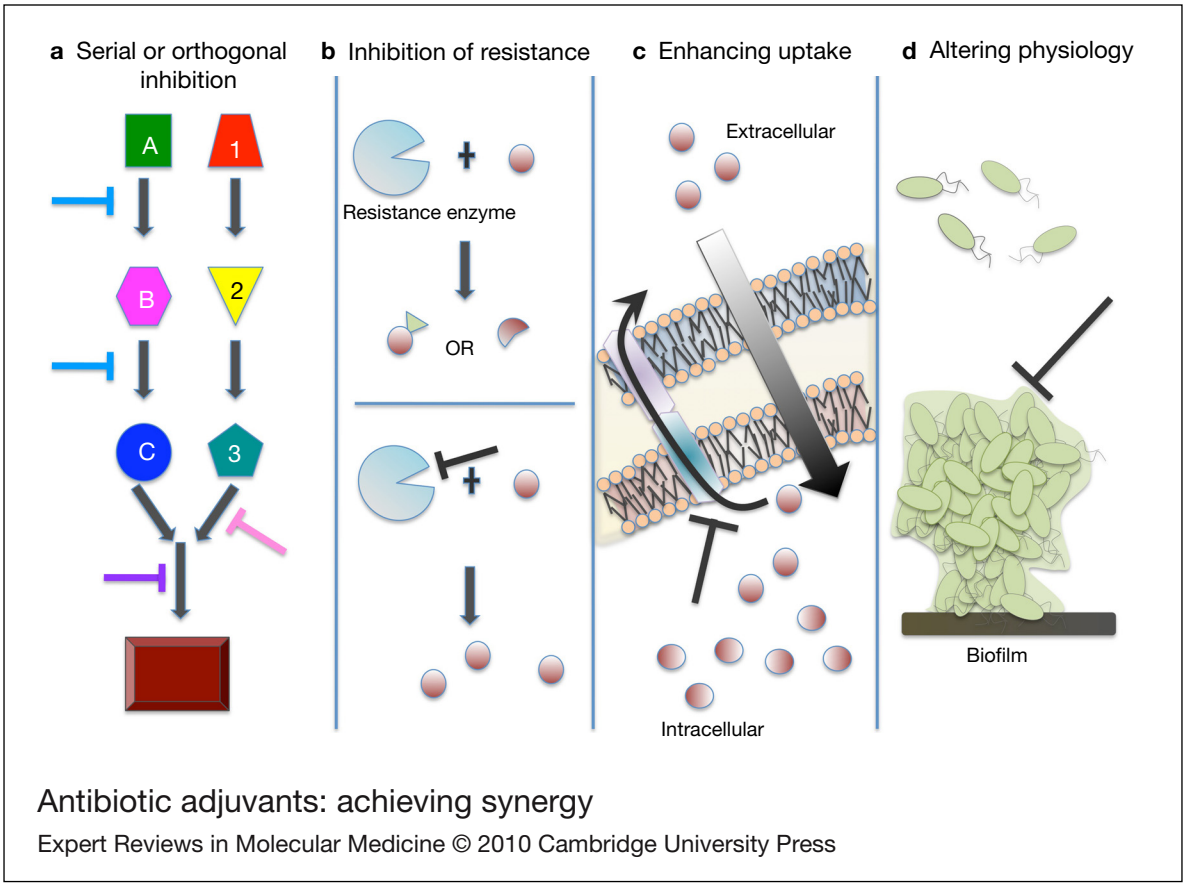

Figure 2. Antibiotic adjuvants: achieving synergy. Potentiation of antibiotic activity can occur through several mechanisms. (a) Synergistic activity between two compounds (antibiotic + antibiotic; antibiotic + non-antibiotic) through serial or orthogonal inhibition of vital physiological pathways. (b) Inhibition of resistance enzymes that degrade or covalently modify an antibiotic to a nonactive form. (c) Compounds that block antibiotic efflux or enhance uptake into the cell. (d) Dispersal of a biofilm to planktonically growing cells, resulting in increased susceptibility to antibiotics.

antibiotic action in physiological states of slow growth, such as biofilms, are yet another example of adjuvant technology. In this review, we discuss established and theoretical antibiotic adjuvant technology in order to demonstrate the proven and potential applications of this approach.

Antibiotic combinations

Combinations of antibiotics have long been used in the clinic. Here, the tactic is the combination of single agents with established antimicrobial activity. These 'like-plus-like' (antibiotic + antibiotic) combinations have traditionally been discovered in an ad hoc fashion in an effort to improve activity against particular pathogens or to achieve broad-spectrum coverage when the nature of the infectious organism is unknown. Antibiotic combinations have four possible outcomes: synergy, additivity, antagonism and autonomy. These are dependent on the mode of action of the antibiotics and the genetic networks in the specific bacterial species and strains tested. In vivo the most desirable positive interaction is that of synergy, where the effect of two drugs in combination is significantly greater than either drug alone, whereas additivity is simply the sum of the effects of each drug, assuming they do not interact with each other. When the observed effect is equal to the most active drug, autonomy exists; however, when a combination has a significantly smaller effect than either drug alone, they are deemed antagonistic (Ref. 20). Although it could be argued that like-plus-like antibiotic combinations are not adjuvants, where synergy arises, the compounds clearly enhance their

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antimicrobial activities and therefore we qualify them as 'adjuvant-like' in their activity.

To assess the effect of any given antimicrobial combination, the fractional inhibitory concentration (FIC) is determined in the laboratory. The most common method used is the checkerboard array, which refers to the pattern formed when two antimicrobials are serially diluted to concentrations above and below their known minimal inhibitory concentration (MIC) in a perpendicular fashion to each other. For example, if the assay is performed in microtitre plates, a dilution series of drug A would be plated in each column, whereas a dilution series of drug B would be plated in each row (Fig. 3a). Each well will have a different concentration of drugs A and B to be assayed against the test organism (Ref. 20).

FIC is determined by examining the pattern formed at the inhibition boundary, which is called the isobologram (Fig. 3b). Synergy is then determined mathematically by dividing the inhibitory concentration of drug X in a given row by the MIC of drug X. The FIC index is calculated by adding the separate FICs for each drug in a well (Fig. 3c). An FIC index of ≤ 0.5 indicates synergy and a value ≥ 4.0 indicates antagonism. Additivity and autonomy lie somewhere in between and cannot always be distinguished; therefore, they are usually classified as 'no interaction'.

The advantages of the checkerboard array approach include its ease of implementation in most laboratory settings and the straightforward interpretation of results. The approach, however, does not distinguish between bactericidal and bacteriostatic combinations, and further testing is required to determine the ultimate impact of combinations on cell growth. In its traditional form, which measures only MIC, the checkerboard array is not capable of measuring dose response relationships and makes the assumption that each antibiotic has a linear dose response. However, because most labs now have access to microplate readers, this drawback is readily overcome and quantitative dose response is readily measured.

Other methods provide a more qualitative view of synergy. Variations on established diffusion assays such as the Kirby–Bauer disc diffusion assay assess interactions by placing drug-soaked discs on an agar plate inoculated with the test organism (Fig. 3d). The discs are placed at a distance from each other equal to the sum of each inhibitory radius of the drugs alone. If synergy occurs, enhanced killing will be observed at the junction of the two zones, or killing will only be apparent where the two drugs are combined. If in vitro methods are not sufficient, serum tests can be performed, where blood is drawn at different time points following antibiotic administration and tested against the infectious organism (Ref. 21).

Specific examples of antibiotic combinations are described below.

Sulfamethoxazole and trimethoprim

Drug combinations that act on a single pathway can give rise to synergy by the sequential inhibition of steps, resulting in a more complete shutdown of downstream biochemistry or physiology (Fig. 2). The combination of sulfamethoxazole and trimethoprim, often referred to as cotrimoxazole, has been available since 1969 under various trade names such as Septra® and Bactrim®, and is an example of this mechanism (Refs 22, 23). The two antibiotics act synergistically by inhibiting sequential steps in the folic acid biosynthetic pathway (Fig. 4).

Folic acid is an essential component of bacterial C1 metabolism. In particular, this cofactor is required by thymidylate synthase that produces dTMP, which is necessary for DNA synthesis. Sulfamethoxazole is a competitive inhibitor of dihydropteroate synthase, which catalyses the production of dihydropteroic acid from *p*-aminobenzoic acid. Furthermore, it can act as an alternative substrate producing an adduct that is a dead-end pathway inhibitor (Fig. 4). Mammalian cells cannot produce folate and acquire it through diet. Dihydrofolate reductase is potently and competitively inhibited by trimethoprim, blocking the conversion of dihydrofolic acid to tetrahydrofolic acid. Although mammals possess a functional dihydrofolate reductase, trimethoprim has been shown to be some 60 000-fold more selective for the bacterial enzyme, resulting in a favourable toxicity profile in humans (Refs 24, 25). Accumulation of dihydrofolic acid also inhibits folylpoly-γ-glutamate synthetase – a key enzyme that modifies folates by the addition of glutamate residues, which ensures that folates are retained within the cell (Ref. 26). The impact of sulfamethoxazole and trimethoprim coadministration is therefore combinatorial inhibition of folate biosynthesis (Fig. 4).

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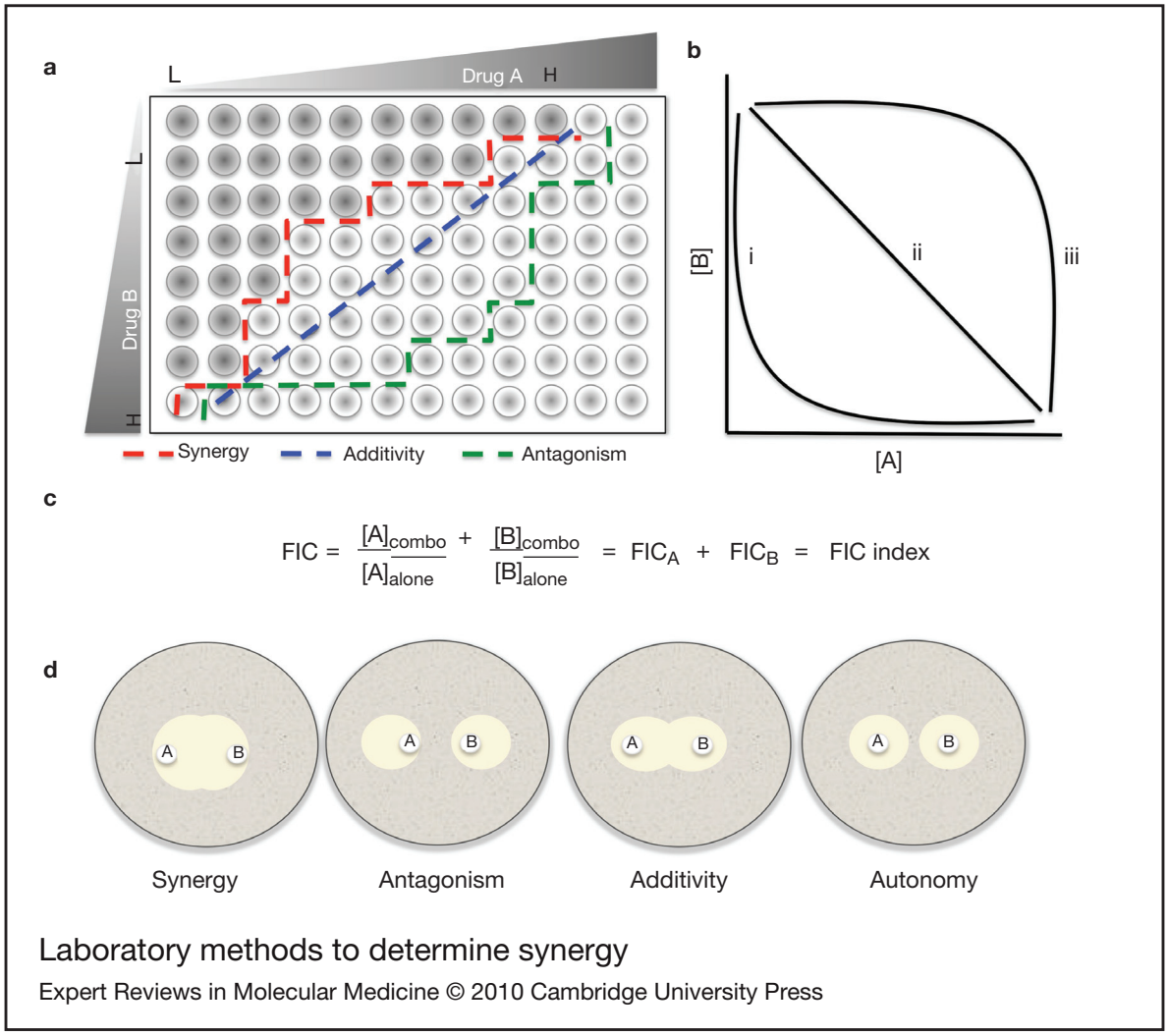

Figure 3. Laboratory methods to determine synergy. (a) Checkerboard assay to determine FIC. Two drugs are serially diluted and arrayed perpendicular to each other in a microtitre plate so that each well has a unique combination. (b) The test organism is inoculated and each combination of drug is scored for growth and plotted to create an isobologram. i, synergy; ii, additivity; and iii, antagonism. (c) The equation used to quantitatively assess combinations. MIC values of A and B alone and in combination are used to determine FIC. Qualitative assessment is determined as in (d), where each drug is soaked onto a paper disc and placed near the other on a plate inoculated with the test strain. Zones of inhibition indicate the type of interaction. Abbreviations: FIC, fractional inhibitory concentration; MIC, minimal inhibitory concentration.

Antibacterial synergy by cotrimoxazole arises as a result of the complete shutdown of the folate pathway in bacteria. This results in cessation of cell division and in many species in cell death. Sulfamethoxazole or trimethoprim administered alone do not completely impede biosynthesis and individually they are bacteriostatic. Resistance to cotrimoxazole is often the result of mutations in the target dihydropteroate synthase and dihydrofolate reductase genes. Often these genes are clustered on mobile genetic elements such as plasmids, which spread horizontally through bacterial populations. This results in production of enzymes with reduced susceptibility to the antibiotics, thereby effectively bypassing the endogenous drug-sensitive pathway.

Aminoglycosides and penicillins

Synergy can also result from interference with genetically connected but orthogonal pathways: for example, where one drug augments access to

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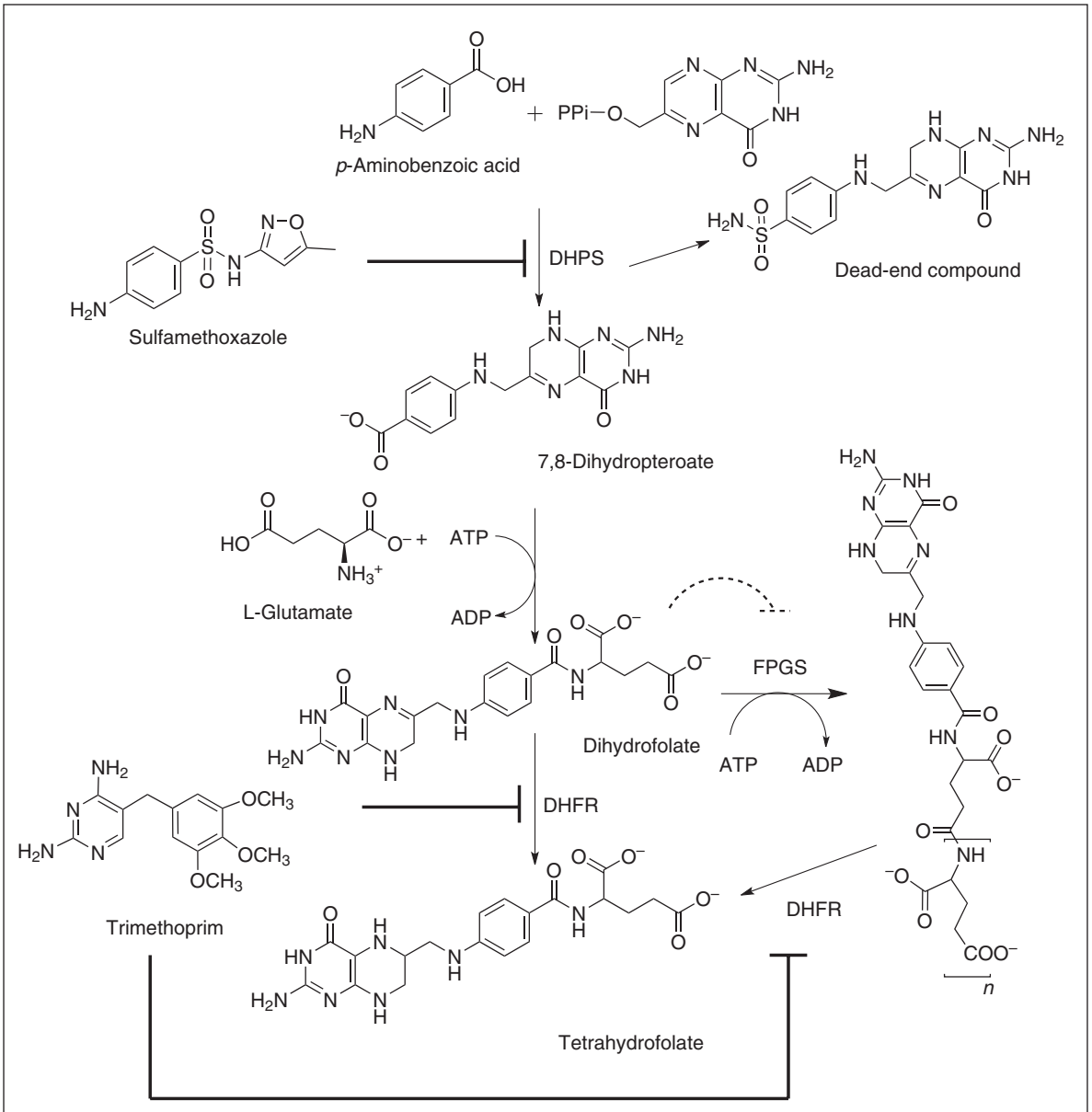

Mode of action of sulfamethoxazole and trimethoprim

Figure 4. Mode of action of sulfamethoxazole and trimethoprim. Synergy occurs by sequential inhibition of folic acid biosynthesis enzymes. Sulfamethoxazole inhibits the enzyme DHPS, which catalyses the production of dihydropteroic acid from *p*-aminobenzoic acid. Trimethoprim competitively inhibits the enzyme DHFR and blocks conversion of dihydrofolic acid to tetrahydrofolic acid. Accumulation of dihydrofolate results in inhibition of synthesis of polyglutamylfolates by FPGS. Furthermore, sulfamethoxazole can act as a surrogate substrate for DHPS, resulting in a compound that cannot be further metabolised to dihydrofolate. Abbreviations: DHFR, dihydrofolate reductase; DHPS, dihydropteroate synthase; FPGS, folylpoly-γ-glutamate synthetase.

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a second drug's target site. Such is the case with the β-lactam and aminoglycoside antibiotics. Aminoglycosides are broad-spectrum antibiotics that bind the A-site on the 30S subunit of the bacterial ribosome, causing incorporation of noncognate aminoacyl tRNAs. Although mRNA translation is not halted, the cell cannot function properly with the aberrant protein expression, and death ultimately occurs. Penicillin, however, irreversibly binds to transpeptidases and bifunctional transpeptidase-transglycosylases, so-called penicillin-binding proteins (PBPs), responsible for stitching the web of peptidoglycan surrounding a bacterial cell together. The effect is impaired cell division and growth.

Weinburg and Moellering examined the mechanism of action of this synergistic combination in *Enterococci* sp. (Refs 27, 28). Using ^{14}C-labelled streptomycin, antibiotic uptake into the bacterium was observed to increase threefold in the presence of penicillin. This effect was delayed when cotreated with both antibiotics, but seen immediately when the cells were pretreated with penicillin before the addition of streptomycin. Furthermore, the same effect was observed with other antibiotics that act on the cell wall – bacitracin, vancomycin and cycloserine – all of which inhibit different steps of cell wall synthesis. The mechanistic interpretation of these results is that impairment of cell wall synthesis by penicillins and other agents has an orthogonal positive impact on aminoglycoside entry (or alternatively inhibition of efflux), resulting in antibiotic synergy.

Aminoglycoside and penicillin synergy only occurs in cells that are intrinsically sensitive to aminoglycosides alone because increased concentration of the drug is not enough to overcome the mechanism of resistance. More recently, studies have been conducted that also associate a synergistic increase in reactive oxygen species in cells treated with a combination of the β-lactam ampicillin and the aminoglycoside gentamicin (Ref. 29), providing yet another contribution to cell death, and highlighting the importance of systematic screening of antibiotics in combination to uncover cryptic interactions.

Although synergistic interactions between β-lactams and aminoglycosides have been well documented in the literature (Refs 30, 31, 32), there are certain combinations that result in antagonism. Gentamicin can be inactivated by carbenicillin when incubated in serum at 37°C. Noone and Pattison (Ref. 33) documented this phenomenon and went on to show that slow inactivation occurred under the same conditions with cloxacillin, ampicillin, methicillin and benzylpenicillin. It should be noted that the concentrations required for significant inactivation are rarely achieved in vivo, such that the only precautionary measures are to ensure independent administration of the two antibiotics when dosing intravenously. Antagonism arises from attack of the chemically vulnerable β-lactam ring of the penicillin by an amino group of the aminoglycoside, resulting in the formation of an amide adduct with no antibiotic activity (Ref. 34).

Antibiotic combinations for Mtb and other *Mycobacteria*

The resurgence of Mtb infection, and in particular extensively drug-resistant tuberculosis (XDR Mtb), has prompted a need to identify new therapies. Since the 1950s, tuberculosis has been treated with combination antibiotic therapy. Triple therapy with 4-aminosalicylic acid, isoniazid and streptomycin in the 1950s and 1960s cured >90% of infections, but increased use of monotherapy, and lack of alternative drugs quickly led to widespread resistance. In 1971, the US Food and Drug Administration (FDA) approved the use of rifampicin, a semisynthetic derivative of the natural product rifamycin SV, produced by *Amycolatopsis mediterranei*. Rifampin proved most efficacious in combination with isoniazid and improved the rate and time of cure. For many years, rifampicin combined with isoniazid provided excellent clinical outcomes. Isoniazid inhibits fatty acid synthesis and specifically mycolic acid synthesis required for the mycobacterium cell wall (Ref. 35). Rifampicin, however, inhibits the β-subunit of bacterial RNA polymerase to block protein synthesis (Ref. 36). Unfortunately rifampicin resistance, especially during monotherapy, arises rapidly. In fact, as early as 1975, rifampicin-resistant Mtb isolates were identified in patients treated with rifampicin alone (Ref. 37). Strains identified as being resistant to both rifampicin and isoniazid were deemed multidrug-resistant Mtb.

In 2005, XDR-TB was first described (Ref. 38) and is now a global problem. Patients must be

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treated with at least four effective drugs, chosen by in vitro susceptibility testing. The prevailing message in the history of TB treatment is that monotherapy is not effective. Sequential treatment with different drugs leads to sequential selection and accumulation of mutations, causing resistance to even more drugs. Although new drugs are in the discovery pipeline, the lengthy time it takes to reach the market will not address current needs. An alternative strategy is to investigate novel combinations and new uses of currently available drugs. The recent finding that β-lactam antibiotic resistance can be overcome with known and clinically proven inhibitors of β-lactamases (see below) is one such approach (Ref. 39).

Vancomycin and oxacillin

Synergy can also overcome antibiotic resistance. Vancomycin, once considered a drug of last resort, is now routinely used as a first-line defence against MRSA. As the first strains of vancomycin-resistant MRSA begin to emerge, treatment options have become very limited. The β-lactam insensitivity of MRSA is the result of expression of an alternative penicillin-binding protein, PBP2a, with very low affinity for β-lactam antibiotics. Vancomycin-resistant isolates synthesise alternative peptidoglycan precursors ending in D-alanine–D-lactate rather than D-alanine–D-alanine. Vancomycin binds tightly to D-alanine–D-alanine; however, precursors ending in D-alanine–D-lactate boast a 1000-fold decrease in binding affinity (Ref. 40). Three *vanHAX* genes, controlled by a two-component regulatory system, encode the biochemical machinery necessary to restructure the cell wall in this manner. Paradoxically, despite the presence of two distinct resistance mechanisms, synergy between the β-lactam antibiotic oxacillin and vancomycin has been reported (Ref. 41). This unique mechanism of synergy is fascinating, because it does not occur in strains that are sensitive to one or both drugs (Refs 41, 42). The mechanism of synergy is the result of PBP2a, encoded by the *mecA* gene. PBP2a is insensitive to β-lactam antibiotics; however, it is unable to accommodate peptidoglycan precursors ending in D-alanine–D-lactate. The presence of oxacillin induces the production of PBP2a, whereas the presence of vancomycin induces the production of the cell precursor containing the alternative D-alanine–D-lactate (Ref. 41). Consequently, a cell cannot support expression of both the *mecA* gene and the *vanHAX* cluster simultaneously, which results in drug synergy.

Inhibition of antibiotic-resistance elements

One of the major causes of antibiotic failure is acquired resistance. Inhibitors of resistance mechanisms can therefore rescue antibiotic activity and, as a result, qualify as antibiotic adjuvants. Enzymes that confer drug resistance are especially amenable to this strategy (Ref. 43). The identification of clavulanic acid in 1976 by researchers at Beecham Pharmaceuticals remains one of the most important discoveries in this area and set the precedent for the discovery of inhibitors of resistance from antibiotic producers themselves (Ref. 44). Clavulanic acid is a β-lactam produced by *Streptomyces clavuligerus*, which also produces several β-lactam antibiotics. Clavulanic acid has modest antibiotic activity but potent anti-β-lactamase activity.

β-Lactamases are enzymes that hydrolytically inactivate β-lactam antibiotics; these include penicillins, cephalosporins, carbapenems and monobactams. There are two general classes of β-lactamases: those that use a catalytic serine residue that covalently participates in β-lactam ring opening during hydrolysis and those that use an active site metal to activate the hydrolytic water molecule. These are further classified based on substrate specificity and inhibitor sensitivity (Ref. 45). Both mechanisms are clinically important, although serine-based enzymes are dominant. Clavulanic acid has no activity against metallo-β-lactamases, but does block the activity of several Ser-β-lactamases. The inhibitor acts as a suicide substrate of the enzyme. Serine attacks the clavulanate β-lactam ring, resulting in covalent modification of the enzyme. With other β-lactam substrates, this would be rapidly followed by hydrolytic cleavage of the newly formed ester; however, in the case of clavulanic acid, ring opening triggers a series of internal bond rearrangements that result in complex products associated with irreversible enzyme inactivation (reviewed in Ref. 46). The combination drugs Augmentin® and Timentin® are mixtures of clavulanic acid with amoxicillin and ticarcillin, respectively,

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which have found extensive clinical use since their deployment in the early 1980s. As noted above, there continues to be interest in clavulanate as a component of combination treatment with meropenem for the treatment of XDR-TB (Ref. 39).

Other clinically approved inhibitors of β-lactamases include the penicillin sulfones sulbactam and tazobactam, which in combination with ampicillin and piperacillin, respectively, give rise to the drugs Unasyn® and Zosyn®. Like clavulanic acid, the sulfones inhibit Ser-β-lactamases by first forming an acyl-enzyme intermediate with the active site serine followed by rearrangement to products that result in enzyme inhibition. Not surprisingly, microorganisms have responded with the evolution of β-lactamases with low affinity for these molecules. The pharmaceutical industry has countered this with the development of several new inhibitors, including novel non-β-lactam chemical scaffolds (Refs 43, 46).

None of these β-lactamase inhibitors have significant antibiotic activity (although clavulanic acid does have intrinsic weak antibiotic activity) (Ref. 44). Their adjuvant activity is the result of relief of antibiotic resistance. This is a strategy that should be applicable to many resistance enzymes. Inhibitors exist for aminoglycoside-modifying enzymes (Refs 47, 48, 49) and erythromycin *ribosomal* methlyases (Erms) (Refs 50, 51, 52) for example; however, none has been deemed sufficiently potent for further development as antibiotic adjuvants. Nevertheless, screens of small molecules against resistance enzymes remain a viable enterprise. Because environmental organisms are the source of most resistance genes and antibiotics (Refs 15, 53), screens of bacterial natural products might be more productive in this regard, as the discovery of clavulanic acid has proved. It is highly unlikely that clavulanic acid is unique.

The challenge in this strategy is the vast number of resistance enzymes. For example, there are hundreds of β-lactamases, many with distinct substrate profiles and at least two distinct chemical mechanisms. Similarly, aminoglycoside modification resulting in resistance can occur through several strategies: phosphorylation, acetylation or adenylylation, each with dissimilar enzyme structures and mechanisms. This diversity makes the task of identifying broad-spectrum inhibitors virtually impossible. Nevertheless, coupled with good diagnostics and epidemiological studies, inhibitor–antibiotic combinations can be very important, as proved by the success of β-lactamase inhibitors in the past 20 years.

Enhanced antimicrobial entry and inhibition of efflux

Molecules that enhance antibiotic entry into cells or prevent them from being removed once inside are excellent candidates for antibiotic adjuvants. Colistin (polymyxin E) is a cyclic cationic polypeptide antibiotic that permeabilises the outer membrane of Gram-negative bacteria. Toxicity concerns have limited its clinical use; however, recently it is seeing significant increased use (Ref. 54). This is the result of the increase in multidrug-resistant Gram-negative infections caused by *A. baumannii*, *Pseudomonas aeruginosa* and carbapenemase-producing *K. pneumoniae*, for which there are few therapeutic options (Refs 10, 55). Adjuvant combinations of colistin with antibiotics not normally used against Gram-negative bacteria have the benefit of reducing colistin toxicity while expanding the spectrum of other compounds. For example, synergistic combination of colistin with rifampin (Ref. 56) and vancomycin (Ref. 57) has been reported.

Colistin permeabilises the outer membrane by binding to and interfering with the structural integrity of the lipopolysaccharide-containing outer leaflet. Resistance to colistin results in detrimental effects to the cell (Ref. 58). High-level colistin resistance arises from mutations in endotoxic lipid A, a core element of the lipopolysaccharide. The negatively charged lipid A facilitates the binding of positively charged colistin to the outer membrane and loss of this charge interaction leads to resistance (Ref. 59). Although this phenomenon can occur by modification of the lipid A head groups to reduce net negative charge, a recent study discovered a link between impaired lipid A biosynthesis and colistin resistance (Ref. 58). Mutations to the *A. baumannii lpxA* gene abolish both lipid A and lipopolysaccharide synthesis, rendering the cell colistin resistant but hyperpermeable. The net result is increased susceptibility to other antibiotics, such as teicoplanin, which is normally only active against Gram-positive organisms. This validates

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inhibition of lipid A and core lipopolysaccharide components such as ADP-heptose as new targets for antibiotic adjuvants. An in vitro screen of small molecules against a reconstituted ADP-heptose biosynthetic pathway identified new inhibitors of the HldE kinase necessary for the synthesis of lipopolysaccharide (Ref. 60). A further screen of 40 000 chemicals identified other inhibitors of this enzyme (Ref. 61) that are good leads as antibiotic adjuvants.

The reciprocal of facilitating entry is blocking efflux. Multidrug-resistance pumps such as the AcrAB–TolC and MexAB–OprM, both belonging to the resistance nodulation division family of pump proteins, are a major problem in some Gram-negative infections (Refs 62, 63, 64). There are numerous examples of efforts to identify and exploit efflux pump inhibitors (EPIs) (Refs 65, 66). The small molecules resperine (Refs 67, 68), arylpiperidines, arylpiperazines (Refs 65, 69, 70), and even trimethoprim and epinephrine have been shown to inhibit efflux pumps (Ref. 71).

Examples of antibiotic synergy with EPIs are also found in nature. Interactions occur between 5-methoxyhydnocarpin (5′-MHC), a multidrug-resistance EPI, and cationic berberine alkaloids, which are both produced by the medicinal plant *Berberis fremontii* (Ref. 72). Berberine resembles and functions similarly to DNA-intercalating compounds such as ethidium bromide. The antibiotic activity of berberine in pathogenic organisms such as *S. aureus* is weak because of the presence of multidrug-resistance efflux pumps; however, in the presence of 5′-MHC, the MIC of berberine is significantly decreased. 5′-MHC does not show antimicrobial activity on its own, and this study was able to exploit the fluorescent properties of DNA-bound berberine to show that 5′-MHC efficiently inhibits berberine efflux from the cell (Ref. 72).

Other naturally derived EPIs include the flavonolignan silybin (Ref. 72), methoxylated isoflavones (Ref. 73), polyacylated neohesperidosides (Ref. 74), and many other plant- and microbe-derived examples (Ref. 75). A recent screen of a library of plant-derived compounds identified ellagic and tannic acids as potentiators of antibiotics, probably as a result of inhibition of efflux (Ref. 76). Although none of these compounds is currently developed for clinical use and the data are still preliminary, the difficulties in fighting multidrug-resistant infections make exploiting the adjuvant activities of EPIs a very attractive option. Moreover, the genetic potential of both plant and microbial sources to produce small molecules with efflux inhibitory activity has not been fully exploited in this area of research. The challenge in this approach mirrors that of resistance enzyme inhibition: there are numerous efflux systems with different affinities for EPIs, often in the same organism. Identification of compounds with sufficient affinity to block a broad collection of clinically relevant efflux has not proved straightforward.

Physiology

Bacterial strategies to resist antibiotics are not always dependent on acquiring genetic elements, but instead, an alteration in lifestyle can impact drug susceptibility. For example, the physiological changes associated with planktonic growth versus growth within a biofilm result in reduced sensitivity to antibiotics. Bacteria form biofilms by attachment to a solid surface and grow as dense complex communities surrounded by biopolymers called extracellular polymeric substances. The matrix that holds the biofilm together comprises ~90% of the biomass and the bacteria make up the remaining ~10%. The hallmarks of the biofilm lifestyle include extensive cell–cell signalling, the formation of microcommunities and, most importantly, protection from antibiotics (Ref. 77). Biofilms are a major concern in the lungs of cystic fibrosis patients, and it has been estimated that a cell in a biofilm is 1000 times more resistant to antibiotics than the planktonic form (Ref. 78). The basis for this resistance is dependent on several factors, including reduced penetration of drugs through the matrix. This is the case for some aminoglycosides (Refs 79, 80); however, fluoroquinolones such as ciprofloxacin can penetrate quickly (Ref. 81). Regardless of penetration, neither of these two drugs readily kills cells within a biofilm, even if planktonic bacteria are susceptible. After 100 hours of *P. aeruginosa* biofilm treatment with either ciprofloxacin or tobramycin, the log reduction in viable bacteria was found to be <1.5, but sensitivity returned when biofilms were resuspended and treated with the drug (Ref. 82). Most antibiotic activity occurs at the air–biofilm interface, suggesting that low metabolic activity and oxygen content contribute to apparent

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resistance (Ref. 82). Furthermore, there is a population of cells within the biofilm that are intrinsically tolerant to antibiotics (Ref. 83). These so-called persister cells are genetically identical to antibiotic-susceptible cells in the biofilm, but are impervious to antibiotics as a result of phenotypic differences (Ref. 84). The production of toxin components of toxin–antitoxin modules has been associated with persistence (Refs 85, 86).

Mechanisms that inhibit or disperse biofilms or otherwise resensitise the cells within the matrix to antibiotics are therefore highly desired (Ref. 87). Mixtures of D-amino acids have been shown to disperse biofilms of Gram-positive and Gram-negative bacteria (Ref. 88). A high-throughput screen of 66 095 molecules for inhibition of *P. aeruginosa* biofilm formation identified several candidate molecules (Ref. 89). Similarly, screens of synthetic and natural-product-inspired libraries have identified small-molecule inhibitors of biofilm formation and enhancers of dispersal (Refs 90, 91, 92). Some of these show synergy with antibiotics and are great leads as antibiotic adjuvants (Ref. 93).

Nonantibiotic combinations

The desire to achieve synergy against pathogenic organisms with combinations of molecules does not require two known antibiotic molecules. There is ample evidence that nonantibiotic molecules have the ability to enhance the activity of antimicrobial agents. There have also been examples of synergy occurring between antibiotics and other drug classes such as anti-inflammatory (Ref. 94), cardiovascular (Ref. 95), psychotropic and newly discovered natural products (Refs 96, 97, 98, 99). Diclofenac is a nonsteroidal anti-inflammatory drug that shows antimicrobial activity on its own against mycobacteria, even multidrug-resistant strains. More interesting is the synergy between diclofenac and streptomycin observed both by checkerboard assay and in vivo, with a $\log_{10}$ reduction in colony-forming units in the lungs and spleen (Refs 100, 101). There have been other efforts to uncover synergistic interactions between known and non-antimicrobial molecules, much of it in the antifungal field. Some examples include marked synergy between azole antifungals and a group of small molecules called citridones, produced by *Penicillium* sp. (Refs 102, 103).

This is a strategy with tremendous potential to expand antimicrobial chemical space. There are thousands of known bioactive compounds, and systematic investigation of their potential to enhance the activity of antibiotics would no doubt identify several unanticipated interactions and even synergy.

Clinical implications and outstanding research questions

Combination therapy has two desired outcomes: (1) improved treatment efficacy and (2) reduction in the rate of mutations that result in resistance. The two, however, are often not necessarily directly correlated. Mathematical models of infection illustrate that improved efficacy with synergistic pairs can result in a higher risk of selecting for drug-resistant mutants (Ref. 104). The reason for this trade-off is dependent on the starting population of organisms and frequency of mutation within it. Synergistic combinations effectively inhibit the wild-type population, alleviate competition for resources and can allow single resistant mutants to proliferate. This population then has the opportunity to become double-resistant mutants. In such a situation, a 'synergy ceiling' of drug interaction occurs, above which higher synergy does not affect efficacy, but still increases the risk of resistance, owing to a higher frequency of mutation. Importantly, this theory applies to populations that compete for resources – a situation that is not always clinically relevant. When the population size is low and competition is weak, the wild-type cells are killed quickly and the risk of resistance is decreased. Although this study is thus far entirely theoretical, it raises important considerations for deployment of antibiotic adjuvant combinations.

Another consideration is the requirement to match pharmacological profiles of compound pairs. Determination of synergy or potentiation in vitro might not be reflected in vivo because of failure to achieve synergistic levels of drugs in the desired tissue, differences in plasma protein binding and drug metabolism. Design of appropriate clinical trials can also be challenging, and because each compound must also be investigated for safety and efficacy independently, trial costs will be higher than those with single agents. However, there are many examples of successful combination

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drugs, and these are templates for success in bringing antibiotic adjuvants to the clinic and to market.

Despite these important considerations, the advantages of investigating antibiotic adjuvants are considerable. They effectively expand antibiotic chemical space dramatically. Furthermore, although this review has concentrated on combining compounds with known antibiotic activity with other molecules to achieve an enhanced effect, it is possible to consider matrices of bioactive molecules that might not necessarily have demonstrable antibiotic activity, but in combination with another molecule could have cryptic antimicrobial effects. A matrix of only 1000 molecules results in 1 000 000 possible pairs, which represents a rich source of chemical space. Higher-order combinations (such as those that have seen such success in Mtb and HIV treatment) would greatly expand this potential.

We are in an era of tremendous clinical need for new antibiotics, perhaps not seen since the mid-20th century. The combination of molecules to block resistance, enhance activity and counter insensitivity to antibiotics represents a highly promising and clinically proven strategy that can contribute to this need.

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Further reading, resources and contacts

Pillai, S., Moellering, R.C. and Eliopoulos, G.M. (2005) Antimicrobial combinations. In Antibiotics in Laboratory Medicine (Lorian, V. ed.), Lippincott Williams & Wilkins, Philadelphia
This chapter in Antibiotics in Laboratory Medicine reviews methods to assess combinations and examples of known antagonistic, additive and synergistic combinations.

Torella, J.P., Chait, R. and Kishony, R. (2010) Optimal drug synergy in antimicrobial treatments. PLoS Computational Biology 6, e1000796
This is a very interesting paper discussing the effect of antagonism versus synergy on antibiotic resistance selection using mathematical models.

De Pascale, G. and Wright, G.D. (2010) Antibiotic resistance by enzyme inactivation: from mechanisms to solutions. Chembiochem 11, 1325-1334
Piddock, L.J. (2006) Multidrug-resistance efflux pumps – not just for resistance. Nature Reviews. Microbiology 4, 629-636
These reviews discuss mechanisms of antibiotic resistance due to enzymatic inactivation and the role of efflux pumps, respectively.

Features associated with this article

Figures
Figure 1. Mechanisms of antibiotic resistance.
Figure 2. Antibiotic adjuvants: achieving synergy.
Figure 3. Laboratory methods to determine synergy.
Figure 4. Mode of action of sulfamethoxazole and trimethoprim.

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