Using Antibacterial Compounds in Synergy

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Abstract

In an effort to contain the ongoing increase in antibiotic resistance, research has been focusing on finding novel ways of employing existing antibiotics. One such line of research has emerged around the concept of drug combination to achieve synergistic effects such as enhanced antibacterial activity, resistance prevention and overcoming resistance. These objectives can be achieved through either combining antibiotics or combining an antibiotic with an antibiotic rejuvenating compound known as an adjuvant. Antibiotic combinations can be used to decrease the change of resistance development through increasing the amount of targeted gene products. Whereas adjuvants are usually used to overcome obtained or intrinsic resistance mechanisms, such as: inhibition of β-lactamases, inactivating antibiotic efflux pumps, or increasing the outer membrane permeability. This last example is of particular interest because it can be used to make previously ineffective antibiotics bactericidal against gram-negative bacteria. One major challenge is the translation of *in vitro* laboratory results into clinical practice. So far clinical success has been achieved with: isoniazid + rifampin against *M. tuberculosis* resistance, sulfamethoxazole + trimethoprim for enhanced antibacterial activity, and several β-lactam antibiotic + β-lactamase inhibitor combinations. But a lot of potential remains, demonstrated by the fast amount of synergistic combinations known from *in vitro* testing.

Keywords: synergetic usage, antibiotics, resistance

Using Antibacterial Compounds in Synergy

Antibiotics are one of the greatest success stories in medicine. Not only did they drastically decrease the mortality rate of bacterial infections, but they also enabled other medical treatments like surgery, that otherwise would be too dangerous to execute due to the risk of bacterial infections (Hedrick, Smith, Gazoni, & Sawyer, 2007). Because of the magnitude of the achievement it is all the more worrisome that this success is at risk of being undone by a growing population of antibiotic resistant bacteria (Gould, 2009). It is estimated that within the EU and EEA alone, approximately 33.000 deaths can be attributed to antibiotic resident pathogens annually, with the burden in disability-adjusted life-years being comparable to that of influenza, tuberculosis and HIV combined (Cassini et al., 2019). With such numbers it is all the more concerning that the development of new antibiotics has been stalling since the end of the antibiotic golden age in the 1960s (Hutchings, Truman, & Wilkinson, 2019). One important reason for the lack of development is the lack, to the pharmaceutical industry, of financial incentives to invest. Antibiotics are a curing drug, so require no chronic usage. To combat the antibiotic resistance new antibiotics are likely to be used as a last resort, further decreasing the potential sales. Luckily, policy makers have been made aware of this problem and several countries have made plans to incentivise the development of new antimicrobial compounds (Gotham et al., 2021). Even with financial incentives the development of new antimicrobial compounds remains a cumbersome task (Butler & Paterson, 2020). Novel strategies to use the pre-existing repertoire of antibacterial agents is therefore necessary. One such strategy could be to employ antibacterial agents in combination, which can lead to synergy.

Synergy is defined by the Cambridge dictionary as: “the combined power of a group of things when they are working together that is greater than the total power achieved by each working separately” (‘SYNERGY | meaning in the Cambridge English Dictionary’, n.d.). In

other words, when synergy occurs, the whole is greater than the sum of its parts. In the context of antibacterial agents there is synergy when two or more antibiotics together exhibit greater activity than would be expected through the simple additive activity (Brennan-Krohn & Kirby, 2019). The idea that drug combinations can lead to enhanced activity is not novel, having already been proposed by Paul Ehrlich in 1913 (Ehrlich, 1913). A famous example in antibiotic treatment is the combination of β-lactam antibiotics with β-lactamase blockers. By blocking one β-lactam resistance pathway the drug is more likely to remain effective (Tooke et al., 2019). Many more combinations are currently used, but a lot of potential remains. In the present review, the current state of antibiotic drug combinations will be discussed. More specifically, the focus is directed towards the mechanisms of these combinations, how they can be tested for, and which are currently already in use. The conclusion provides an outlook towards the potential future of synergetic usage in the specific context of challenging the antibacterial resistance crisis.

Overview of the mechanism of antibiotics

In order to understand how synergy can arise from combinational usage of antibacterial agents, a general understanding of the functioning of these agents is required. The following paragraph aims to provide the necessary insight into the functioning of antibiotics.

The aim of antimicrobial compounds is to kill or inhibit growth of bacteria while afflicting minimal harm to the host organism. This last aim means the drug/compound has to be *selective*. This selectivity is what sets antibacterial compounds apart from disinfectants, which instead kill indiscriminately. Whereas disinfectants often cause deadly events leading to necrosis of the bacterium, antimicrobials often cause deadly processes leading the bacterium to induce apoptosis (Baquero & Levin, 2021). As mentioned, these deadly processes are required to be selective, in order to minimize the harm done to the host cells, and maximize

the harm done to bacterial pathogens. This selectivity can be achieved by for instance targeting a metabolic pathway that is only present in the pathogen (Graham, 2017). This is how, for instance, Sulphonamides obtain their selectivity, by targeting tetrahydrofolate synthesis (Graham, 2017). The most common way in which selectivity is obtained is by targeting enzymes or structures in the bacterium that are unique or significantly different from eukaryotic cells, such as the cell wall or ribosomes respectively (Graham, 2017).

Besides being selective, antibacterial compounds need to be sufficiently *disruptive* to the targeted bacteria. Five major mechanisms can be identified for such antibacterial activity (Graham, 2017):

1. *Inhibition of cell metabolism.* Antibiotics that use this mechanism are known as antimetabolites. A famous example of an antimetabolite are the Sulfonamides. They block tetrahydrofolate synthesis by competitive inhibition.
2. *Inhibition of bacterial cell wall synthesis.* Selectivity is obtained by the absence of a cell wall in eukaryotes. A famous example of this mechanism are penicillins. They block the formation of crosslinks between the peptidoglycan via covalent inhibition.
3. *Disruption of the plasma membrane.* These compounds are especially useful against gram negative bacteria. By disrupting the membrane they increase the permeability for instance ions.
4. *Disruption of protein synthesis.* These drugs can disrupt several possible steps (depending on the drug) in the translation process, causing misreads and/or premature termination. Because proteins stand at the basis of many cellular processes, this can lead to bactericidal effects.
5. *Acting on the nucleic acid transcription.* Disrupting this earlier step in the central dogma, just like disrupting translation can cause bactericidal effects

Unfortunately bacteria are becoming increasingly resistant against these conventional antibiotic mechanisms, by enzymatically modifying the drug, changing the drug target, or preventing the drug from reaching its target via physical barriers or efflux pumps (Blair, Webber, Baylay, Ogbolu, & Piddock, 2015). One way to overcome these resistance mechanisms is by drug combinational usage.

Mechanisms of antibacterial synergy

As the mechanisms of antibacterial compounds have been discussed, it is now possible to focus on how combinations of these individual compounds can lead to synergy. There are two major synergetic possibilities from drug combinations: enhanced antibacterial activity and resistance prevention (Brennan-Krohn & Kirby, 2019).

Prevention of resistance

When one drug has multiple targets, the likelihood of development of resistance to that drug decreases (Silver, 2007). This phenomenon can be explained by the *multi-target hypothesis*: drugs that are susceptible to the single-step development of high- level endogenous resistance are the ones which interact with a single gene product (Silver, 2007). In other words, when a drug interacts with multiple gene products, the emergence of endogenous resistance is less likely, because the change of simultaneous development of resistance in multiple gene products is far less likely than either isolated event. Of course the same reasoning applies to combinational drugs when their targets are separate gene products. As long as the presence of one resistance pathway does not benefit the bacteria, because the two individual antibiotics are bactericidal, there will be no selective pressure for either individual resistance mechanism. When two events do not influence each other's outcome, (i.e., they are independent) the probability of both events taking place, is the product of the probabilities of the independent events. This brings us back to the definition of synergy, where the product is more than the mere additive (i.e., sum) effect of its parts. Because the

probability of simultaneous resistance is equal to the product instead of to the sum of the probabilities of the individual resistance mechanisms, this can be considered to be a synergistic effect of the drug combination. It is important to note that the multi-target hypothesis only applies to obtained resistance through mutations, but not to resistance obtained through horizontal gene transfer.

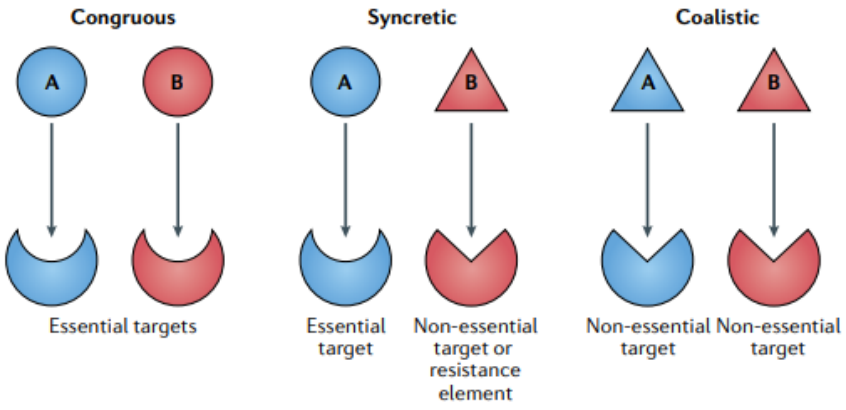
Enhanced antibacterial activity

How drug combination can lead to enhanced antibacterial activity cannot be generalized in the same manner as how resistance can be prevented, due to a large number of different possibilities. However, a way of navigating these diverse scenarios is via categorization based on drug type and drug target. This categorization can be helpful in illustrating the mechanisms underlying enhanced antibacterial activity.

Types of drug combinations

In antibacterial combinational usage two types of drugs can be defined: the first group contains the antibacterial compounds that show intrinsic bactericidal activity. These will be referred to as antibiotics. The second group contains compounds that only show antibacterial activity in combination with another drug. These two types can be combined in three groupings with defined nomenclature (Tyers & Wright, 2019).

Figure 1: *Classification of Synergetic antibiotic combinations. Taken from: Tyers & Wright, 2019*



When two antibiotics are combined, the resulting combination is called *congruous*. An example of a congruous combination is when penicillin is combined with the aminoglycoside streptomycin to treat *Enterococcal* infections (Plotz & Davis, 1962). Currently, congruous combinations are the most commonly used in clinical practice, although established doses remain rare (Tyers & Wright, 2019). As discussed before this type of drug combination can be used to prevent the emergence of resistance.

The second type of combination, instead, involves one antibiotic in combination with a drug that only exerts bactericidal activity in combination with the antibiotic. This type is referred to as: *syncretic*. The helper drug in this context is known as the antibiotic adjuvant. A well-known example of a syncretic drug combination are the penicillin and β-lactamase blocker combinations. β-lactamase blockers do not have antibacterial activity on their own and are therefore the antibiotic adjuvant in this example. Antibiotic adjuvants can be divided into two types. *Class I* adjuvants target the bacterial metabolism or physiology, while *class II* adjuvants target the host (i.e., patient) biology (Tyers & Wright, 2019). Just as in the example, syncretic combinations are often used to overcome a resistance mechanism. In this case the β-lactamase blocker blocks the enzyme that hydrolyzes penicillin, reviving the penicillin's bactericidal activity. Resistance mechanisms can also be endogenous. Think about how gram negative bacteria block the entry of many antibiotics with their outer membrane (Klobucar & Brown, 2022). Also here adjuvants can play an important role by facilitating the diffusion of antibiotics through the outer membrane (Klobucar & Brown, 2022).

The last combination type combines two drugs that only have bactericidal activity when combined. When the drugs are administered independently no antibacterial effect should be observed. This combination is known as *coalistic* (Tyers & Wright, 2019). Because bactericidal activity from coalistic drug combinations is emergent in itself the strength of the activity is less relevant for the definition of synergy.

Drug-Target combinations

In a 2007 review on Multi-target therapeutics, Zimmermann, Lehár, & Keith identified three types of target combinations (Zimmermann, Lehár, & Keith, 2007). In the first type the drugs have different targets. These can be in one sub system, like one metabolic pathway, or target completely separate systems all together. In the second combination type, one drug influences the ability of the other drug to reach its target. Examples of this could be: influencing membrane permeability or blocking an enzyme from deactivating the other drug. This target combination is often employed in syncretic drug combinations with class I adjuvants. In the last type the two drugs allosterically affect the same target, like for example different sites of the ribosomes. If the target is one gene product and not strongly conserved, this target combination might be relatively susceptible to endogenous resistance (Silver, 2007). All these types of target combinations can lead to synergistic effects in which many combinations of targets and drugs are possible.

How to test for synergy

Once established how synergy can arise, another important aspect to be considered is how its presence can be assessed. Because the definition of synergy depends on the mechanism of the drug combination, different types of drug combinations require different tests or thresholds, to determine if the combination is indeed synergetic. Different tests yield different results and thresholds can be arbitrarily applied (Brennan-Krohn & Kirby, 2019). Bacteria are often considered to be dead when they can no longer replicate, because they no longer form colonies on a plate. This perception is however flawed because the individual cell can still be performing metabolic processes and in some cases the ability to replicate can even be regained (Baquero & Levin, 2021; Lewis, 2007). It is therefore important to be cautious when interpreting the implications of different test results, and when comparing results from different tests.

Testing for congruous synergy

Because the two antibiotics in congruous combinations have Minimal Inhibitory Concentrations (MICs) of their own, a combination is only synergetic when both MICs are significantly lower when the drugs are combined. Maybe the simplest way of testing this is with the *checkerboard array method*. This method makes use of a checkerboard array on a 96 well plate in which different concentrations of each antibiotic are present in the length and width of the plate respectively. This ensures different combinations of concentrations in each well. The concentrations of the antibiotics are expressed as fractions of their individual MIC values. These fractional inhibitory concentrations (FICs) of both antibiotics are summed up for each well. If the combined FIC is lower than 0.5 the antibiotic combination is considered to be synergetic (Brennan-Krohn & Kirby, 2019). The benefit of this method is that it does not require any expensive laboratory equipment and can therefore be readily performed in any standard microbiology laboratory. It is however important to keep in mind that MIC values do not guarantee bactericidal activity as much as inhibition to replicate (Baquero & Levin, 2021).

Another approach to measure growth inhibition, makes use of *antibiotic diffusion gradients*. These diffusion-based methods use the antibiotic gradient from disks or diffusion strips on an agar plate, to determine in which antibiotic concentration bacteria can grow (White, Burgess, Manduru, & Bosso, 1996). The area around the disks or strips where the antibiotic concentration is too high for growth is known as the inhibition zone. For the disk-based method, synergy can be determined when the inhibition zone between the two antibiotic containing disks increases where the gradients overlap. When there is a decreased inhibition zone the antibiotic combination is antagonistic. For a less perception-based gradient method, antibiotic diffusion strips can be used. By placing the strips consecutively on the plate, an overlapping gradient is made from which the concentrations at the inhibition zone can be determined. The strips can also be placed perpendicular, intersecting at the MIC values of the

individual antibiotics. The advantage of the diffusion methods compared to the checkerboard array method, is that FIC values can be determined on a gradient and not at set concentrations. A downside is that the results are more open to interpretation rather than a clear presence or absence of bacterial growth.

Time kill assays are another approach to determine synergy (Brennan-Krohn & Kirby, 2019). Where the previous methods only test for growth inhibition, time kill assays actually assess bactericidal activity over time. Liquid cultures are made with predetermined concentrations of antibiotics. One for each antibiotic, one combined and one negative control without antibiotics. Samples are taken from the cultures over time and if need be diluted before being spread on agar plates. The next day the viable bacterial concentration of each sample at each time point can be calculated from the colony counts. This way the bactericidal activity can be determined over time. An antibiotic combination is considered synergistic when the decrease in viable bacteria in the combined culture is more than a 100X (2 log) more than in either individual antibiotic culture with the same concentration (White et al., 1996). Even though this method provides a lot of information about the bactericidal activity of the combination, the downside is that it is relatively laborious. Because of this the amount of antibiotic concentrations that can be tested is more limited compared to the previously mentioned approaches.

Testing for syncretic and coalistic synergy

Because antibiotic adjuvants do not have intrinsic antibacterial activity at low concentrations, the same testing as used for congruous synergy, cannot always be directly applied for testing for syncretic synergy. One approach uses ¼ of the antibiotic MIC. After which the rescue concentration of the antibiotic adjuvant can be established by testing methods described before (Tyers & Wright, 2019). The rescue concentration is the concentration of the adjuvant from which the bacteria are again susceptible to the antibiotic.

Another method is to use the MIC of the adjuvant and apply the same synergy criteria as used for congruous combinations. The MIC of the adjuvant will often lay higher than conventional antibiotics, but will inevitably exist, as the famous quote attributed to Swiss physician Paracelsus goes: “The dose makes the poison” (Chen, Giesy, & Xie, 2018).

In coalistic synergy none of the individual compounds have intrinsic antibacterial activity at low concentrations. This complicates microbiology laboratory testing significantly. Because the coalistic combinations in natu re often exceed two compounds, ordinary checkerboard arrays are not enough (Tyers & Wright, 2019). It is possible to make checkerboard screens with 3 compounds in 3D, by altering the concentration of the 3rd compound in each plate (Yu, Felegie, Yee, Pasculle, & Taylor, 1980). But this is labor intensive and still limited to only 3 compounds. It is more likely that combinations need to be directly determined from nature (Challis & Hopwood, 2003). Or that promising combinations need to be identified from modelling (Sun, Vilar, & Tatonetti, 2013).

In conclusion, determining the occurrence of synergy is a challenging procedure, which also varies greatly depending on the compounds involved. While research continues to investigate and improve testing methodologies, specific compound combinations are already being developed and employed in clinical practice.

Currently employed combinations

Many drug combinations are being investigated to achieve synergistic antibacterial effects. The following section addressed three core aspects surrounding currently researched combinations: which objectives can be achieved via different combinations, at which stage of development and employment these combinations are, and which drawback the different approaches present.

Congruous synergy

Combining antibiotics for congruous synergy can be used to achieve the following main objectives: resistance prevention and enhanced antibacterial activity. This latter objective, in turn, allows for the reduction in the drug dosage, which can, in some cases, lead to a diminished risk of toxicity, an additional reachable objective.

Probably the most famous application of congruous synergy with the objective to avoid resistance is in the treatment of tuberculosis. After the introduction of streptomycin as a tuberculosis treatment drug, it soon became apparent that *M. tuberculosis* could quickly develop resistance against the antibiotic (Crofton & Mitchison, 1948). Because *M. tuberculosis* quickly develops resistance against any single antibiotic, nowadays combinations of antibiotics are used instead (Brennan-Krohn & Kirby, 2019). The WHO advises combinations of isoniazid, rifampin, ethambutol and pyrazinamide against drug-susceptible *M. tuberculosis* isolates (World Health Orgnaization, 2017). The combination of isoniazid and rifampin alone has also been shown to prevent the emergence of resistance, in line with the multi-target hypothesis (Moulding, Le, Rikleen, & Davidson, 2004).

With regards to the objective of enhancing antibacterial activity, an array of diverse examples can be discussed. Specifically, the different combinations employed achieve this goal with different mechanisms. One such combination involves sulfamethoxazole with trimethoprim (Masters, O'Bryan, Zurlo, Miller, & Joshi, 2003). Both of the antibiotics inhibit the synthesis of tetrahydrofolic acid. But by targeting two different enzymes the antibiotics inhibit two consecutive steps in the process, which leads to enhanced bactericidal activity. However, over years of usage, the emergence of resistance has rendered the combination less attractive as first line therapy. Because of this the combination is currently only employed in specific cases, rather than as a general approach (Masters et al., 2003). Another congruous combination that can be used to enhance antibacterial activity is that of penicillin with

gentamicin (Plotz & Davis, 1962). Differently from the previous example, in this combination, penicillin inhibits cell-wall synthesis, while gentamicin inhibits translation. The synergy is thought to arise from the enhanced permeability of the cell wall to gentamicin (Plotz & Davis, 1962). Another example of enhanced antibacterial activity through increased permeability is the combination of aminoglycosides with carbapenems (Yadav et al., 2017). This time the aminoglycoside disrupts the outer-membrane, to allow for the carbapenem to reach its target (Yadav et al., 2017). The mechanism of physical outer-membrane disruption is analogous to how outer membrane targeting antibiotic potentiators enhance antibacterial activity, which will be discussed later in this review. A potential advantage of aminoglycosides over outer membrane disrupting adjuvants, is their intrinsic antibacterial activity which adds an additional gene product necessary to overcome to obtain resistance to the combination (Silver, 2007).

Finally, as mentioned, congruous synergy can sometimes lead to reduced toxicity to the host, thanks to the possibility to administer a reduced dosage of the individual antibiotics. An example of a combination in which reduced toxicity was observed is the combination of colistin with bacteriocins (Naghmouchi et al., 2013). Currently, this combination has provided evidence of reduced toxicity to the host in terms of reduced levels of DNA damage in mammalian cells in *in vitro* conditions (Naghmouchi et al., 2013).

It is important to note that from the previously described combinations only the sulfamethoxazole trimethoprim combination and tuberculosis treatment combinations have seen clinical application.

Syncretic synergy

Just as with congruous combinations, some syncretic combinations are currently used clinically. Most clinical success has been made with the adjuvant β-lactamase inhibitors, with

a lot of potential remaining as can be judged from the presence of β-lactam β-lactamase inhibitor drug combinations in different stages of clinical trials (Butler & Paterson, 2020). Two other promising adjuvant groups are efflux-pump inhibitors (EPIs) and outer-membrane disruptors. With the latter playing a potentially important role in potentiating antibiotics against gram negative bacteria that would otherwise have endogenous resistance.

β-lactamase inhibitors

Due to the great success of β-lactam antibiotics, and therefore their widespread usage, there has been strong selective pressure on many pathogenic strains to evolve resistance against these antibiotics. One of the most prevalent mechanisms of resistance is by expressing enzymes that deactivate the antibiotics by hydrolyzing the strained β-lactam ring. These enzymes are called β-lactamases and can be categorized in three groups based on their structure and enzymatic mechanisms. Groups A, C and D use a serine-based hydrolysis mechanism that is closely related to that of the PBP enzyme that β-lactam antibiotics target, whereas group B uses two zinc ions to activate a water molecule as nucleophile. The members of this last group are known as metallo β-lactamases (MBLs). Although the A, C and D serine β-lactamases (SBLs) use the same mechanism of hydrolysis, their sequences are significantly different (Tooke et al., 2019). There are more than 4300 unique sequences of β-lactamases known, a number that is rapidly increasing (Tooke et al., 2019). This large diversity makes targeting all of them simultaneously difficult.

Because the serine β-lactamases are closely related to PBPs it should come as no surprise that the first β-lactamase inhibitors are closely related to the β-lactam antibiotics. The first clinically approved β-lactamase inhibitor is clavulanic acid and shares structural similarities with penicillin. It was admitted to the market, in combination with the β-lactam antibiotic amoxicillin, by the FDA in 1984 (Jalde & Choi, 2020). This combination is known as Augmentin and can be used against infections with *S. pneumoniae, H influenzae* and *M.*

catarrhalis. Other penicillin-based inhibitors, sulbactam and tazobactam followed soon after, with the latter being admitted in 1993 (Jalde & Choi, 2020). Sulbactam has even been shown in specific cases to have bactericidal activity of its own (Higgins, Wisplinghoff, Stefanik, & Seifert, 2004). The penicillin-based inhibitors are mostly active against class A β-lactamases, so the search for more broad spectrum inhibitors continued. The second group of inhibitors are based on diazabicyclooctane. With clinical examples being: avibactam and relebactam, approved in 2005 and 2019 respectively. These inhibitors show a broader spectrum inhibition, being generally active against most categories of serine β-lactamases. Several new diazabicyclooctane based inhibitors are in development with some like nacubactam showing intrinsic antibacterial activity (Morinaka et al., 2016). Utilizing the intrinsic antibacterial activity of β-lactamase inhibitors there is even one combination in clinical development of the penicillin based inhibitor sulbactam with the diazabicyclooctane based inhibitor durlobactam, without any antibiotic (Durand-Réville et al., 2017).

Even though the diazabicyclooctane based inhibitors cover a relatively broad spectrum of β-lactamases none of them have shown any activity against the metallo β-lactamases. A combination of avibactam with the antibiotic aztreonam is currently undergoing clinical trials against metallo β-lactamases (Sader et al., 2018). But here aztreonam is the compound that inhibits the metallo β-lactamase. A promising group of broad spectrum β-lactamase inhibitors are the boronic acid-based inhibitors. Boron compounds have been shown to be potent inhibitors of other serine proteases, which caused interest to investigate these compounds (Jalde & Choi, 2020). In 2017 vaborbactam became the first clinically available boron acid-bases inhibitor, in combination with the antibiotic meropenem (Jalde & Choi, 2020). Vaborbactam inhibits class A and C β-lactamases and some class A carbapenemasese, but shows no activity against metallo β-lactamases. There are some promising boronic acid-based inhibitors in development that do inhibit metallo β-lactamases, like: taniborbactam and

QPX7728. Both of which also show broad spectrum activity against the serine β-lactamases (Jalde & Choi, 2020). This makes the boronic acid-based inhibitors the most promising candidates for broad spectrum β-lactamase inhibitors.

Efflux pump inhibitors

One resistance mechanism of particular concern is that of the bacterial efflux pumps. Because these pumps often have a broad spectrum activity they can render antibiotics of several categories ineffective simultaneously, leading to the multi drug resistance phenotype (Sharma, Gupta, & Pathania, 2019). There are five classes of efflux pumps known in bacteria: the ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multidrug and toxic compound extrusion (MATE) family, the small multidrug resistance (SMR) family and the resistance nodulation division (RND) family (Sharma et al., 2019). The last one plays a prominent role in multi drug resistance in gram negative bacteria (Li, Plésiat, & Nikaido, 2015). Efflux pumps also seem to be involved in bacterial biofilm formation, making potential efflux pump inhibitors all the more interesting (Alav, Sutton, & Rahman, 2018). Several promising efflux pump inhibiting adjuvants have been identified. These can be categorized into two main modes of action: inhibition through target binding and inhibition through energy dissipation. The first mode works by either competing with the substrate for the binding site or inducing a conformational change that inactivates the pump. The second mechanism works by removing the energy source of the pump (Sharma et al., 2019).

Some examples of inhibitors through direct binding are: phenylalanyl arginyl β-naphthylamide (PAβN) and 1-(1-naphthylmethyl)-piperazine (NMP). PAβN was the first EPI that showed activity against the clinically relevant RND family of efflux pumps. It has shown to potentiate antibiotics such as levofloxacin, sparfloxacin, chloramphenicol and erythromycin in *P. aeruginosa*, but had limited activity against other RND substrates: tetracycline and carbenicillin (Lomovskaya et al., 2001). The exact mechanism of action is still disputed, but if

PAβN would be a competitive inhibitor this could mean that the RND pump has different binding sites for its substrates. Hence that would explain why PAβN does not inhibit all RND substrates (Opperman & Nguyen, 2015). NMP has shown to be able to lower the MICs of several antibiotics across species such as: *E. coli, E. aerogenes, K. pneumoniae* and *A. baumannii.* Yet despite this success it has little therapeutic potential, because of low potency and suspected host toxicity (Opperman & Nguyen, 2015). Carbonyl cyanide-m-chlorophenylhydrazone (CCCP) is an example of an EPI that works via energy dissipation. It disrupts the proton motive force by facilitating proton leakage across the membrane. Besides the ABC superfamily, which uses ATP hydrolysis as energy source, the efflux pumps use the proton or sodium motive force to transport their substrates across the membrane (Sharma et al., 2019). Therefore proton decouplers like CCCP could in theory inhibit a broad spectrum of efflux pumps. Again host toxicity prevents therapeutic usage of CCCP as EPI (Sharma et al., 2019).

The idea of rejuvenating antibiotics by inhibiting efflux pumps has been around since the 1990s, but so far no adjuvant antibiotic drug combination has become commercially available. And with not a single efflux pump inhibitor currently undergoing clinical testing, it does not look likely there will be any combination commercially available any time soon (AlMatar, Albarri, Makky, & Köksal, 2021). Several commercial and practical reasons have been suggested for the lack of clinical success, such as: complicated synthesis mechanisms, host toxicity, and a lack of interest by the pharmaceutical industry (AlMatar et al., 2021).

Outer membrane targeting antibiotic potentiators

One of the major challenges in the field of antibiotics is the endogenous resistance of gram negative bacteria. Because the outer membrane functions as a physical barrier to hydrophobic compounds, many antibiotics don’t work on gram negative bacteria, even though the antibiotic targets are present. These antibiotics can in theory be rejuvenated if they are

combined with an adjuvant that removes this barrier. There are two main approaches for adjuvant membrane disruption. The adjuvant can physically disrupt the membrane, or the adjuvant can inhibit the membrane synthesis. Both mechanisms have successful examples in vitro, but physical disruption appears to be the more common approach.

One group of interest are the antimicrobial peptides (AMPs). AMPs are often cationic which attracts them to the anionic outer membrane. By inserting themselves in the outer membrane they can either form pores, cause micellization or just increase the membrane fluidity (Luo & Song, 2021). The result is that the co-administered antibiotic can then reach its previously inaccessible target and exert bactericidal activity. One problem with peptides is their bio-instability. In case of oral administration they could simply be digested by peptidases. Several adjustments are however possible to improve biostability, like peptidomimetics (Baker et al., 2019). Cationic Steroidal Antibiotics are another alternative. These compounds, related to steroid hormones, do show similar outer membrane activities as AMPs, but vastly improve on the biostability (Lai et al., 2008). Another more general problem, of physical membrane disruption, is the issue of selectivity. If the adjuvant disrupts both bacterial and mammalian membranes this can cause toxicity to the host. It is generally the case that when a peptide shows activity towards bacterial outer membranes, but not towards mammalian cells, it also does not have activity on the bacterial inner membrane (Klobucar & Brown, 2022). Because most hydrophobic antibiotics can diffuse across the inner membrane this does however pose a limited problem in antibiotic combinational usage. One notable exception is the AMP SLAP-S25, which both disrupts the outer membrane by lipopolysaccharide binding and the inner membrane by binding phosphatidylglycerol (Luo & Song, 2021). Because these targets are specific to bacteria, selectivity is maintained. Showing that selectivity is possible while also disrupting the inner membrane. Besides peptides, peptidomimetics and steroidal analogs, there are various other compounds that have shown

outer membrane disrupting abilities but they are beyond the scope of this review (Klobucar & Brown, 2022).

Inhibitors of outer membrane synthesis are less numerous than the physical outer membrane disruptors, but there are some examples in the literature, like: PF-5081090 & MAC13243(García-Quintanilla et al., 2016; Muheim et al., 2017). An advantage of this approach is the increased selectivity, because of the absence of the enzymatic targets in mammals. PF-5081090 inhibits LpxC which limits lipid A synthesis. Though PF-5081090 has limited antibacterial activity of its own, it is effective as adjuvant in combination with rifampin, vancomycin, azithromycin, imipenem and amikacin against *A. baumannii* (García-Quintanilla et al., 2016). The other example, MAC13243, works by binding the periplasmic chaperone LolA. Because LolA plays a role in the transportation of multiple lipoproteins, its inhibition has various effects. At high concentrations of MAC13243 these effects are bactericidal. But because of the low stability in aqueous environments these concentrations are not likely to be achieved. MAC13243 however maintains sufficient activity at subinhibitory concentrations to allow other antibiotics such as novobiocin and erythromycin bypass the outer membrane barrier. However no synergy was observed with rifampicin and vancomycin (Muheim et al., 2017).These two examples illustrate that when inhibitors of outer membrane synthesis don’t have enough bactericidal activity of their own, they can still have great potential as an adjuvant.

Conclusion and Future of synergetic usage

The purpose of this review was to provide insight into how synergetic effects in drug combinational usage can be used to combat the challenge of antibiotic resistance.

Congruous combinations have shown clinical success with the combinations of: isoniazid with rifampin and trimethoprim with sulfamethoxazole, to combat resistance and

enhance antibacterial activity respectively. The now prevalent resistance against the later combination goes to show that multitarget therapeutics are not immune to resistance. The absence of other established congruous synergetic combinations in clinical practice shows that there is difficulty in translating *in vitro* testing results into clinical practice. Established combinations are important, because microbiology laboratory testing takes too much time, while quick drug administration is essential. There is also little known about translating *in vitro* results into *in vivo* application (Brennan-Krohn & Kirby, 2019). Drug combinations often undergo clinical trials without comparison to single drug treatments, which leaves the presence of *in vivo* synergy dubious (Brennan-Krohn & Kirby, 2019).

Syncretic antibacterial combinations look promising, because of the clinical success of β-lactamase blocking adjuvants antibiotic combinations, such as Augmentin. Especially broad spectrum β-lactamase inhibitors such as boronic acid based taniborbactam have great potential in rejuvenating β-lactam antibiotics. Efflux pump inhibitors seem to have more challenges in their development with no clinical application so far. With several challenges holding it back, like: a lack of broad spectrum inhibitors and host toxicity. But because of the role of efflux pumps in biofilm formation and multi drug resistance, there is a lot of potential for a successful inhibitor. Another more promising approach of syncretic synergy is adjuvant induced disruption of the outer membrane. This does not only potentiate antibiotics against intrinsic resistance in gram negative bacteria, but also can rejuvenate antibiotics that have encountered obtained resistance through reduced outer membrane permeability or efflux pumps. Especially the field of physical outer membrane disruption has seen the introduction of many candidates, like AMPs and Cationic Steroidal Antibiotics. The main challenges lay in the biostability and selectivity, but these hurdles can be overcome.

In conclusion, drug combinational usage has great potential in combating antibiotic resistance, with some clinically successful applications. Nevertheless, many challenges

remain especially in the clinical application of *in vitro* laboratory results. Here lays a task for policy makers and the pharmaceutical industry.

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