Dual-acting hybrid antibiotics: a promising strategy to combat bacterial resistance

Varvara Pokrovskaya & Timor Baasov

Importance of the field: The emerging and sustained resistance to currently available antibiotics and the poor pipeline of new antibacterials urgently call for the development of new strategies that can address the problem of growing antibacterial resistance. One such strategy is the development of dual-action hybrid antibiotics: two antibiotics that inhibit dissimilar targets in a bacterial cell covalently linked into one molecule. The possible benefits include: i) activity against drug-resistant bacteria, ii) expanded spectrum of activity and iii) reduced potential for generating bacterial resistance.

Areas covered in this review: In this article, we detail the recent activity in the design and development of dual-action hybrid drugs with a non-cleavable linker. We explore newly developed synergistic and antagonistic hybrid compounds with emphases on their potential to reduce resistance development.

What the reader will gain: Recently developed synergistic and antagonistic antibacterial drug–drug interactions and the impact of such interactions on the evolution of antibiotic drug resistance are described. Additionally, we discuss the implications of the latter observations on the development of hybrid antibiotics with the emphases on whether their synergistic or antagonistic effect will be more efficient at forestalling/reducing the development of new resistances.

Take home message: The approach of dual-acting hybrid antibiotics holds significant current promise in overcoming existing resistance mechanisms, as three of such compounds are entering clinical trials. However, the key challenge in this area should be a broader experimental demonstration of whether the “synergistic effect” or the “antagonistic effect” of the developed hybrid drug is better at preventing/reducing the evolution of resistance. This fundamental challenge must be overcome before yielding a successful drug.

Keywords: bacterial resistance delay, dual-action drugs, heterodimer antibiotics, hybrid antibiotics


1. Introduction

Antibiotic resistance (both endogenous and acquired) has been an important determining factor in the historical development of antibiotics as indispensable therapeutic agents for the treatment of infectious diseases [1]. Only a year after penicillin (a fungal product) was introduced for use by the general public, the first report of penicillin-resistant strains of Staphylococcus aureus appeared [2]. At that time the biochemical mechanism of resistance was not known. This alarming situation, unprecedented for those times, stimulated broad research in different scientific fields of antibacterial therapy. A major breakthrough came with the discovery of the synthetic derivative of penicillin, methicillin, less susceptible to hydrolysis by
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**Article highlights.**

- There is a crucial and urgent need to develop novel antibacterial agents and more advanced strategies that can address the concerns of growing bacterial resistance.
- Combination therapy/cocktail of drugs has been used with some clinical success to address the growing bacterial resistance problem.
- Kishony and co-workers showed that while the “synergistic” antibacterial drug combinations have developed multidrug resistance by sequential single-resistance steps, suppressive or “antagonistic” drug combinations have slowed the evolution of resistance.
- “Hybrid (heterodimer) antibiotics” strategy has several advantages versus simple cocktails including greatly reduced potential for generating bacterial resistance.
- Although several synthesized hybrids overcome the existing resistance mechanisms of MDR pathogens by addressing two different targets, none of these “synergistic” antibacterials have delayed evolution of bacterial resistance.
- Fluoroquinolone-aminoglycoside “antagonistic” hybrids provided the first demonstration of the ability of a hybrid structure to delay the emergence of resistance development in both Gram-positive and Gram-negative bacteria.

This box summarizes key points contained in the article.

β-lactamases, introduced in 1961 for the treatment of penicillin-resistant *S. aureus*. But again, only a year later, the methicillin-resistant *Staphylococcus aureus* (MRSA) strains appeared [3]. Streptomycin, discovered by Selman Waksman in 1944, was the first aminoglycoside antibiotic to be isolated from a bacterial source and was the first effective therapeutic for tuberculosis (TB), caused by *Mycobacterium tuberculosis*. Soon after streptomycin was introduced for the treatment of TB, it was found that bacterial resistance often developed due to spontaneous mutants of *M. tuberculosis* arising during the course of the therapy with an antibiotic [1]. Kanamycin, another member of aminoglycoside family of antibiotics, was isolated in Japan in 1957 and rapidly became the antibiotic of choice in that country. The first example on enzyme-mediated kanamycin resistance was reported in 1967 [4]. Because such resistance is transferable and largely spreads via R-plasmids, transposons and integrons, a high level of aminoglycoside resistance rapidly spread internationally. The natural aminoglycosides (tobramycin and gentamicins) and the semi-synthetic derivative of kanamycin, amikacin, all extremely effective antibiotics against kanamycin-resistant bacteria, were introduced in the early 1970s [1]. Consequently, novel enzyme-mediated resistance mechanisms that conferred high-level resistance to this newest generation of aminoglycosides began to appear on the scene very soon after their introduction in clinical use [5,6].

Although the above-mentioned examples illustrate how development of resistance to antibiotics has had a profound impact on the clinical utility and medicinal chemistry of antibacterials, they also demonstrate that once a new antibiotic is introduced into the clinic, whether it is a novel chemical entity acting at distinct bacterial targets or a semi-synthetic derivative that counters the resistance to its parent drug, it is only a short matter of time until new resistance will yet again emerge and create a public health problem [8]. Thus, even though this continuous battle between humans and bacteria has resulted in several millstone drugs, the situation today is more severe due to the emergence of serious multidrug-resistant (MDR) bacterial strains that are highly resistant to the majority of currently available antibiotics [9,10]. Gram-positive pathogens of particular concern include MRSA, VRE, vancomycin-resistant *S. aureus* (VRSA) and penicillin-resistant *Streptococcus pneumoniae*. Several Gram-negative pathogens, such as *Klebsiella pneumoniae*, *Antibacter baumannii* and *Pseudomonas aeruginosa*, that are resistant to extended-spectrum β-lactam antibiotics (aminoglycosides, fluoroquinolones and other classical antibacterials) are also on the rise. Nosocomial infections associated with these latter organisms are usually hard to treat and are often associated with considerable morbidity and mortality. Of equal concern is the spread of MDR strains of *M. tuberculosis* and the risk of dissemination of such resistant pathogens is a serious disease control threat [11]. Thus, there is a crucial and urgent need to develop novel antibacterial agents and more advance strategies that can combat the problems of growing bacterial resistance.

### 1.1 Combination therapy as a solution to resistance development

One approach that has been used to address the growing bacterial resistance problem with some clinical success is a combination therapy (Figure 1) [12]. Four principal modes of action by which two compounds in combination (a cocktail) can enhance the activity of each other are classified where a second compound (an adjuvant): i) prevents the degradation or modification of the primary drug (an antibiotic); ii) allows
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The clinical effectiveness of combination therapy to slow the emergence of new resistance has been proven with particular success in the treatment of TB-causing *M. tuberculosis*. Currently, a standard course treatment for TB is a combination of isoniazid, rifampicin, pyrazinamide and ethambutol or streptomycin for 2 months, then isoniazid and rifampicin for a few further months [14]. The drugs in this particular combination are acting in synergy by weakening the cell membrane and disrupting protein synthesis. Isoniazid is never used as a monotherapy since resistance emerges rapidly [11]. Thus, apart from the synergistic action of multiple drugs in combination for the treatment of TB, such cocktails also slow the emergence of resistance. However, whether the synergistic drug combinations are “always” associated with the delay in development of resistance or not, is not definitive. Of particular concern in this regard are recent reports suggesting that synergistic drug combinations can actually enhance the development of resistance rather than slowing it down [15,16]. To clarify this issue, we use different models of drug–drug interactions, outlined in Figure 2.

Generally, two drugs interacting in combination can be classified into three main types: additive (no interaction), synergistic (greater than the additive effect) and antagonistic (less than the additive effect) [17]. Since synergistic combinations generate increased efficiency at lower doses, clinicians have for a long time taken advantage of the clinical and biological benefits of synergy, whereas antagonistic combinations have been completely ignored [18]. Recently, mathematical models that predict and explain the emergence of resistance have been formulated to explain and promote combination therapy [16,19,20]. Establishing the basic concepts of drug interactions within a particular cocktail, Kishony and co-workers showed that some antagonistic combinations have a potential advantage to delay and even reverse development of bacterial resistance [16]. The laboratory experiments and theoretical modeling used in their study are selected for doxycycline resistance. Using a direct competition assay between doxycycline-resistant and doxycycline-sensitive *Escherichia coli*, it was shown that in the presence of either doxycycline alone or the synergistic pair doxycycline–erythromycin, the resistant strain outcompetes its wild-type strain. By contrast, in the environment of an antagonistic pair of doxycycline–ciprofloxacin, the sensitive strain wins under certain drug ratios, indicating selection against mutants resistant to one of the two drugs. The following explanation was provided: when the combination of two drugs (A + B) is less inhibitory to the bacterial growth than each drug alone (A and B separately, Figure 2C, antagonistic drug–drug interaction), then there is no benefit for the bacteria to develop resistance against either A or B alone. In such a situation the resistant strain will face a more hostile environment than in the case of A + B; the strain that acquires resistance to one drug (either against A or B) in the cocktail loses in competition with the sensitive strains because either drug alone is a stronger antibiotic than the combination. Consequently, a large delay in resistance development occurs. On the contrary, synergistic combinations (Figure 2A), while increasing antibacterial potency against both sensitive and resistant strains, also increases the relative selection for resistance, because resistance mutations to either drug (A or B) are more favorable for the bacterium [15]. However, one important consideration against the use of antagonistic drug combinations, also mentioned by the authors, is the reduced efficacy. This may lead to significantly longer treatments for infectious clearance and subsequently increase the chance for the resistance to develop. Nevertheless, this critical discrepancy, between the current thinking in antibiotic drug discovery toward synergy and the above-mentioned recent

![Figure 1. Two distinct strategies, combination therapy and hybrid antibiotics, to combat bacterial resistance.](image-url)
reports of Kishony and co-workers stressing the benefits of antagonism, should definitely be weighed out when combination therapy is being considered with the goal of minimizing the emergence of resistance. The limited success of combination therapy to date within the antibiotic field for only the treatment of TB further supports this statement.

1.2 Hybrid antibiotics as an emerging solution to resistance development

Unfortunately, combination therapy has several serious disadvantages; the effect of a particular combination therapy in vitro does not always lead to a clear response in vivo due to different pharmacokinetic properties of the drugs in combination [12,21]. Consequently, there is the necessity of fine tuning the formulation to ensure that the in vivo concentrations are the same as in the tablet. Furthermore, this strategy cannot address the problem of those MDR strains exhibiting the developed resistance for both drug families in combination, and thus necessitates employment of other families of drugs in combination. The small number of powerful antibacterial drug families largely limits the application of this approach for that purpose. An emerging strategy, with the potential to address some of the above limitations of combination therapy, is the "hybrid (heterodimer) antibiotics" strategy. According to this strategy, two pharmacophores that inhibit dissimilar targets in a bacterial cell are covalently linked into one molecule (Figure 1). Covalent connection between two drugs makes the pharmacokinetics of the resultant hybrid molecule more predictable. It is also possible to use the penetration capacity of one antibiotic moiety in a hybrid molecule to boost the bioactivity of the second antibiotic, and importantly, by a rationally designed linkage between the two drug motifs, better inhibit both targets, thus overcoming the existing resistance mechanisms against either or both drugs. Furthermore, a potential reduction in the toxicity of a drug due to hybridization with another drug might be observed. Finally, the rational design, syntheses, and systematic development of novel hybrid entities should be much more inspiring and beneficial for the medicinal chemist (the important player in drug design and development) both academically and practically than configuring "ideal" drug cocktails. All these possible advantages of hybrids versus simple cocktails, in conjunction to their greatly reduced potential for generating new resistance, surely make the development of dual-action hybrid drugs a highly promising strategy to combat hostile MDR bacterial strains.

A covalent linkage between two drugs in a hybrid can be a cleavable moiety, which can actually be considered a prodrug approach. Alternatively, the linkage can be a chemically and metabolically stable entity, making the hybrid structure act as a dual-action drug. Similar to cocktails, the partner drugs in a hybrid can exhibit either a synergistic or an antagonistic antibacterial effect. It is apparent to date, that the main inspiration in hybrid drug research has/is toward the development of novel chemical entities with synergistic activity (superior to the sum of the constituent agents) [22]. Additional important advantages to hybrid drugs that have been addressed in the literature include: activity against drug-resistant bacteria, an expanded spectrum of activity and increased duration after the onset of resistance, and reduced likelihood for generating bacterial resistance [13,23,24]. These new and challenging tasks have introduced fresh research avenues into the field of antibiotics research. In this article, we discuss recent examples of dual-action hybrid drugs with a non-cleavable linker. Additionally, we explore newly developed synergistic and antagonistic hybrid compounds with emphases on their potential to reduce resistance development.

2. Fluoroquinolone-containing hybrid compounds

Undoubtedly the most comprehensively represented hybrid compounds to date are those that contain the fluoroquinolone class of antibiotic linked to another antibacterial agent. To explain such a broad utilization of fluoroquinolones, several
reasons might be mentioned. In general, fluoroquinolones target two enzymes inside the bacterial cell, topoisomerase IV and DNA gyrase, thereby inhibiting DNA replication and transcription. Since these compounds target two dissimilar but essential enzymes in bacteria. Some disturbance in binding to one of the targets, for example, due to steric hindrance imposed by a linker or a second partner, might be compensated by targeting the other enzyme without a significant reduction in antibacterial potential. Another major benefit is the widely studied and well-defined structure-activity relationship of fluoroquinolones that emphasized the basic amine group in the C-7 piperidino moiety as a readily acceptable and tolerant point for attachment of various bulky substituents through carbamate formation, N-alkylation, quaternary salt formation and more. Fluoroquinolones are also stable under a wide variety of synthetic conditions making them ideal candidates for hybrid formation and development. It is noteworthy, however, that because of their very strong bactericidal activity (very low MIC values) and the spontaneous mutations of targeted enzymes as the major resistance mechanism, very fast rates of resistance development have been reported for this type of antibiotic [25,26].

One of the most successful series of fluoroquinolone-based dual-action hybrid compounds, which has been extensively described in both the scientific and patent literature, is the fluoroquinolone-oxazolidinone (eperezolid) hybrids [27-31]. The oxazolidinone class of antibiotics inhibit protein synthesis by binding at the P-site of the ribosomal 50S subunit [32]. Due to their limited ability to penetrate Gram-negative membrane, oxazolidinones exhibit relatively lower activity against Gram-negative bacteria. Therefore, the linking of a lipophilic fluoroquinolone (ciprofloxacin) to an oxazolidinone (eperezolid) in a single entity was expected to be highly beneficial. Since both eperezolid and ciprofloxacin possess a piperazinyl substituent at positions 4 and 7, respectively, they were merged at these two positions with a variety of heterocyclic linkers (including piperazinyl, pyrrolidinyl, azetidinyl, piperidinyl and others) to give a library of hybrid compounds (exemplified by compound 1, Figure 3). Several hybrids showed superior activity than both the parent drugs and their 1:1 combination as measured against both resistant and susceptible strains of S. aureus (MRSA and methicillin-sensitive S. aureus [MSSA]), S. pneumoniae, Enterococcus faecalis and Enterococcus faecium. Both enantiomers of the most active hybrid (containing a 3-hydroxyethyl pyrrolidinyl linker) exhibited improved antibacterial activity against bacterial strains resistant to either ciprofloxacin or oxazolidinone alone and showed a balanced dual-mode of action on both biological targets in vitro [29]. However, while the authors clearly mentioned the potential of the newly developed hybrids toward a lowered propensity for the development of bacterial resistance, no data have been shown to justify this hypothesis. In the more recent patent literature, another hybrid compound from this series containing a 4-hydroxy piperidine linker (MCB-3681) was highlighted as the most active fluoroquinolone-oxazolidinone hybrid and claimed activity against Bacillus anthracis bacteria (MIC value of 0.03 µg/ml), the causative of anthrax (Figure 3) [28]. In addition, MCB-3681 exhibited synergistic antibacterial activity against various bacterial strains, including linezolid- and fluoroquinolone-resistant Gram-positive (MRSA, VRE, methicillin-resistant S. pneumoniae, glycopeptide intermediate S. aureus) and Gram-negative (but not P. aeruginosa) bacteria. MCB-3681 has progressed into human clinical trials under the guise of a more water soluble phosphate ester produg (MCB-3837) that hydrolyzes in vivo to give the active agent [31].

The fluoroquinolone pharmacophore has also been utilized for the synthesis of fluoroquinolone-aminouracil hybrid compounds [33-35]. 6-Aminouracils are non-traditional antibiotics that selectively bind and inhibit the bacterial DNA polymerase IIIC that is absolutely essential for the DNA replication process in Gram-positive bacteria and mycoplasmas. In addition, the inhibitors of the bacterial DNA polymerase IIIC have demonstrated bactericidal activity. However, anilinouracils alone are not always effective as DNA polymerase inhibitors, probably due to lack of penetration, removal of compound by efflux pumps, or alteration of the sensitivity of the target enzyme [36]. The connection of the fluoroquinolone moiety to this promising class of antibacterial agents via its secondary amine group at piperazine ring, could improve their penetration, and broaden and improve its antibacterial spectrum by targeting two distinct steps in the DNA replication process. Various fluoroquinolone compounds were linked to the N-3 position of 6-aminouracils to afford a library of new hybrid compounds that demonstrated up to 64-fold improvement in inhibition of DNA polymerase and Gram-positive bacterial growth (exemplified by compound 2, Figure 3) [34]. But, the hybrids showed a lack of activity against Gram-negative bacteria (E. coli) and exhibited significantly reduced DNA gyrase and topoisomerase IV inhibitory activities in comparison to parent fluoroquinolone compounds. More recently, one of the best representatives of this series, compound 2 (251D) (Figure 3) was tested in a variety of in vitro assays, including DNA polymerase and topoisomerase/DNA gyrase enzyme assays, antibacterial, bactericidal, and mammalian cytoxicity assays [35]. The activity of compound 2 generally was significantly higher than that of the parent 6-aminouracil component in a wide variety of Gram-positive organisms tested, including both sensitive and resistant strains (e.g., MRSA, VRE and others). This hybrid structure was however significantly less potent against most sensitive Gram-positive organisms (fourfold less active against Bacillus subtilis and E. faecalis, and twofold less active against Bacillus cereus, Bacillus thuringiensis, S. aureus) in comparison to the fluoroquinolone component alone, yet, the hybrid maintained potent activity against those organisms that were resistant to either fluoroquinolone or to both fluoroquinolone and anilinouracil components (more than 20-fold improvement against MRSA and vancomycin-resistant E. faecalis (VREF)). Thus, in terms of the synergistic effect of this particular hybrid compound against Gram-positive bacteria, the synergy was only clearly
Dual-acting hybrid antibiotics: a promising strategy to combat bacterial resistance

Figure 3. Structures of fluoroquinolone-containing hybrid compounds.
demonstrated in those instances where the organism was already highly resistant to the fluoroquinolone component. Since anilinouracil compounds do not significantly inhibit the growth of Gram-negative bacteria, an attempt to enhance such activity by the preparation of anilinouracil–fluoroquinolone hybrids was not particularly successful; the hybrid 2 was only moderately active against most Gram-negative strains tested (e.g., *P. aeruginosa*, *K. pneumoniae*, *E. coli*), but displayed 32-fold greater activity in comparison to the fluoroquinolone partner against the ciprofloxacin-resistant *E. coli* strain. Again, the synergistic effect of the hybrid 2 has been demonstrated only under conditions where the organism is resistant (poorly active) to each partner alone. Furthermore, unlike hybrid 2, no synergistic effect is observed when an equimolar combination of each of the partner compounds alone were tested either against sensitive or resistant Gram-positive organisms. It has been suggested that the greater uptake of the hybrid 2 or its enhanced inhibitory activity against DNA polymerase IIIC due to the increased binding provided by the fluoroquinolone moiety, could explain the observed synergistic effect of 2 versus the lack of synergy in the cocktail. Finally, when the frequency of spontaneous (single step) resistance development was tested in *S. aureus*, the individual components developed resistance, but not the hybrid compound, within a single passage. In multi-passage experiments, however, the hybrid 2 developed resistance at a rate comparable to those of the partner components (16-fold increase in MIC values after 17 successive passages). Thus, even though the hybrid 2 displayed synergistic effects against resistant strains, the expected propensity to delay the resistance development is not truly demonstrated. Note that the latter resistance development experiments were performed on the *S. aureus* (Smith 13709) strain against which the hybrid 2 neither demonstrated a clear synergistic nor an antagonistic effect (the MIC values of 0.313, 5 and 0.078 µg/ml for the hybrid 2, anilinouracil, and fluoroquinolone components, respectively). Importantly, the hybrid 2 demonstrated a lack of *in vitro* toxicity and good inhibitory activity of the targeted enzymes. In a separate study the authors also demonstrated the *in vivo* efficacy of this class of hybrids when given intravenously in a murine staphylococcal infection model, confirming its potential as novel anti-infective agent [34]. A recent patent application from Microbiotics (MA, USA) highlights several produgs of this type of hybrid where the carboxylic acid group at position 3 of the fluoroquinolone moiety has been esterified [37]. The compounds were tested *in vivo* against lethal infections. The lead morpholino-ester produrg showed a half effective dose (ED$_{50}$) of 18 mg/kg (intravenous dose) for protection of mice from the intraperitoneal injection of *S. aureus* (Smith strain) infection.

Another type of novel hybrid design involves an analog of the fluoroquinolone class linked to macroyclic core of rifamycin [38,39]. The biological activity of rifamycins relies on the inhibition of DNA-dependent RNA polymerase leading to a suppression of RNA synthesis and cell death [40]. Rifamycins are utilized globally for the treatment of TB in combinations with other agents and against a variety of Gram-positive bacteria. However, the liability of high resistance development observed in bacteria treated with rifamycins, limits their approved use to combination regimes [41]. Antibiotic therapy with a combination of rifampicin and ciprofloxacin has been shown to be a reasonable treatment option for biofilm-associated staphylococcal infection [42]. In order to improve the antibacterial spectrum and bacterial resistance development properties of rifamycin, workers at Cumbre Pharmaceuticals prepared a series of hybrids in which rifamycin SV was covalently connected to a quinolone pharmacophore derived from the 4*H*-4-oxo-quinolizine subfamily of fluoroquinolones. 4*H*-4-oxo-quinolizines were recently designed by replacing nitrogen with a carbon between ring carbons C-4 and C-5 to overcome bacterial resistance to fluoroquinolones. The lead compound 3 (CBR-2092) contains a fluorinated 4*H*-4-oxo-quinolizine at the 3-position of the rifamycin core, linked via a hydrazone linkage (Figure 3). The detailed biochemical evaluation of compound 3 shows its excellent antibacterial activity against Gram-positive pathogens, including rifamycin- and ciprofloxacin-resistant strains, and that it is equipotent to the parent compounds’ inhibitory activities against targeted enzymes [39]. However, antibacterial activity against Gram-negative bacteria (*E. coli* ATCC 25922) was only comparable to that of rifampin (rifamycin), and was significantly lower than that of ciprofloxacin [43]. In its favor, compound 3 displayed superior efficacy in multiple *in vitro* models of staphylococcal biofilm states and in an *in vivo* standard rodent infection and rabbit endocarditis models, caused by MRSA. Although the frequency of spontaneous mutation in the presence of 3 is at a low level, the comparative multistep passage resistance selections between 3 and the parent antibacterials have resulted in the isolation of *S. aureus* strains with enormous resistance levels after the course of 2 – 15 passages in all compounds (MIC value of 3 increased up to 500-fold after 15 passages). In contrast to fluoroquinolone compounds, the obtained resistant strains showed that mutational activation of efflux pumps was not a contributory factor in the development of resistance to the hybrid 3. In light of the increasing prevalence of efflux-mediated resistance traits in Gram-positive cocci, the authors pointed to the observed data as a promising advantage of hybrid agents over cocktail combinations.

Recent work by Sriram et al. reveals a series of hybrids containing various fluoroquinolones linked to tetracycline derivatives as promising anti-HIV and antimycobacterial agents, and as inhibitors of HIV-1 integrase [44]. Tetracyclines inhibit the protein synthesis in bacterial cells by binding to the 30S ribosomal subunit and preventing the docking of aminoacylated tRNA. In addition, tetracyclines have demonstrated promising anti-HIV inhibitory activity, especially those containing bulky substituents at C-2 position of carboxamine moiety [45]. The design of tetracycline–fluoroquinolone hybrids with a robust, non-cleavable linker at this position was envisioned as a potential strategy to ensure that the pharmacokinetics, pharmacodynamics, and tissue distribution of the composite...
pharmacophores are matched. As such, bulky aryl fluoroquinolones were linked to several tetracycline derivatives to give a library of 12 hybrid compounds. Two pharmacophores were connected by reacting appropriate tetracyclines, formaldehyde and secondary amino (piperazino) group of fluoroquinolones using microwave irradiation (exemplified by compound 4, Figure 3). One of the lead hybrids, compound 4, was also found to be active against the HIV-1 replication process and non-toxic to the CEM cells. Importantly, the MIC value of hybrid 4 was more than 30-fold lower than the parent compounds against tetracycline-resistant Mycobacterium tuberculosis. This increased antimycobacterial potency of the hybrid compound has been explained as a possible synergistic dual-mode of action by inhibiting both biological targets of the parent compounds.

One of the most recent patents described a new series of hybrid compounds in which either 4-quinolones or 4H-4-oxo-quinolizines have connected with benzyl pyrimidines as potential antimicrobial agents [46]. Benzyl pyrimidines target the production of tetrahydrofolic acid by inhibiting the dihydrofolate reductase enzyme that reduces dihydrofolate to tetrahydrofolate in bacterial, parasitic and epithelial cells. One of the representatives of this class, trimethoprime is a widely used antibiotic with a broad spectrum of action. A combination therapy comprising the administration of a trimethoprime and ciprofloxacin cocktail has not been successful due to the different pharmacokinetic properties of the two antibacterial agents [47]. To address this problem a series of hybrid compounds, in which benzyl pyrimidines connected at the C-7 position of 4-quinolones or 4H-4-oxo-quinolizines with different linkers, were designed and synthesized (exemplified by compound 5, Figure 3). This approach of targeting two individual steps in the same target (DNA synthesis) offered several possible benefits, such as a synergistic effect in terms of efficacy, lowered resistance selection propensity, activity against resistant bacteria, and reduced susceptibility to efflux pumps and toxicity in comparison to a cocktail of the two drugs. Indeed, the lead compound 5 (BP-4Q-002) was 30-times more potent than ciprofloxacin alone or its 1:1 cocktail with trimethoprim, and four-times more potent than trimethoprim against Staphylococcus aureus NRS 19 (resistant to ciprofloxacin). The antibacterial activity of 5 against susceptible Escherichia coli and Bacillus subtilis bacteria was similar to trimethoprim, but significantly lower than ciprofloxacin. Additional analogs showed antibacterial activity lower than ciprofloxacin and similar to or better than trimethoprim. Unfortunately, no bacterial resistance development tests have been reported for these compounds.

3. Aminoglycoside-containing hybrid compounds

Aminoglycoside antibiotics are long-known antibacterial agents, which are active against a wide variety of Gram-positive and Gram-negative bacterial strains. The main molecular target of aminoglycosides is the decoding site (A-site) of 16S rRNA in the 30S bacterial ribosome subunit. Binding of aminoglycosides at this site decreases the fidelity of protein synthesis during the translation process that leads to bacterial cell death. However, the appearance of bacterial strains resistant to these drugs and their relative toxicity are critical problems that largely limit their intensive clinical use [48-50]. Generally, aminoglycosides suffer from resistance enzymes that modify their hydroxyl or amino groups rendering the resulting products inactive. One of the recent efforts to solve these problems attaches another pharmacophore to the aminoglycoside molecule resulting in heterodimer/hybrid compounds.

The first example of an aminoglycoside-based hybrid structure was published by Mobashery and co-workers in 2001 [51]. The β-lactam moiety of cephalosporin was exploited to develop a dual-action compound by linking an aminoglycoside at the C-3 position of the β-lactam partner (exemplified by isepamicin–deacetylcephalothin hybrid 6, Figure 4). Several previous studies have indicated that a combination of aminoglycoside and β-lactam antibiotics is frequently synergistic [52,53]. Accordingly, the design principle involved the “expulsion” of aminoglycoside on the C-3 position of cephalosporin antibiotic in the presence of β-lactamases, or synergistic action on two biological targets as a hybrid antibiotic in the absence of β-lactamases. Significant reduction in toxicity and better uptake of the highly polar aminoglycoside were expected after its fusion with the cephalosporin moiety. Although the synthesis of such hybrid compounds was pointed out to be very challenging due to the complicated chemistries of both β-lactams and aminoglycosides, several hybrid compounds, such as 6 were synthesized (Figure 4). Preliminary biological evaluation of compound 6 indicated good substrate activity with β-lactamase and reduced toxicity to the mammalian host in comparison to the aminoglycoside component, isepamicin [51]. However, no antibacterial activity of the resultant compounds has been reported.

More recently, Yu and co-workers reported on the development of a series of hybrids containing an aminoglycoside (neomycin B) linked to chloramphenicol or oxazolidinone (linezolid) partners with different linkers [54-57] (Figure 4). Chloramphenicol and oxazolidinone antibiotics bind to the 23S rRNA of the 50S ribosomal subunit; chloramphenicol inhibits the peptidyl transferase activity and oxazolidinone inhibits the initiation step of protein synthesis. Similar to aminoglycosides, these antibacterial agents have broad spectrum of activity against Gram-positive and Gram-negative bacterial strains. In order to improve RNA binding and specificity of aminoglycosides and to reduce their toxicity, the loop-binding compounds chloramphenicol and linezolid were linked to the stem-binding neomycin B, providing heterodimers with a potential to recognize both RNA stem and loop motifs present on the ribosome of the pathogenic organisms. Some of the designed hybrids displayed enhanced affinities to specific RNAs with dissociation constants significantly lower than that of neomycin B. In addition, the results of footprinting and mutation studies suggested that the affinity of hybrids is RNA sequence-specific.
The neomycin-linezolid heterodimer 7 showed a more than 20 times improvement in affinity to models of bacterial 16S and human 18S A-sites in comparison to neomycin B, whereas the neomycin–chloramphenicol 8 showed the highest discrimination factor between bacterial and human A-sites. Unfortunately, antimicrobial activities of 7 and 8 against a panel of 20 standard pathogenic strains, as well as their IC₅₀ values in translation inhibition assays in vitro, have not correlated well with their dissociation constants and were lower than those of neomycin B in all cases.

The recent development of aminoglycoside–fluoroquinolone hybrids by Pokrovskaya et al. highlights the potential of antagonistic heterodimer compounds to delay the emergence of bacterial resistance [58]. By linking an aminoglycoside (neomycin B) and a fluoroquinolone (ciprofloxacin) via different 1,2,3-triazole containing spacers a series of 17 hybrid compounds with a common structure 9 were prepared (Figure 4). Although none of the synthesized compounds showed higher activity than ciprofloxacin, the majority of hybrids was significantly more potent than neomycin B against a panel of...
of Gram-positive (e.g., MRSA resistant to neomycin B) and
Gram-negative bacteria (neomycin-susceptible and neomy-
cin-resistant *E. coli* strains). Furthermore, selected hybrid
compounds were also able to overcome most prevalent type
of aminoglycoside resistance associated with APH(3′)-Ia,
APH(3′)-IIIa, and AAC(6′)/APH(2″) resistance enzymes.
Selected hybrids inhibited bacterial protein synthesis with
potencies similar to or better than that of neomycin B, and
were up to 32-fold more potent as inhibitors than ciprofloxa-
cin for the fluoroquinolone targets, DNA gyrase and topo-

doanase IV, indicating a balanced dual-mode of action.
Perhaps the most important discovery of this study was the
case of multi-passage experiments of one of the hybrids
showing a significant delay of resistance development in

![Figure 4. Structures of aminoglycoside-containing hybrid compounds (continued).](image-url)
both Gram-negative (E. coli ATCC 35218) and Gram-positive (B. subtilis ATCC 6633) bacteria in comparison to that of each parent antibiotic separately or their 1:1 mixture; although the MIC value of the tested hybrid remained unchanged (3 µg/ml) in both bacteria after the 1st and 15th passages, the MIC values for ciprofloxacin, neomycin B and their 1:1 combination increased significantly corresponding to 75-, 4- and 20-fold resistance development in E. coli, respectively, and 37.5-, 8- and 7.6-fold resistance development in B. subtilis, respectively. To our knowledge, this study provided the first demonstration of the ability of a hybrid structure to delay the emergence of resistance development in both Gram-positive and Gram-negative bacteria. It is of particular note that the MIC values, as measured against E. coli for the hybrid, ciprofloxacin and neomycin B, were 3, 0.01, and 12 µg/ml, respectively, and against B. subtilis were 3, 0.02 and 0.75 µg/ml, respectively, demonstrating antagonistic activity of the tested hybrid structure in comparison with each partner drug separately. Therefore, the observed data in this study of the hybrid antibiotic correspond well to Kishony and co-workers results [15,16] with antibacterial drug combinations, and support the idea that the antagonistic effect of drug combinations, whether as a simple cocktail or as a hybrid structure, can lead to the delay of resistance development.

Very recently, novel aminoglycoside–peptide conjugates have been reported with a 1,2,3-triazole-containing linker (Figure 4) [59]. Short amphiphilic peptides were linked to an aminoglycoside (neomycin B and kanamycin A) partner via click chemistry to afford a variety of hybrid structures. The hypothesis was that conversion of aminoglycoside antibiotics into cationic amphiphiles would enhance antibacterial activity against resistant strains and slowdown the development of resistance rate. In addition, the aminoglycoside amino groups of two hybrid compounds were also converted to guanidines in order to explore the effects of basicity. Evaluation of the antibacterial activity in this series of hybrids showed a strong influence dependant on the nature of the peptidic component. Although the majority of compounds showed enhanced antibacterial activity against neomycin B-, kanamycin A-resistant MRSA, kanamycin A-resistant methicillin-resistant Staphylococcus epidermitis (MRSE) and gentamicin-resistant P. aeruginosa, the most potent compounds were conjugates of dipetides with a tryptophan unit where the aminoglycoside part contained a guanidine instead of amine (compounds 10 and 11, Figure 4). However, reduced antibacterial activity against neomycin B- and kanamycin A-susceptible strains has been demonstrated. In vitro toxicity measurements show little hemolytic activity against mammalian erythrocytes at low MIC concentrations, but significant hemolytic activity at higher concentrations. The observed concentration-dependent hemolytic activities of the aminoglycoside–peptide hybrids and previous studies with other cationic amphiphiles have confirmed a membranolytic mode of action of the resultant compounds. Unfortunately, no resistance development tests have been reported.

4. Other hybrid compounds

In this section we combined a series of hybrid structures reported recently that do not contain fluoroquinolone and aminoglycoside as partner pharmacophores. One family of such novel hybrid molecules was created by merging similar aromatic motifs of oxazolidinone and chalcone, which may represent the simplest/smallest hybrid structures reported to date (exemplified by compound 12, Figure 5) [60]. Chalcones are products of condensation of simple or substituted aromatic aldehydes with acetophenones in the presence of alkali, possessing a wide range of biological activities including antimicrobial potency. It was found that α, β-unsaturated ketofunctional group is the structural feature responsible for antibacterial activity of chalcones. Numerous regioisomeric chalcone–oxazolidinone hybrids were synthesized with the goal to obtain compounds with synergistic antibacterial activity. To achieve this goal, a series of different substituents that span from strong electron donating to strong electron withdrawing natures, were introduced onto the aromatic ring of the chalcone part. However, synthesized hybrids exhibited only trace or no activity against a panel of Gram-positive organisms tested (MSSA, MRSA, VREF, E. faecalis). By introducing a fluorine atom onto the aromatic ring and converting the acetamido group to the corresponding thiocarbamate, several more active compounds were designed and synthesized. One of them, the hybrid 12, showed activities similar to linezolid and vancomycin against susceptible S. aureus and MRSA bacterial strains, and exhibited a more than 30-fold improvement against VREF and E. faecium in comparison to vancomycin. Since no antibacterial activity of the chalone component has been reported, it is difficult to draw a conclusion about the synergistic properties of the resultant hybrid 12.

In another study, Breukink and co-workers described hybrid compounds containing vancomycin and nisin fragments connected via different spacers [61]. Even though these two antibiotics have different modes of action, they both target the essential cell wall precursor lipid II. Vancomycin is a glycopeptide that inhibits cell wall biosynthesis in Gram-positive bacteria by forming a tight complex with D-Ala-D-Ala-containing peptidoglycan precursors that presents in lipid intermediate II. Nisin (1 – 12) demonstrates high-affinity binding to lipid II leading to pore formation and thus disrupting the cell wall synthesis pathway. Subsequently, connection of these two into one molecule was claimed to increase affinity to lipid II through a bivalency or chelate effect that could restore vancomycin activity against resistant strains, such as VRE. The lengths of the spacers and the attachment site(s) were predicted by computer modeling. By using click-chemistry reactions, three hybrid compounds were synthesized and evaluated for their antibacterial potency against susceptible and vancomycin-resistant strains of enterococci and K. pneumoniae, and against Moraxella catarrhalis resistant to both vancomycin and nisin (exemplified by
Figure 5. Structures of various hybrid compounds.
compound 13, Figure 5). The lead compound 13 exhibited higher antibacterial activity than parental agents against all resistant strains, but was slightly less active than vancomycin against susceptible enterococci. Thus, the synergistic activity of the hybrid was only demonstrated against the resistant strains, being ~40 times more active than its components against VRE. It was suggested that, since in the vancomycin-resistant strains the vancomycin part of the hybrid should lose most of its affinity for the available D-Ala-D-Lac binding sites, the nisin part that retains affinity for lipid II in these resistant strains allows the hybrid to disrupt the cell wall biosynthesis pathway.

A similar strategy was applied to the design of vancomycin and β-lactam hybrid compounds [62,63]. These hybrid structures were developed to inhibit Gram-positive bacterial cell wall biosynthesis by simultaneously targeting both
cellular targets of vancomycin (lipid intermediate II) and \( \beta \)-lactams (transpeptidase domain of penicillin binding protein). Due to the physical proximity of two targets and their sequential role in the cell wall biosynthetic pathway, enhanced potency and bactericidal activity were expected. A series of vancomycin–cephalosporin heterodimers were synthesized by connecting two antibacterial agents at several attachment positions with a range of linking moieties. The cephalosporin core was derivatized at three positions: at the C3 pyridinium substituent by a methyl amino moiety (exemplified by compound 14, Figure 5), at the oxime (exemplified by compound 15, Figure 5) and at aminothiazole (exemplified by compound 16, Figure 5). In addition, three different attachment points of vancomycin were explored via the amide bond formation: the vancosamine amino group of the terminal sugar (compound 16), the carboxyl terminus (compound 15), and the 4' resorcinol-like position on the aromatic side chain of amino acid 7 (compound 14). The combination of vancomycin and cephalosporin attachment points resulted in the preparation of nine amide-linked hybrids. The compounds were screened against a panel of susceptible and resistant Gram-positive human pathogens. Interestingly,
all the tested hybrids exhibited synergistic antibacterial activity (> 15 times higher activity than the parent antibiotics) against tested bacterial strains, including MRSA and VREF. In view of the fact that the specific attachment position of the two drugs’ moieties appeared to have little effect on the potency profile, it was concluded that the two active components of the hybrids do not bind simultaneously at both cellular targets but synergistically inhibit cell wall synthesis. In addition, compounds 14 and 15 exhibited superior in vivo activity in a mouse model of MRSA infection. Compound 15 was selected as a clinical candidate (TD-1792) that successfully completed the Phase II clinical trial for the treatment of complicated Gram-positive skin and skin structure infections. Additional patents from Theravance, Inc. (CA, USA) describe a series of other hybrids of different β-lactams and glycopeptides [64,65]. The compounds of this invention, consist of 2 – 10 ligands of the β-lactam and glycopeptide classes of antibacterial agents covalently connected by a variety of linkers, were claimed to interfere with the synthesis/metabolism of the cell wall. The lead compound from this series showed significantly higher antibacterial activity than vancomycin against MRSA and MRSE (MIC value of < 0.1 μg/ml). In vivo studies with neutropenic mice showed a half effective dose (ED₅₀) of < 0.20 mg/kg (intravenous dose) for the protection of mice from S. aureus (MRSA 33591) infection, whereas the ED₅₀ of vancomycin was 9 mg/kg.

Cumbre Pharmaceuticals (TX, USA) has recently published a patent that describes rifampicin–metronidazole hybrid compounds with potent synergistic activity against Gram-positive and Gram-negative pathogens (exemplified by compound 17, Figure 5) [66]. Metronidazole is a nitroimidazole antibiotic used particularly for anaerobic bacteria and protozoa (Trichomonas infections). After permeation by diffusion, metronidazole undergoes reduction in vivo and the resultant intermediate exhibiting cytotoxicity interacts with the host cell DNA, resulting in DNA strand breakage and fatal destabilization of the DNA helix [67]. In order to achieve synergy, minimize the resistance development to these antibiotics and make the pharmacokinetic profile more predictable, rifampicin was covalently connected to an antibacterial pharmacophore from the nitroimidazole, nitrothiazole, or nitrofurantoin class of compounds. The hybrids demonstrated significant enhancement of activity in comparison to their simple (unlinked) combinations. The lead compound of this series, hybrid 17, exhibited antibacterial activity higher than the parental agents against Gram-positive bacteria including susceptible Clostridium difficile, metronidazole-resistant S. aureus, metronidazole-resistant M. tuberculosis, and M. tuberculosis resistant to both parental agents. In addition, 17 showed synergistic activity (higher than both metronidazole and rifampin alone) against Gram-negative organisms, including susceptible and resistant to rifampin Helicobacter pylori and susceptible Bacteroides fragilis. Although no resistance development tests have been performed, the data of the study clearly indicated desired synergistic character of the hybrid 17 with both susceptible and resistant bacterial strains.

A large number of aminoquinoline-based hybrids were recently described in a patent application by Palumed SA (France) and CNRS (France) [68]. 4-Aminoquinoline and 8-aminoquinoline compounds were covalently combined with penicillin (represented by compound 18), cephalosporin (represented by compound 19), fluoroquinolone (represented by compound 20), streptogramine (represented by compound 21), macrolide (represented by compound 22), glycopeptide (vancomycin) (represented by compound 23) and oxazolidinone (represented by compound 24) (Figure 6). Aminoquinolines are primarily known as antimalarial agents [69], but in addition they are known as inhibitors of efflux pumps in MDR Gram-negative bacterial clinical isolates [70]. It has been shown that aminoquinolines, by inhibiting the AcrAB-ToLC efflux pump, restore the susceptibility of MDR clinical strains to structurally unrelated antibiotics such as norfloxacin, tetracycline and chloramphenicol [70,71]. Therefore, the covalent connection between aminoquinoline and different antibacterial agents could overcome an inhibitory effect on the efflux pumps of resistant bacteria and enhanced activity of a hybrid could be observed. The resultant hybrids were tested against a panel of susceptible and resistant bacterial strains. The hybrid 18, designed as a prodrug, contains a penicillin moiety with a protected carboxylic acid and was expected to convert to the free carboxylate in vivo. However, it exhibited twofold lower antibacterial activity than that of penicillin G against the MSSA bacterial strain in vitro; no MIC data were reported for the free carboxylate derivative. More extensive data were reported for the aminoquinoline-cephalosporin hybrid compounds. The lead compound 19 exhibited similar or slightly lower activity than cephalosporin ceftriaxone against a panel of susceptible strains, including S. aureus, S. pyogenes, S. pneumoniae bacteria, and showed high synergistic activity against S. aureus and S. pneumoniae resistant to both parental agents and their 1:1 cocktail. In addition, 19 demonstrated low oral toxicity and low binding to human serum, and exhibited enhanced in vivo activity in a murine model of septicemia due to MSSA. Aminoquinoline-fluoroquinolone heterodimer 20 demonstrated improved antibacterial activity against sensitive S. aureus, E. faecalis, B. subtilis, B. thuringiensis Gram-positive bacteria, and ciprofloxacin-resistant S. aureus. However, lower potency than ciprofloxacin was reported against all tested Gram-negative bacteria, such as sensitive E. coli, H. influenza, and P. aeruginosa. The lead compounds of aminoquinoline hybrids with streptogramin (compound 21), macrolide (compound 22), vancomycin (compound 23) and oxazolidinone (compound 24) exhibited higher antibacterial activity than parental agents against a panel of susceptible and resistant Gram-positive bacterial strains, including MSSA, and S. pneumoniae. To conclude, most of the prepared hybrids showed high synergistic antibacterial activity against susceptible and resistant Gram-positive bacteria. However,
Dual-acting hybrid antibiotics: a promising strategy to combat bacterial resistance

Figure 6. Structures of aminoquinoline-containing hybrid compounds.

their activity was lower when tested against Gram-negative bacteria, probably due to the fact that aminoquinolines do not exhibit antibacterial activity by themselves but exhibited inhibitory properties with efflux pumps in Gram-negative *E. aerogenes* and *K. pneumoniae* bacteria only.

5. Expert opinion

This abbreviated overview illustrates that the combined efforts over the past several years between academy and industry have significantly advanced our understanding of how chemical
Redesign of the existing antibacterial drugs can evade the resistance mechanisms that have evolved in pathogenic bacteria to these drugs. This knowledgebase has prompted the development of hybrid antibiotics as an emerging option in antibiotic therapy with the goal to control the growing army of resistant pathogens. The primary goal of the hybrid antibiotics approach to overcoming the existing resistance mechanisms of MDR pathogens, by addressing two different targets either in parallel or subsequently, has been accomplished in a number of examples; the three hybrid molecules currently investigated in human clinical trials, TD-1792, MCB-3837 and CBR-2092, have been reported with impressive activity against MDR strains exhibiting resistance to both of the partner drugs alone, and with superior efficacy relative to a simple combination of partner drugs. Therefore, we would like to encourage the continuation of hybrid antibiotics development, even though the optimization course toward clinically successful hybrids might be more complex.

The other scene of hybrid antibiotics development, however, that relies on their anticipated ability to avoid/reduce the emergence of new resistance development still awaits further elucidation and validation. Although the strategy of hybrid antibiotics development is principally based on the “multivalent therapy” approach [23,72], a strategy with a hypothesized advantage in slowing down the emergence of spontaneous target-related resistance development relative to “monovalent therapy”, the reality is that this issue in the antibiotics field has recently faced some controversial observations [15,16,19], requiring further careful revision, especially when drug–drug combinations are considered. Thus, although the primary goal of the hybrid antibiotics approach, to justify their development for clinical use, has been a demonstration of synergy superior to that provided by the simple combination of partner drugs, whether such synergistic hybrids can avoid/reduce the emergence of new resistance development remains to be demonstrated conclusively. Indeed, surprisingly, none of the synergistic hybrids reported either in the scientific or patent literature have been tested for their ability to delay, or if they even enhance, resistance development in those resistant strains on which their superior antibacterial efficacy have been demonstrated. Only in a few examples, were the hybrids that mainly demonstrated the synergy in resistant bacteria tested for resistance development in wild-type bacteria by using multipassage experiments. Although the results obtained in these experiments were not particularly encouraging, no clear explanation has been provided as to whether the contested behavior of the hybrid drugs – the reduced propensity for resistance development – can in principle be achieved by the “synergistic hybrids” or if that challenge ought to be directed in another direction, e.g., “antagonistic hybrids”.

The most recently developed system biology approaches, equipped with theoretical models and subsequent in vitro experimental data, have provided very clear and justified
arguments on this issue: while the “synergistic” antibacterial drugs combinations have developed multidrug resistance by sequential single-resistance steps, suppressive or “antagonistic” drug combinations have selected against resistant bacteria and slowed the evolution of resistance [15, 16, 19]. Even though this fundamental concept of drug–drug interaction has only been demonstrated in vitro and it is still unclear if it will also be operational in vivo, this fresh and extremely intriguing observation in the antibiotic arena, together with a recent report in which an aminoglycoside–fluoroquinolone “antagonistic hybrid” (compound 9, Figure 4) that exhibited considerable antibacterial efficacy also demonstrated significant reduction in resistance development in comparison to partner drugs and their cocktail, clearly support the above notion that the main challenge with regard to hybrid antibiotics development should be carefully revised.

After all, and in with applause to the medicinal chemists, while the main challenge toward the hybrid drug development has been designated the development of “synergistic hybrid drugs” due to reduced potential for generating bacterial resistance, the main motivation, ambition, and encouragement have been directed toward the achievement of “synergy” rather than the essentiality of demonstrating the delay of resistance development. Therefore, the key challenge should definitely be a clear and broader experimental demonstration whether the “synergistic effect” or “antagonistic effect” of the developed hybrid drug is better at preventing/reducing the evolution of resistance. Although many additional issues can be counted as limiting factors for successful hybrid drug development for use in a clinical setting [23, 24, 73], it is clear that this fundamental issue of drug–drug interaction must be solved before these drugs will find their way into the clinic. The hybrid antibiotics should provide excellent model systems for answering this question at both in vitro and in vivo levels.

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Affiliation
Varvara Pokrovskaya & Timor Baasov†
†Author for correspondence
Technion – Israel Institute of Technology, The Edith and Joseph Fischer Enzyme Inhibitors Laboratory, Schulich Faculty of Chemistry, Haifa 32000, Israel
Tel: +972 4 829 2590; Fax: +972 4 829 5703; E-mail: chtimor@tx.technion.ac.il