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REVIEW ARTICLE

Hybrid Compounds as Anti-infective Agents

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Abstract: Hybrid drugs are multi-target chimeric chemicals combining two or more drugs or pharmacophores covalently linked in a single molecule. In the field of anti-infective agents, they have been proposed as a possible solution to drug resistance issues, presumably having a broader spectrum of activity and less probability of eliciting high level resistance linked to single gene product. Although less frequently explored, they could also be useful in the treatment of frequently occurring co-infections. Here, we overview recent advances in the field of hybrid antimicrobials. Furthermore, we discuss some cutting-edge approaches to face the development of designed multi-target agents in the era of omics and big data, namely analysis of gene signatures and multitask QSAR models.

Alan Talevi

Keywords: Hybrid drug, hybrid molecule, chimeric molecule, anti-infective, antimicrobial, antibiotic, antifungal, antiviral, antimicrobial resistance, multi-target drug, multi-target agents.

1. INTRODUCTION

The relative lack of innovation constitutes a general reality in the drug discovery field, where increasing spending on research and development has not translated into a higher number of new approved drugs per year [1, 2]. However, the issue of stagnation gets deeper in the field of anti-infective development, where not only the number of approved drugs has been declining through the years but also the number of novel active scaffolds introduced into the clinics [3]. At first sight, this could be surprising having in mind that infectious diseases are still among the most frequent causes of death worldwide [4]. However, if causes of death are segregated by country income group it becomes clear that infectious diseases have at present much more impact on low- and lower-middle income countries, which are less lucrative markets for pharmaceutical companies. And there are some other factors of strictly economic nature that make anti-infective agents less attractive targets for development than other drug classes [5, 6], at least in the frame of the traditional drug discovery model. First, in contrast with other drug classes, anti-infective agents are frequently administered during a short time period, until the infection resolves. Second, in some cases (i.e. broad spectrum antibiotics) the use of newly approved anti-infective drugs is restricted to the treatment of serious infections, in order to prevent resistance development. Finally, many infections (prominently, those caused by protozoa and helminths) are associated to poor and often marginalized communities. Even if we leave aside a purely humanitarian perspective, the previous market economy viewpoint might well prove too narrow when taking into consideration the highly dynamic epidemiology of communicable disorders: in a globalized world characterized by constant human migrations, the endemic label of some infectious diseases could reveal itself obsolete. As an example we can mention Chagas disease, which not much time ago was endemic to Latin American; presently, however, the estimated patients with Chagas living only in USA rise to 300,000 [7]. The previous analysis underlines some market flaws in relation to drug research and the need of public policies addressing and correcting such flaws; in particular, despite poor revenue, anti-infective agents are extremely valuable to society and infectious diseases should be thought as a universal menace. Fortunately, this perspective seems to be gaining weight in the international community and the neglected condition of some infectious diseases appears to be reversing during the last decades.

Still, can we identify some non-economic explanation to the sharp decline in anti-infective drugs productivity? Some authors and specialized organizations mention regulatory problems, such as inadequacy of trials in proving non inferiority and the lack of clear antibiotic approval pathways [8, 9]. Interestingly, the prevalence of target-driven approximations that have dominated the drug discovery arena during the previous decades (which can be summarized under the

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reductionist "one gene, one target, one drug" premise) in detriment of other presumably "irrational" approaches, has been suggested as one possible explanation to the lack of innovation in the field of anti-infective medications [8]; what is more, the use of single and selective agents and single type of interventions against infectious diseases may have favored the appearance and spread of drug-resistant strains [10].

Single-target agents have, in theory, many practical advantages that should pave the way for reduced drug development timelines [10 and refs therein]: they allow the definition of structure-based virtual screening and drug design campaigns; they can be identified through simple biochemical assays which are easily integrated into high-throughput screening platforms; they are presumably safer since they are conceived to avoid off-target interactions and; their mechanism of action should be more easily established (because there is, from the very beginning of the drug discovery process, a starting hypothesis for the mechanism of action and the molecular target).

However, there also exist some limitations to target-driven approximations that have resulted in a renewed interest in phenotypic screening within the drug discovery community, with more emphasis in certain therapeutic categories (among them, anti-infective agents). First, it should be highlighted that, even if a drug candidate has been identified through a target-centered scheme, there is no guarantee that it will hit solely the intended target [8]. There are, of course, a number of precautions that one can take to favor the selection of highly selective agents. For instance, a correlation between a number of molecular features (molecular weight, lipophilicity, complexity, topology) has been identified and it is theoretically possible to examine such features to discriminate between selective and non-selective drugs [10]; still, this strategy has not been extensively adopted within the drug discovery community yet. It is also always possible to screen (either *in silico* or *in vitro*) a candidate of interest against a panel of targets (i.e. target fishing), in order to progress with exquisitely selective agents; however, it is difficult to imagine that such a panel could be exhaustive.

Probably, though, the main limitation of target-based approaches is that promising results at the *in vitro* level usually fail to translate when testing the hits against more complex systems, e.g. phenotypic (cellular and *in vivo*) models. Reasonably, single-target agents usually fail to treat multifactorial disorders with polygenic origin and/or a strong environmental component [11-13]. As clearly stated by Silver in her extensive but insightful and comprehensive review, target-based screening has produced many hits and leads within the antibacterial field, but none have yet been fully developed [8]. For example, an active compound might be incapable of overcoming permeability barriers or subjected to efflux systems [14]; on the other hand, due to compensatory mechanisms and escape routes biological systems are usually resilient to single-target interventions [15]. Other significant limitation specific to anti-infective drug development is that it has been hypothesized, on the basis of retrospective observations and theoretical considerations, that single-target agents are more prone to single step high-level mutations [8, 16, 17]. These limitations of single-target agents explain the renewed interested in phenotypic screening (in which drug candidates are selected by their inhibitory effect on infectious organisms of interest, e.g. pathogens, without regard to their mechanism of action, thus allowing detecting multi-target agents and hitting previously undescribed targets). For instance, the multiple mechanisms of action of the recently approved antibiotic Oritavancin confer it activity against vancomycin-resistant organisms as well as rapid killing versus actively growing, stationary phase, and biofilm-producing Gram-positive bacteria [18]. Besides the advantages that multi-target agents could have against resistance development, they could also be beneficial to approach co-infections/multiple infections since they could imply a broader and even tailored spectrum of activity (see, for instance [19]).

In the discussion on target- versus phenotypic-based strategies, tailored (or designed) multi-target agents can be regarded as the dialectical synthesis that pick the best out of each paradigm. Tailored multi-functional agents are deliberately devised to selectively modulate a number of chosen targets, usually relying on computer-aided design and data analysis applications and reducing the impact of the cost- and time-expensive target deconvolution process. Whereas theoretically multi-target agents are equivalent to combined therapies with different single-target agents, they are however advantageous in terms of diminished probability of drug interactions, simpler pharmacokinetics and improved patient compliance [20], with other possible advantages related to drug management, e.g. simplified prescribing and dispensing. Note that some, but not all, of these features are also shared with fixed-dose combination therapies/multicomponent drugs.

Hybrid agents are a particular type of multi-target agents obtained when covalently linking two (or, rarely, more) drugs or pharmacophores in a single molecule (a kind of fragment-based approach), in order to attack two or more molecular targets simultaneously [21]. Beside the already mentioned expanded spectrum of activity and reduced probability of developing resistance, hybrid agents could lead to synergic activity and reduction of the potential toxicity for a constituent agent. Conjugation of already known active scaffolds could also prove advantageous from an intellectual property perspective, since the linked moieties provide a novel structure. It has been argued, though, that the pharmacodynamics of the components of a hybrid drug should be compatible [8] that is, the ratio of activities at the different targets should usually be adjusted, which could prove complicated [22]. It has also been pointed out that the compounds obtained by joining two non-overlapping pharmacophores often display unfavorable biopharmaceutic features (e.g., compounds that violate more than two of the Lipinski's rules) [23, 24], and that this strategy could have a negative impact on binding efficiency metrics [23].Consequently, it is advised to carefully watch the physicochemical properties related to druglikeness of the resulting hybrid when designing hybrid molecules; alternatively, the overlapping or merging [25] approach can be useful to overcome the previously mentioned limitations of this type of drugs. It has been proposed that ideal fragments should follow the 'rule of three' (molecular weight < 300, calculated logP < 3, the number of hydrogen bond donors and acceptors < 3 and the number of rotatable bonds should be < 3).

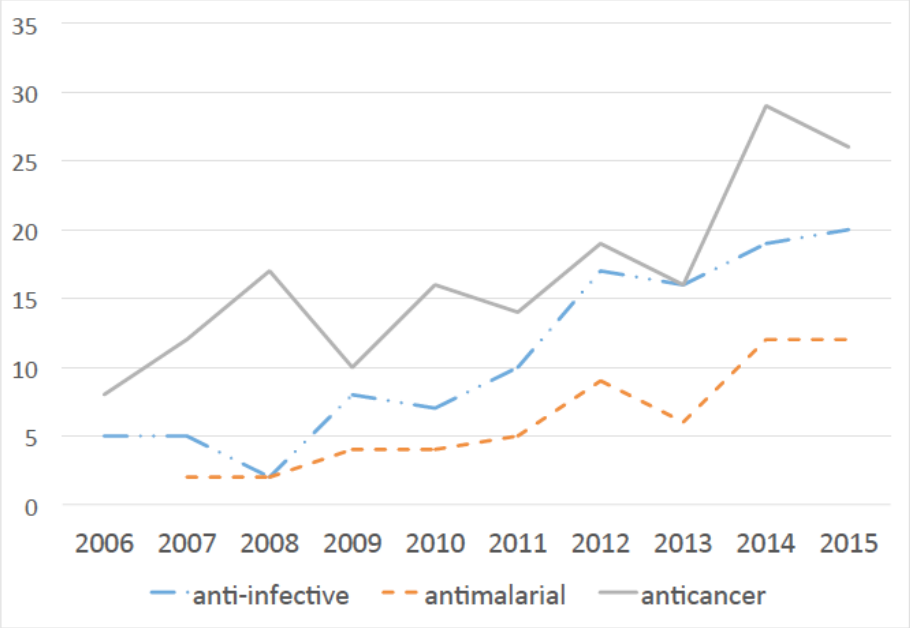

Fig. (1). Number of articles containing the query terms *versus* time. Despite the enormous differences between investment (and revenue perspectives) in anticancer and anti-infective agents, a considerable number of scientific articles refer to the development of anti-infective hybrid drugs candidates. The search was performed in titles, abstracts and keywords of scientific articles indexed by Scopus. Search criteria where: for anti-infective, "hybrid molecule" and "anti-infective" or "antibiotic" or "antifungal" or "parasite" or "antibacterial" or "antimicrobial" or "antiviral"; for antimalarial, "hybrid molecule" and "malaria" or "plasmodium" or "antimalarial"; for anticancer, "hybrid molecule" and "cancer" or "anticancer" or "antineoplastic" or "tumor" or "antitumor".

As can be appreciated in Fig. (**1**), the development of hybrid drugs has had great impact in the field of anti-infective drugs, particularly in the case of antimalarial agents (the applications of this concept in other parasitic diseases such as neglected tropical infections has been far more modest). Note that (Fig. **1**) presents a comparison with the use of hybrid molecules as potential drug candidates to treat cancer. With about a 100-billion-dollar market, cancer is the fastest-growing area of pharmaceutical research and drives many companies' pipeline investment; accordingly, cutting edge technology and novel approaches to drug discovery are usually applied to anticancer drug discovery in the first place and are later adopted for other drug classes. It is thus interesting to compare the time-evolution by which the hybrid molecule approach has been adopted in the anti-infective field. The search criteria have been included within the figure caption.

It can be appreciated that, despite the declining interest in anti-infective drug discovery, hybrid drugs have attracted a tremendous interest in the last ten years.

This review will overview some recent advances in the burgeoning field of hybrid anti-infective agents. Furthermore, we will also discuss some approaches which can be used to develop multi-target anti-infective drugs in the era of *omics* and *big data*. We have overviewed some recent developments of antibacterial, antiparasitic, antifungal and antiviral hybrid drugs. In the cases of antiparasitic agents, we have focused on malaria and trypanosomatid-caused infections (i.e. Chagas disease, African trypanosomiasis and Leishmaniasis).

2. RECENT EXAMPLES OF HYBRID DRUG CANDIDATES WITH ANTI-INFECTIVE ACTIVITY

2.1. Anti-Bacterial Hybrid Molecules

As stated in the introduction, the spreading resistance to antibiotics and the scarce number of new antibacterial drugs in the pipeline demand the incorporation of novel approaches to antibiotic discovery.

With this in mind, Wang and coworkers merged the morpholinyl group of 3-aryl-4-(2-morpholinoethoxy) furan-2(5H)-ones and the piperazinyl group of fluoroquinolone (FQ) moieties as we show in Fig. (**2**). Using such approach, they synthesized 27 3-arylfuran-2(5H)-one-FQ hybrids and evaluated their biological activities against resistant strains (*Escherichia coli* -ATCC 35218-; *Bacillus subtilis* -ATCC 6633- and *Staphylococcus aureus* -ATCC 25923) as well as the inhibitory effect against DNA gyrase and tyrosyl-tRNA synthetase (TyrRS) [26]. Their results showed compounds with excellent antimicrobial activities and outstanding inhibitory activities against DNA gyrase and TyrRS. Authors reported the minimum inhibitory concentration (MICs) observing substantial activity against all test microorganisms; some of the hybrids were more potent than the reference drug, Ciprofloxacin (CPX). It was proposed that such potent effect could be due to the introduction of the 3-arylfuran-2(5H)-one moiety, which can inhibit TyrRS and possibly improve the inhibitory effect against DNA gyrase. The most effective hybrid (Fig. **3**) presents values of MIC_{50} (μM) of 0.16 against *B. subtilis*, 0.35 against *S. aureus* and 0.2 against *E. coli*, which was about 30-fold, 36-fold and 51-fold more potent than CPX, respectively.

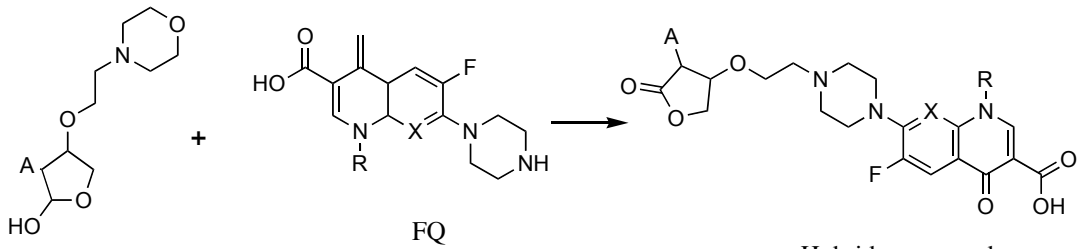

Fig. (2). The hybrid scaffold obtained by linking the morpholinyl group of 3-aryl-4-(2-morpholinoethoxy) furan-2(5H)-ones and the piperazinyl group of FQ; the resulting compounds display inhibitory effects on DNA gyrase and TyrRS.

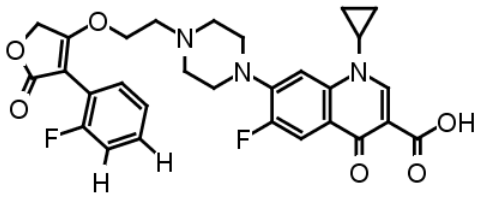

Fig. (3). Molecular structure of the more potent hybrid compound obtained by the combination between 3-aryl-4-(2-morpholinoethoxy) furan-2(5H)-ones and FQ.

In another interesting work, Plech and coworkers employ CPX to synthesize some hybrid drug candidates, with the general idea of expanding the spectrum of activity to both Gram-positive and Gram-negative bacteria [27]. In order to achieve their purpose, they decided to combine the CPX with a 1,2,4-triazole system, since this fragment has antibacterial effects against Gram-positive bacteria only. 18 hybrid compounds were prepared (Fig. **4** shows the proposed scaffold). The antibacterial activity of the compounds was tested against Gram positive bacteria (*Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 10876, *Micrococcus luteus* ATCC 10240) and Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Proteus mirabilis* ATCC 12453, *Pseudomonas aeruginosa* ATCC 9027). A drug-resistant strain of *S. aureus* (methicillin-resistant *S. aureus* ATCC 14001) was used. The measured activity was compared with that of CPX; in the case of the resistant strain Vancomycin was used as control.

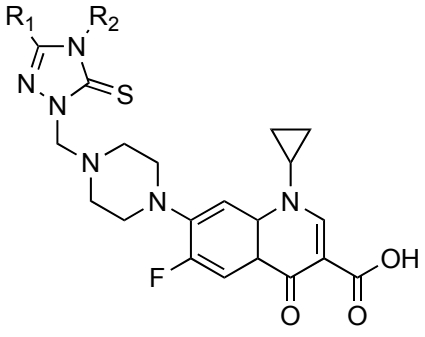

Fig. (4). Scaffold of the eighteen synthesized hybrids by Plech *et al.*

11 out of 18 hybrids showed enhanced antibacterial effect compared with CPX, against both Gram-positive and Gram-negative bacteria. The best result (about 8.5 times more potent than the reference antibiotic on *S. aureus*-ATCC 25923, CPX sensitive) was found for the hybrid with a 3-OH-Ph substituent in R1 and a 2,4-diCl-Ph substituent in R2 (Table **1**) [27].

In 2015, the same group published a related report in which they studied the structure-activity relationship of a series of 1,2,4-triazole derivatives [28]. Again, hybrids from CPX and different 1,2,4-triazoles were obtained and the synthesized compounds proved more potent than the reference antibiotic. At this point the authors decided to evaluate the cytotoxicity using human embryonic kidney cells (HEK-293), finding that in all cases the toxic concentrations were much higher than those needed to elicit antimicrobial effects. Interestingly, the authors observed a weakening of affinity (in comparison with CPX) towards the main molecular targets of FQ (type II topoisomerases), suspecting that additional (unspecified) mechanisms may take part in the antibacterial effect of the novel compounds.

Similarly, antibiotics combining the nucleoside and peptidyl moeities from Nikkomycins and Polyoxins (two peptidyl nucleoside antibiotics) have been obtained and some of the hybrid compounds display enhanced bioactivity and stability than the parent antibiotics [29].

Back in 2012, Karoli *et al.* developed a "chimeric approach" using click chemistry where the pharmacophores from different drugs are overlapped into a smaller drug-like molecule [30]. Such an approach is in line with the partially and fully overlapping pharmacophores described by Morphy *et al.*, which results in a smaller molecular size and improvement of other biopharmaceutically relevant molecular properties, in contrast to the traditional hybrid molecules. The authors chose the benzyl pyrimidine and the FQ, two classes of antibiotics with broad spectrum activity against both Gram-negative and Gram-positive bacteria and without antagonistic effect. The X-ray crystal structures of benzyl pyrimidines and FQs in complex with their targets are available as well as extensive SAR information. The obtained series of compounds include candidates showing (moderate) activity against the targets of both trimethoprim (dihydrofolate reductase), and FQs (DNA gyrase and topoisomerase IV). Whereas the observed *in vitro* activity was modest, the compounds showed no cytotoxicity and the work opens up the possibility of using the same strategy in the discovery of other promising molecules for infectious diseases.

Table 1. MIC values of the more potent hybrid obtained by Plech *et al.* vs. CPX and Vancomicyn.

	Minimum inhibitory concentration (μM)								
	Gram positive						Gram negative		
	S. aureus ATCC 25923	*S. epidermidis*	*B. subtilis*	*B. cereus*	*M. luteus*	*S. aureus* ATCC 14001	*E. coli*	*P. mirabilis*	*P. aerugi-nosa*
Hybrid	0.35	0.35	0.18	0.18	1.44	0.35	0.044	0.022	0.088
CPX	2.96	1.48	0.09	0.36	5.88	1.48	0.024	0.045	0.72
Vancomicyn	-	-	-	-	-	0.68	-	-	-

Remarkably, some hybrid antibiotic compounds have already reached clinical trials. An example is Cadasolid (ACT-179811), an oxazolidinone and quinolone hybrid that is under development as an oral treatment for *Clostridium difficile* infection, which is the most common infectious cause of antibiotic-associated diarrhea [31, 32, 33]. At present, two Phase 3 multi-center, randomized, double-blind studies are in phase of recruiting to compare the efficacy and safety of Cadazolid vs. Vancomycin in subjects with *Clostridium difficile*-associated Diarrhea [34, 35].

TD-1792, a Vancomycin–Cephalosporin hybrid, has been reported as an antibacterial drug with superior efficacy relative to a simple combination of the parent drugs in multidrug resistance (MDR) infections [30]. It is presently being evaluated against skin infection caused by Gram positive microorganisms. This candidate drug has completed Phase II clinical trials, but results are not available yet [36].

If, with any luck, some of these advanced drug candidates are able to gain approval and reach the pharmaceutical market in the forthcoming years, we will be able to stablish if availability of designed multi-target agents results in an enhanced capability to control bacterial infections and a reduced frequency of multi-drug resistance strains.

2.2. Hybrid Drugs for Trypanosomiasis

Neglected Tropical Diseases (NTDs) are a diverse group of diseases that prevail in tropical and subtropical conditions in 149 countries and affect more than one billion people, costing developing economies billions of dollars every year. They principally affect populations living in poverty, without adequate sanitation and in close contact with infectious vectors and domestic animals and livestock [37]. Additionally, drug companies have historically shown reluctant to finance the development of new treatments because of their low expected return [38], though fortunately this scenario seems to be reverting at present.

Among the NTDs, there is a group of diseases (Human Trypanosomiasis Africana, Chagas disease and Leishmaniasis), whose etiological agents belong to the Trypanosomatid family and are responsible for infections that mainly affect rural areas of the planet.

Existing therapies for these complex pathologies are based mainly in chemotherapy. However, in most cases they rely on outdated and toxic drugs that were identified decades ago, such as the arsenical Melarsoprol for HAT and the Pentavalent Antimonials for Leishmaniasis. In the case of Chagas disease, the only available drugs are Benznidazole and Nifurtimox, which are associated to significant and frequent adverse effects (such as peripheral polyneuropathy, depression of bone marrow, and allergic dermopathy) that limit adherence to treatment, and are not effective in the chronic stage of the disease. These limitations of the few existing treatments for these parasitic infections, together with growing resistance development, justify the urgent medical need for the development of new trypanocidal agents. The identification of such agents, which should ideally display good oral availability and broad-spectrum activity against several of these parasites, is a highly topical area of research [39].

Taking into account that molecular hybridization is a powerful approach for the design of new compounds, da Silva Júnior and coworkers, in 2008, searched for new quinone derivatives as potential anti-*T. cruzi* compounds [40]. They proposed that molecules having a quinone core have favorable biological activity and that naphthoquinones, which are involved in several oxidative processes, have a broad distribution in the plant kingdom. Thus, from the heartwood of *Tabebuia* trees (Bigoniaceae) the researchers extracted lapachol (2-hydroxy-3-(3′-methyl-2-butenyl)-1,4-naphthoquinone), and prepared about 20 derivatives which were screened for their trypanocidal effect, using the infective bloodstream form of *T. cruzi* (strain Y). Besides lapachol, another quinone was obtained by Hooker oxidation [41], the nor-lapachol (2) (2-hydroxy-3-(2-methyl-propenyl)-[1,4]-naphthoquinone), and five substituted ortho-naphthofuranquinones were prepared, a non-substituted para-naphthofuranquinone, a new oxyrane and an azide. The trypanocidal activity of the naphthofuranquinones was variable, though two of the molecules synthetized, compounds A and B, were more active than the reference drug Benznidazole (EC_{50} : 86.3 μM±4, 88.2±6.7 μM, 103.6±0.6 μM respectively) (Fig. **5**). In conclusion, the substituted arylamino quinones and substituted naphthoquinones appear as interesting new prototype compounds though more experiments are needed to investigate their mode of action. These compounds could possibly open new perspectives for the development of more potent and selective trypanocidal drugs.

In 2010, Nava-Zuazo and his team, inspired in molecular hybridization, synthesized a new series of quinoline tripartite hybrids from the antiprotozoal drug Chloroquine (CQ) and the anti-mycobacterial drugs Ethambutol and Isoxyl [42]. 9 compounds were obtained and tested *in vitro* against five

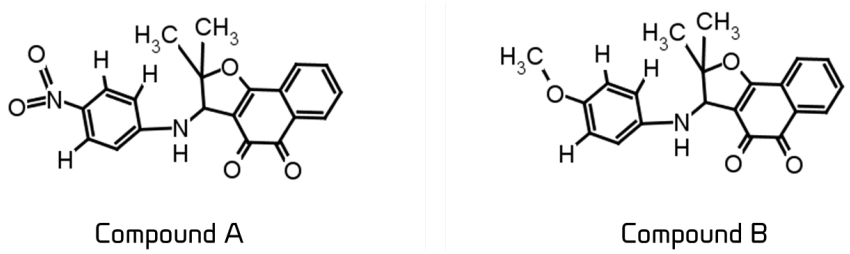

Fig. (5). Compounds A and B, the more potent naphthoquinones of the lapachol derivates synthetized by da Silva Junior *et al.*

Table 2. Activity of the most potent hybrid synthesized against all parasites tested.

	Compound R: - 4-BuOPh	CQ	Ethambutol	Isoxyl	Metronidazole	Benznidazole	Pentamidine
MIC (μg/mL) ***M. tuberculosis* H37Rv**	2	8	4	4	-	-	-
IC_{50} (μM) ***G. intestinalis***	0.01	0.40	-	-	5.36	-	-
IC_{50} (μM) ***T. vaginalis***	8.44	30	-	-	0.29	-	-
IC_{50} (μM) ***E. histolytica***	3.64	9.58	-	-	0.77	-	-
IC_{50} (μM) ***L. mexicana***	9.90	>50	-	-	-	-	13.32
IC_{50} (μM) ***T. cruzi***	50	>50	-	-	-	34.38	-

protozoa (*Giardia intestinalis, Trichomonas vaginalis, Entamoeba histolytica, Leishmania mexicana* and *Trypanosoma cruzi)* and *Mycobacterium tuberculosis*.

All the screened candidates showed good activity (EC_{50}<1.8 μM) against *G. intestinalis*, being more potent than Metronidazole, the reference drug (EC_{50} = 5.36 μM). The substitution in the R position with a 4-butoxyphenyl (N-(4-Butoxyphenyl)-N0-{2-[(7-chloroquinolin-4-yl)amino] ethyl-urea) generated the most active compound (Fig. **6**) against all parasites tested, as we show in Table **2**. For *L. mexicana*, this was the only compound as active as Pentamidine (second-line anti-leishmanial drug). The remaining compounds were inactive against this kinetoplastid parasite. Moreover, that compound was two-fold more potent than Ethambutol and Isoxyl versus *M. tuberculosis.* Unfortunately, the *in vitro* anti- *T. cruzi* activity was no so good, and only some of the compounds had moderate effects against *T. cruzi* (active at 50 μM); in contrast, Benznidazole (first-line anti-*T. cruzi* drug) showed an EC_{50} = 34.38 μM.

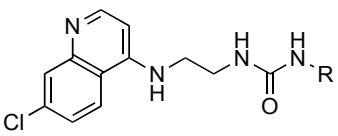

Fig. (6). Scaffold of the most active compound (R: 4-BuOPh) from the tripartite hybridization from the antiprotozoal drug Chloroquine and the anti-mycobacterial drugs Ethambutol and Isoxyl synthetized by Nava-Zuazo and coworkers.

In 2013, Gehrke SS *et al.* developed hybrid compounds by linking the 3-hydroxypyridin-4-one (HPO) of the Deferiprone scaffold to the 4-aminoquinoline ring system present in the antimalarial drug CQ (Fig. **7**) [39]. Deferiprone, an HPO derivative, has anti- parasitic activity and good oral bioavailability [43] and CQ has anti-*Plasmodium* activity. Authors reported inhibitory effects of these novel analogues against four parasitic protozoa: *T. brucei, T.cruzi, L. infantum and P. falciparum*. The innovative HPO derivatives have potent iron chelating ability. Iron chelation has been proposed as a possible new strategy to combat parasitic infections [44]. It has been shown that the use of iron chelators can compromise the activity of Fe^{+3} containing enzymes in parasites, affecting the DNA synthesis. Notably, one of the hybrids, Deferirpone-CQ hybrid (Fig. **7**) shows a clearly enhanced activity against *T. brucei* and *T. cruzi* (>25-fold against *T. brucei* and >15-fold against *T. cruzi* compared to Deferiprone, and about four-fold against both species compared to CQ) [39].

These results show a promising starting point to reconsider the application of iron chelators, in particular of the HPO class, as anti-parasitic agents. They also suggest that, at least against trypanosomes, the hybrid strategy leads to synergistic effects between the HPO and CQ fragments, while retaining reasonable selectivity indices. New modifications of their acid/base properties could represent a tactic for further optimization of the anti-trypanosomal activity of this class of iron chelators [39].

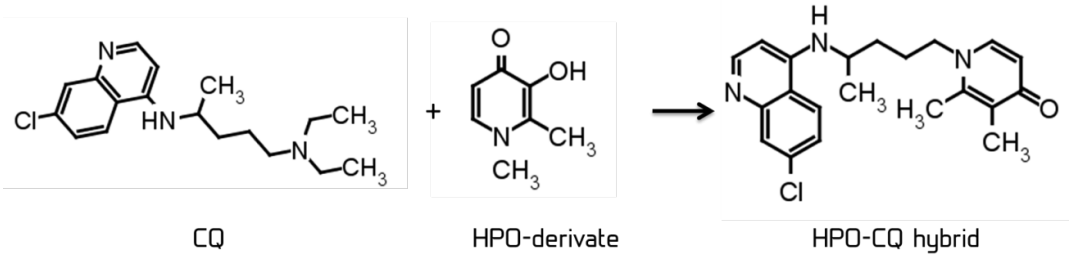

Fig. (7). Hybrid compounds where the HPO scaffold of the Deferiprone has been conjugated to the 4-aminoquinoline ring system present in the antimalarial agent Chloroquine by Gehrke SS *et al*.

Based on previous reports regarding new selenium compounds with *in vitro* antiparasitic activity against *Leishmania infantum* [45], Baquedano *et al.* explored the modification of their previously reported candidates by derivatization of the amine groups. The aim of their research was to enhance some characteristics as polarity and solubility, facilitate cellular uptake, optimize anti-leishmanial activity and reduce cytotoxicity improving the level of selectivity [46]. Accordingly, they designed a new class of diselenide derivatives by molecular combination between 4,40-diselanediyldianiline, as the scaffold, with sulfonamide moieties bound to different rings (Fig. **8**). The 16 new hybrid molecules were evaluated against amastigotes from *L. infantum*. Moreover, in order to exclude hybrids with unfavorable toxicity, they assessed cytotoxicity against a human cell line.

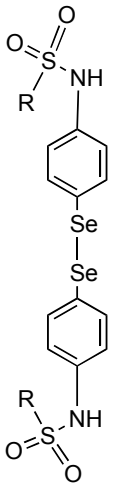

Fig. (8). Hybrid diselenide derivatives scaffold by combination of 4,40-diselanediyldianiline, and sulfonamide moiety developed by Baquedano *et al.*

The effects of the 16 synthesized sulfonamides against axenic and intracellular amastigotes were analyzed in comparison with standard drugs Miltefosine and Edelfosine. The authors proved that 12 of the screened compounds have similar or even higher activity than Miltefosine (EC_{50}=2.84 μM) against axenic amastigotes. They established that the 8-quinolinyl, 2-thienyl and 2-(1-methyl-1H-imidazolyl) analogs display high activity in this assay. Additionally, cytotoxicity against the THP-1 human cell line was assessed and the selectivity index (SI) of the compounds was computed as the ratio of cytotoxicity (EC_{50} value on THP-1 cells) to activity (EC_{50} value on *L. infantum* axenic amastigotes). Seven hybrids outperformed the selectivity index of reference drugs Edelfosine and Miltefosine (SI = 6 and 7 respectively). Because of their activity and selectivity, three compounds with R substituent 4-methylphenyl, 4-fluorophenyl and 8-quinolinyl were selected to test their leishmanicidal activity in amastigote-infected THP-1 cells. The EC_{50} for each compound was 4.1 μM, 2.8 μM and 6.2 μM, in that order, whereas for Edelfosine the measured EC_{50} was 3.1 μM. In conclusion, the authors designed, synthesizedand tested a new series of *in vitro* anti leishmanial drugs. Most of the hybrids exhibited a notable inhibition of *L. infantum* amastigotes growth. Due to its activity on amastigotes and considering its selectivity (therapeutic index > 17), the analog with a 4-fluorophenyl substituent seems to be most promising compound, constituting a suitable lead structure for the development of future antiparasitic drugs.

2.3. Hybrid Drugs for Malaria

Malaria is one of the most widespread parasitic disease; it is caused by a parasite of the genus *Plasmodium;* the parasites are spread to people through the bites of the infected female *Anopheles* mosquitoes. According to figures from the World Health Organization (WHO), in December 2015 there were 214 million cases of malaria which determine around 438,000 annual deaths [47]. Concerted efforts to develop an active vaccine have thus far been fruitless, and consequently chemotherapy remains one of the most important strategies to control malaria [48]. Parasite drug resistance is one of the major obstacles for malaria elimination, and this issue requires sustained investment in drug discovery programs to ensure a future supply of effective treatments [49]. More than 90% of the current drug research projects target the blood stages; today there is still no medication available equally active against all the stages of the life cycle of Plasmodium and against all *Plasmodium* species [50]. Currently, the WHO recommends the use of antimalarials in fixed-dose regiments with partner drugs. Each of these partner drugs should be effective in killing the parasite, with minimal signs of resistance [50, 51].

The synthesis of 4-Aminoquinoline-Pyrimidine hybrids containing flexible linkers has been proposed to overcome the resistance to CQ [52, 53]. The hybrids were combined in a two-step nucleophilic substitution process. All the prepared hybrids were active against two strains of *P. falciparum*: the D10, sensitive to CQ, and Dd2, resistant to CQ. One of the

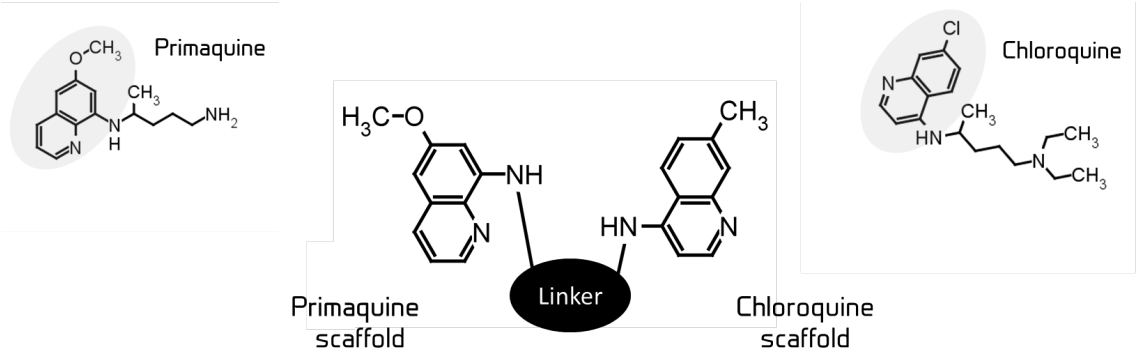

Fig. (9). Linked Primaquine-Chloroquine hybrid templates by Lödige & Hiersch.

compounds was found as potent as CQ and Pyrimethamine against the D10 strain, and possessed a moderately superior potency over CQ against the Dd2 strain (EC_{50}: 0.157 vs. 0.417 μM). The authors proposed that the activity of this compound may be due to the piperazine linker, which is strongly protonated and possibly favors a higher accumulation of the drug in the digestive vacuole of the parasite [54].

Another approach to obtain hybrids was the use of Ferrocene as starting scaffold. Ferrocene is an excellent starting chemical because of its unique properties such as aromaticity, aqueous stability, and redox behavior. An example of a Ferrocene based drug is Ferroquine (SSR97193), an antimalarial highly active against CQ-resistant malaria strains, and currently in phase IIb clinical trials sponsored by Sanofi-Aventis [55]. In this work the authors reported the synthesis and characterization of new Ferrocene-indole hybrids [56] which were obtained by introducing the Ferrocene core in position 3 of the 2-phenylindole scaffold. The potential of the obtained derivatives was tested once again against different strains of *P. falciparum*, but the new derivatives exhibited only weak inhibitory effects vs. the reference drug. Despite these results, Ferrocene is still an attractive candidate though further work is needed to obtain new analogues with improved potency.

Very recently, Lödige & Hiersch reported the design, synthesis, and characterization of novel hybrid molecules consisting of the reference drugs Primaquine and CQ [50]. The novelty of this research lays in the synthesis of hybrids with activity against different stages of the *Plasmodium* infection. Eleven new candidates were prepared and evaluated in liver stages (aiming to reduce the progress of the infection), blood stages (aiming to cure the clinical symptoms), and gametocytes (aiming to inhibit the transmission cycle). The synthetic approach uses a divergent synthetic methodology to link Primaquine-CQ templates in a diversity of ways (Fig. **9**). The reported hybrids showed good to excellent biological activity against the liver stage (*P. berghei*), blood stage (*P. falciparum*, strains 3D7 –a CQ and Pyrimethamine sensitive strain-, Dd2 –a strain resistant against CQ, Mefloquine, and Pyrimethamine-, and K1- a resistant strain against Pyrimethamine and one of the most resistant strains against CQ-), and gametocytes (*P. falciparum*). The most promising hybrid showed remarkably high activity not only reducing the number and the diameter of liver stages but also against blood stages of 3D7, Dd2, and K1 strains (EC_{50} lower than 0.1 μM) and against gametocytes. This promising effects of the compounds against different stage of the parasites are interesting under the perspective of interrupting its transmission cycle. Interestingly, the results of the hybrids are better than the combined activity of the parent drugs. These results are described in a patent application [50].

Dambuza *et al.* have also studied the development of antimalarial candidates using the HPO. The hybrids were synthesized by combining CQ with HPO, and tested *in vitro* and *in vivo* models. The EC_{50} against sensitive *P. falciparum* strains (D10 and 3D7) for candidate 1 were 0.064 and 0.047 μM and 0.041 and 0.122 μM for candidate 2 (Fig. **10**). The values obtained against resistant strains (Dd2 and K1) were 0.505 and 0.463 μM (candidate 1), 0.089 and 0.076 μM (candidate 2), respectively. The mice model indicates that compound 1 was able to reduce parasitaemia levels in *P. berghei*-infected mice when administered intravenously, but regrettably parasites recrudesced 24 h after the administration of the last dose. CQ remained more effective *in vivo* than both compounds; it is possible that further modifications on the chemical structure could improve pharmacokinetic properties, such as oral bioavailability, thus improving efficacy [57].

While an exhaustive revision of hybrid antimalarial drug candidates has not been provided, some of the previously reviewed reports suggest that the hybrid approach could indeed provide improved therapeutic solutions against malaria, with the interesting possibility of treating all the stages of the infection and different *Plasmodium* species with a single drug. Nevertheless, it has to be seen if the more promising results at the early drug discovery stage translate into superior treatments at the clinical trials stage.

2.4. Antifungal Hybrids

It is broadly accepted that fungal pathogens have a huge influence on plant and animal life. While most people will undergo only superficial fungal infections through their lifetime, millions of people will contract life-threatening invasive fungal infections that are much harder to diagnose and treat [58]. A serious concern related to the treatment of fungal infections is the restricted number of successful antifungal drugs, some of which display disadvantages such as narrow therapeutic spectrum, drug resistance, high toxicity and low bioavailability [59]. Undoubtedly, these infections demand urgent discovery and development of novel antifungal molecules.

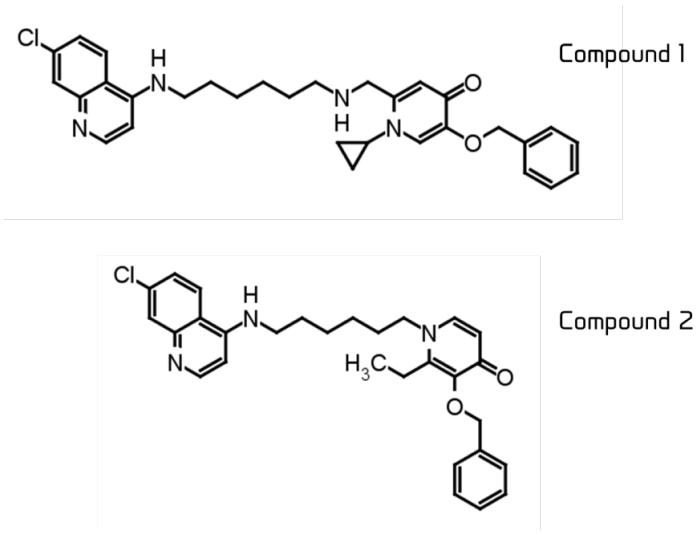

Fig. (10). The scaffold of 2 candidates of the merge CQ-HPO obtained by Dambuza *et al.*

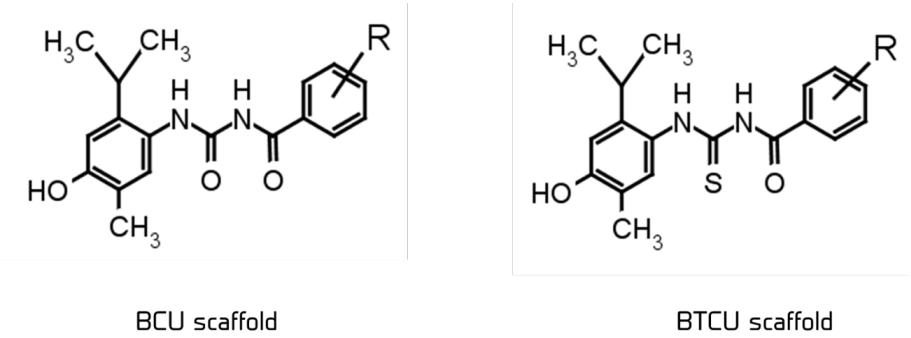

Fig. (11). Benzoylcarvacrylthiourea's (BCTU) and benzoylcarvacryl urea's (BCU) scaffolds obtained by Pete *et al*.

The hybrid strategy has also been used for the synthesis of new and more effective agrochemicals with insecticidal and antifungal properties. A known antifungal monoterpenoid, Carvacrol, was combined with benzoyl urea or thiourea moieties present in commercially used Benzoylphenyl urea (BPU), a class of insecticide [60]. Numerous aromatic plants such as black cumin (*Nigella sativa L.*), marjoram (*Origanum majorana L.*), oregano (*Origanum vulgare L.*) summer savory (*Satureja hortensis L.*) and thyme (*Thymus vulgaris L.*) produce Carvacrol (2-methyl-5-[1-methylethyl] phenol). The antifungal activity of this molecule has been demonstrated against many phytopathogens and human pathogenic fungi. Carvacrol generates membrane damage by diminishing ergosterol content. It also has been proved effective against different insects like *Codiplosis japonensis, Aphis craccivora,* and *Leucania separata*. In this study, authors generated hybrid molecules of Carvacrol and BPU hopping to develop new molecules with two biological activities: insecticidal and antifungal. Two series of compounds benzoylcarvacrylthiourea's (BCTU) and benzoylcarvacryl urea's (BCU) were synthesized (Fig. **11**).

Notably, Carvacrol is used as food additive (approved by the US Food and Drug Administration) as a flavoring agent in different foods. Although at first hybrids were developed as insecticides, the BCTU and BCU derivatives were tested for antifungal activity against different human fungal pathogens: *Candida albicans, C. glabrata and Cryptococcus neoformans*. In general, the BCTU derivatives showed potent antifungal activity (Table **3**), while not all BCU compounds were effective against the tested fungi.

Cellular toxicity of the compounds was checked by the haemolysis assay finding that at MIC concentrations the haemolysis was negligible (<2%) for all the derivatives. In conclusion, these results suggest that the hybrid compounds are more efficacious and safer than BPU's and Carvacrol, although more studies should be done to advance in the characterization of these molecules.

Alwan *et al.* described the synthesis of novel chalcones and Schiff base hybrids of imidazo [2,1-*b*]-1,3,4-thiadiazoles (Fig. **12**) with their subsequent biological evaluation for antibacterial, antifungal and anti-mycobacterial activity. These authors reported the synthesis of 25 novel hybrid compounds, which were tested against standard cultures of *S. aureus* (ATCC25923), *B. subtilis* (ATCC6051), *E. coli* (ATCC35218), *P. aeruginosa* (ATCC27853); moderate activity was observed against these bacteria strains [59]. However, compounds displayed substantial anti-fungal activity with MICs ranging between 1.56-100 μg/mL were obtained when the compounds were tested against three fungal strains: *C. albicans* (ATCC90028), *C. neoformans* (ATCC66031) and *Aspergillus niger* (ATCC16404), and two clinically isolated *C. albicans* and *C. neoformans*.

Table 3. Antifungal effects of BCTU and BCU compounds against fungal human pathogens.

Hybrid compounds	Minimum Inhibitory Concentration (µg/ml)					
	C. albicans NCIM 3557	*C. albicans* NCIM 471	*C. glabrata* NCIM3237	*C. neoformans* NCIM 3541	*C. neoformans* NCIM 3542	*C. neoformans* NCIM 3378
BCTU 1	128	>512	32	16	32	128
BCTU 2	64	>512	64	32	64	>512
BCTU 3	64	>512	32	8	32	32
BCTU 4	128	32	128	64	128	512
BCTU 5	64	>512	64	32	64	128
BCTU 6	256	>512	64	32	64	128
BCT 1	512	>512	>512	>512	256	>512
BCT 2	128	>512	>512	256	128	128
BCT 3	64	>512	32	32	32	32
BCT 4	>512	32	16	<4	16	16
BCT 5	>512	32	16	<4	16	16
BCT 6	>512	32	16	<4	16	16
Carvacrol	128	256	128	128	128	128

NMCI: National Centre for Industrially Important Microorganisms

Fig. (12). Chalcones and Schiff base scaffold of imidazo [2,1-*b*]-1,3,4-thiadiazoles obtained by Alwan *et al*.

2.5. Antiviral Hybrids

Dengue (DENV) is a mosquito-borne viral disease that has quickly spread in recent years; it is mainly transmitted by female mosquitoes of the species *Aedes aegypti.* This disease is widespread throughout the tropics, and has a severe condition known as Dengue Haemorrhagic Fever, which is a potentially deadly complication due to plasma leaking, fluid accumulation, respiratory distress, severe bleeding, or organ impairment [61]. On the other hand, West Nile Virus (WNV) is a member of the *flavivirus* genus and belongs to the Japanese encephalitis antigenic complex of the family *Flaviviridae*. WNV can cause fatal neurological disease, and is commonly found in Africa, Europe, the Middle East, North America and West Asia [62].

There are many DENV and WNV viral proteins that have been targeted for drug discovery, including helicase, methyltransferase, serine protease and viral RNA. Up to now, only a minor number of non-peptidyl inhibitor scaffolds for DENV and WNV proteases have been described and the available structure-activity relationship data are limited.

Recently, 1,2-Benzisothiazol-3(2H)-ones and 1,3,4-oxadiazoles have independently attracted significant interest in the drug discovery field. In this work, Lai *et al.* have described the design, synthesis, and structure-activity relationship studies of a series of novel 1,2-benzisothiazol-3(2H)-one and 1,3,4-oxadiazole hybrid derivatives (Fig. **13**) [63].

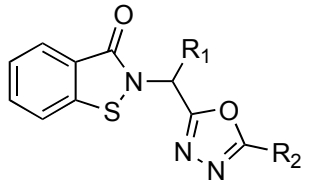

Fig. (13). Molecular structure for hybrid compounds prepared by Lai *et al.*

They synthesized 24 hybrid compounds and investigated their inhibitory activity against DENV and WNV over non-structural proteins. Ten out of 24 analogues showed 50% inhibition against DENV-2 (DENV type 2) and WNV protease. One of the most potent compounds, with a benzyl substituent in R1 and a p-methoxyphenyl substituent in R2, was chosen for further kinetic studies. The IC_{50} values of this compound against DENV2 and WNV proteases were determined to be around 3.75 μM and 4.22 μM, respectively. The authors continued the study of this promising compound with the correspondent kinetic analysis. The apparent Michaelis-Menten constants (Km, app) increased and kcat/Km decreased consistently with increasing concentration of this compound. Thus, the kinetic data provided suggested a competitive mode of inhibition.

Zeng *et al.* designed and synthesized a novel series of diarylbenzopyrimidine analogues (DABPs) and evaluated their activity against HIVs [64]. The *in vitro* evaluation examined the potency of inhibition of the replication of wild-type HIV-1 virus and some mutant strains. The effect on HIV-1 indicated that the new compounds present potent antiviral activity, with EC_{50}values in the nanomolar range. The DABP derivative with a hydrogen atom in the R position and a 2,6-dime-4-cyano phenyl group in the A position, displayed the most potent activity against wild-type HIV-1 (Fig. **14**). This hybrid exhibited an EC_{50} value of 1.8 nM, which was much more effective than the reference compounds Nevirapine (by 731-fold) and Delavirdine (by 266-fold). Also, this compound revealed excellent efficacy against some mutant strains as L100I, K103N, Y188L, and K103N+Y181C with EC_{50} values of 18, 3.6, 36, and 60 nM, respectively. The compound can serve as base for further modification in the search of more effective candidates for improved anti-HIV-1 chemotherapy.

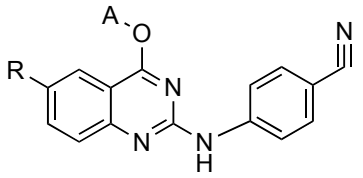

Fig. (14). DABPs scaffold of the most active compound. Substitution with an hydrogen in R position and a 2,6-dime-4-cyano phenyl group in A position, generate the most potent activity against wild-type HIV-1 find by Sen Zeng *et al*.

Another series of antiviral hybrids effective against polyomaviruses has been reported. Presently, 12 human polyomaviruses are known, some of which are associated with disease in immunocompromised individuals. As occur in most of the infectious diseases, available treatments such as Cidofovir target viral DNA non-specifically, and therefore display off-target undesirable side effects [65]. An interesting characteristic is that all polyomaviruses express Large Tumor Antigen (T Ag), which is unique to this virus family and could thus be an interesting drug target. In a previous work, Brodsky *et al.* screened pyrimidinone–peptoid hybrid compounds identifying 2 inhibitors of viral replication and T Ag ATPase activity, MAL2-11B and MAL2-11B tetrazole derivative [66]. In this work, the aim was to synthetize a series of MAL2-11B analogs with improved antiviral activity. The replacement of a flexible methylene chain linker with a benzyl group or, alternatively, the addition of an ortho-methyl substituent on the biphenyl side chain of MAL2-11B yielded an IC_{50} of ≈50 μM. After combining both structural motifs, a new lead compound was identified that inhibited T Ag ATPase activity with an IC_{50} of ≈5 μM. The authors believe that the knowledge gained from the structure–activity relationship and a further refinement cycle of the MAL2-11B scaffold will provide a specific, novel therapeutic treatment option for polyomavirus infections and their associated diseases. The authors were able to produce two side chain modifications to generate a compact new analog, SMAL (Fig. **15**) that inhibited T Ag ATPase activity with an IC_{50} of ≈5 μM.

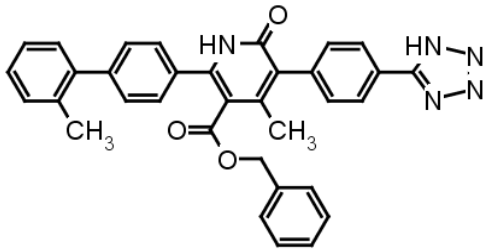

Fig. (15). SMAL, an antiviral hybrid for the Polyomaviruses by Brodsky *et al*., with an IC_{50}≈5.

3. THE REVOLUTIONARY INTEGRATION OF GENE PRODUCT PROFILING TO DRUG DISCOVERY

As insinuated in the introduction when discussing multi-target drugs, it is increasingly clear that both target- and phenotypic-based approaches have their own limitations. Accordingly, there is presently a general trend to integrate both approximations, which can be regarded as complementary. For instance, multi-target drugs represent a more systemic perspective to target-focused drug design. Similarly, phenotypic screening is becoming more and more target and hypothesis directed [14]. The advent of the *omics* era has granted tools to assist target deconvolution in a more high-throughput manner (e.g. DNA microarrays, next generation sequencing). The general assumption here will be that microorganisms will resort to compensatory mechanisms (up and/or downregulation or activation and/or inactivation depending on drug effects and mechanisms of action) to counteract the effects of exposure to an anti-infective agent, which could provide clues on the mechanism of action of the drug. Some of those adaptive effects could be rather unspecific (e.g. overexpression of drug efflux transporter to stimulate drug clearance), while others would be directly related to specific interactions of a drug with a molecular target or a targeted pathway.

Genome-wide gene expression profiling offers a snapshot of globally measured transcript levels in a given cell, tissue or organism at a specific point of time under a certain experimental condition [67], which to some extent can provide a holistic picture of their physiological state. While monitoring protein expression could seem more adequate than monitoring RNA expression, it has traditionally been problematic owing to the absence of highly efficient protein amplification technologies (which have traditionally limit the identification and quantification of low abundant proteins), low through-

put, and the relative heterogeneity of antibody reagents as opposed to the consistency of nucleic acid probes [68]. Monitoring mRNA expression to study the direct effects of a drug has its own and significant limitations [68, 69]: genes can be regulated at multiple levels: transcriptional, translational and post-translational; there is no necessarily simple correlation between mRNA and protein levels; drugs targeting two different proteins of the same pathway might have similar expression profiles. Still, analysis of changes in gene expression in response to small molecules can yield many novel mechanistic insights, i.e. activation mechanisms, cellular transporters and direct protein targets and make connections between perturbagens acting through similar mechanisms (*connectivity mapping*) [70]. Target overexpression confers resistance or sensitivity as a predictable property of drug mechanism; overexpressing putative targets provides a systemic approach to distinguish mechanisms of drug action [71].

Lately, genomics and proteomics approaches have been increasingly applied to gain clues on the mechanism of action of different antibiotics. For example, Kim and coworkers studied the effects of extracts from five herbs (*Houttuynia cordata* Thunb, *Chrysanthemum lavandulifolum*, *Patrinia scabiosaefolia*, *Angelica dahurica* Bentham *et* Hooker and *Agrimonia pilosa*) against *Escherichia coli* O157:H7 [72]. Total RNA of the treated cells was isolated to compare and analyze their gene expression profiling. According to their results, the authors concluded that the multi-target molecular mechanisms of the antibiotic effects of the herbs included bacterial cell wall biosynthesis, DNA replication and repair, protein synthesis and hydroxyl radical damage followed by overproduction of superoxide. More details on the effects of effects of *Chrysanthemum lavandulifolium* on the mRNA signature of *E. coli* O157:H7 were reported by that same group [73]. The ethanolic extract obtained from aerial parts of *C. lavandulifolium* was fractioned with a series of organic solvents; the methylene chloride fraction displayed antibiotic efficacy similar to 5 mM Ampicillin. The gene regulation induced by the methylene chloride fractions was examined. A significant downregulation was observed in a number of genes involved in protein synthesis, DNA replication, cell wall biosynthesis and bacterial noxious factors. Notably, only genes involved in DNA repair were upregulated, suggesting a mechanism of action involving DNA damage. The same group has used the disk diffusion technique and DNA microarrays to examine the molecular mechanism of *Angelica dahurica* antibiotic effect on *E. coli* O157:H7; real-time qPCR was employed to confirm the microarray data [74]. The inhibitory effect of an ethyl acetate fraction of such herb induced changes on the expression levels of genes related to the bacterial cell envelope formation (mostly, upregulation), folate biosynthesis (both up and downregulation depending on the gene), DNA replication (downregulation in all cases) and etiological factors (downregulation, in all cases) of the pathogen. Similarly, *Patrinia scabiosaefolia* ethyl acetate fraction of the ethanolic extract of the plant roots displayed significant upregulation of genes related to the cell motility, protein synthesis, DNA repair, and cell wall synthesis, whereas significant downregulation was particularly observed in genes related to DNA replication [75]. The previous results illustrate the complex pattern of herb extracts antibiotic effect. It is interesting to note that the previous authors have exploited gene profiling tools to the examination of antibiotics of natural origin, which have previously been left somewhat aside in favor of synthetic libraries of drug candidates friendlier to HTS and target-driven approaches [8].

Recent advances in the field of proteomics, in particular, label-free and high-throughput methods, have provided a framework to investigate bacterial behavior in antibiotic stress, including intrinsic and adaptive drug resistance. For instance, Liu and coworkers have recently analyzed the global proteome changes of methicillin resistant and methicillin sensitive *S. aureus* under Oxacillin stress, based on the spectral counting of MS spectra [76]. Remarkably, the authors used subinhibitory doses of the antibiotic to give the cells a chance to respond to the antibiotic and continue to grow, thus allowing proteome profiling in the context of relatively normal cellular processes. It was noted by the authors that higher doses would trigger massive disruptions in the cellular processes as the cells struggle to survive, which can potentially mask proteome changes directly related to antibiotic response, a point which should be considered when analyzing global genomic and proteomic profiling.

Lin *et al.* have combined multiple state of the art quantitative proteomic approaches (isobaric tags for relative and absolute quantitation and sequential windowed acquisition of all theoretical fragment ions) to obtain reliable results on fitness mechanisms of *Aeromonas hydrophila* under Oxytetracycline stress [77]. While this pathogen is more relevant in fishes and amphibians than in man, the approach is interesting since, as the authors state, the entire validation process only took hours instead of at least two months with the more laborious immunoassays. The authors focused on altered proteins with similar tendencies between both methods by using a very conservative set of identification criteria. They observed an enrichment on translation process (which is consistent with the 30S ribosome attacking function of Oxytetracycline) and decreasing abundance on glycolysis/ gluconeogenesis and TCA.

Hesketh and coworkers used high-res mass spectrometry-based proteomic analysis to examine the response to Vancomycin-induced cell wall stress in *Streptomyces coelicolor* A3 [78]. Noteworthy, fractionation of cell extracts into cytosolic and membrane protein components was implemented to contrast changes in the abundance of proteins closely associated with the cytoplasmic membrane to those taking place in the cytosol, thus maximizing the information obtained about the response (Vancomycin alters cell wall biosynthesis). As in previously reviewed reports, bacteria were exposed to a sublethal concentration of the antibiotic. The authors observed the induction of enzymes from the Vancomycin resistance cluster for the redirection of peptidoglycan precursor biosynthesis toward lipid II derivatives terminating in a D-Ala-D-Lac dipeptide in place of D-Ala-D-Ala, and upregulation of the CseBC two-component regulatory system involved in sensing and coordinating a response to cell envelope stress. The membrane-associated subproteome also showed evidence for membrane-specific changes in the abundance of a number of other enzymes required for pepti-

doglycan biosynthesis. Four penicillin-binding proteins involved in the extracellular cross-link formation stage of mature peptidoglycan formation showed a decrease in abundance in the membrane-associated fraction after Vancomycin addition, whereas enzymes for the intracellular biosynthesis of peptidoglycan precursors increased by a similar amount. This observation suggested a recruitment of the precursor synthesis enzymes to the intracellular face of the membrane to accompany the up-regulation in production of D-Ala-D-Lac lipid II derivatives directed by the van resistance cluster enzymes. This and other similar works [79] illustrate the value of transcriptomic and proteomic analysis to study specific cellular responses to antimicrobials; while the effects of Vancomycin on the cell wall synthesis has long been known, the reader can appreciate the role that these technologies could have in the development of new drug candidates with not fully understood mechanisms of action. See, for instance, the proteome analysis in methicillin-resistant *S. aureus* treated with the natural antibiotic candidate Rhodomyrtone [80].

The former examples illustrate how state-of-the-art (and ever expanding) omics tools can be used to assist, in an efficient manner, the important problem of target deconvolution, which could boost the revival of phenotypic-based drug screening and hopefully launch a new golden era of anti-infective drug discovery.

4. MULTI-TASK QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIPS (QSAR)

The basis of the QSAR theory is to infer, from a set of chemical objects, a quantitative generalization (a QSAR model) that might be then used to predict (bio) activity data for independent instances (objects that have not been used to train or calibrate the model).

Classic QSAR models were quite linked to target-driven drug discovery. First, the models were built from a series of homologous chemicals. It was usually stated that, especially in the case of 3D QSAR approaches, all training samples should share a common mechanism of action (and, ideally the same binding mode) [81-83]. It was claimed that 3D QSAR methods had been conceived to describe only one interaction step in the lifetime of ligands [81], a premise which partially supported by the fact that many 3D QSAR methods are highly alignment-dependent (the results depend on the position and orientation of the molecule representation in space).What is more, it was also said that only *in vitro* biological data should be considered, since a *in vivo* data reflect a number of parallel processes (transport, metabolism, binding to multiple targets) and b) by definition it is not possible to reach equilibrium in an *in vivo* system [81, 82].It is true that *in vitro* data is "cleaner" than *in vivo* data, in the sense that interpretation of the test result is more direct and less influenced by confounding factors; on the other hand, living systems undergo significant time-dependent changes. However, very frequently biological data emerging from phenotypic models (e.g. *in vivo o*r cellular models) are used to obtain QSAR models, and in spite of this the models achieve considerable descriptive and predictive ability (see, for example, [85-88]; note that there is an example in the field of anti-tuberculosis drug discovery). A common mechanism could be presumed when compounds of the same chemical series are being considered, but the modeler cannot be truly sure regarding the specific action mechanism explaining the phenotypic observation or the number or identity of the pharmacologically active chemical species. The complex nature of the biological response makes it impossible to describe, *a priori*, a well-defined action mechanism, or to discriminate the influence of other processes on the modeled activity (e.g. transport processes, bioactivation). Additionally, there is really no point in obtaining a model from a homologous series if it is intended to be applied in the virtual screening of large chemical repositories. And, finally, QSAR theory has greatly evolved in the last years with multitasking QSAR models being probably one of the most important steps in the evolution of this technique [89], and possibly the best argument against the requirement of using training examples with a well-defined and ideally single mechanism of action.

Multi-tasking QSAR can be defined as the prediction of multiple outputs with a single model and it is closely related to the more general term multi-tasking learning. Such single model can be used to predict, for example, several mechanisms of actions or activity against different microbial species to any drug. Notably, most of the multi-tasking QSAR models developed so far are based on conformation- and orientation-independent molecular descriptors (e.g. topological indices). Information relative to the class of property to be predicted (e.g. activity against a certain bacteria or strain, mechanism of action) can be introduced inside molecular descriptors, thus having different values for a given descriptor for the same molecule depending on, for instance, a specific response or a defined microorganism. González-Díaz and coworkers pioneered the application of this approach repeatedly, to construct pairs of antiparasite [90, 91], antifungal [92, 93], antiviral [94] and antibacterial [95, 96] drugs with multi-species predicted-activity profile, representing them as complex networks that cluster drugs according to their similarity on multi-species affinity profile.

Multi-task QSAR models thus rise as a valuable tool to assist the development of drug (and even protein-drug) networks and aid the development of in silico screening to detect novel broad spectrum multi-target anti-infective agents.

CONCLUSION

After a long (and scarcely productive) period of target-driven approaches, current anti-infective drug development has progressed to the integration of target- and phenotypic-based approximations. Though phenotypic and natural product screening dominated the Golden Age of antibiotic development, such approaches were progressively left aside in favor of the seemingly more rational target-based paradigm, which was, in addition, compatible with high-throughput screening technologies. However, technology has amazingly evolved and at present there exist high-throughput methodologies that allow phenotypic screening, natural product screening and faster target deconvolution, overcoming some of the previous limitations of phenotypic-based drug discovery. Even if target-focused drug discovery campaigns are being conducted, it is advisable to complement biochemical tests with more complex, cell-based screening. Whereas bio-

chemical tests can provide confirmation on the activity of the drug candidate on the intended target, cell-based assays are more reliable in terms of predicting therapeutic efficacy and, what is more, may provide clues on an unsuspected multi-target nature of the candidate and reveal unforeseen targets: frequently, a candidate selected through target-based screening might be more potent when tested against the whole pathogen than against the intended target. Whereas other explanations to such phenomenon could be possible (e.g. active influx transport) it should be kept in mind that most known drugs have some degree of non-selectivity.

Tailored multi-target agents, including hybrid molecules, allow expanding the target-focused approaches in line with a more modern system pharmacology perspective. Multi-task QSAR models may prove useful to assist the high-throughput *in silico* screening to identify multifunctional drug candidates. Although sometimes discouraged by some conservative QSAR modelers, the possibility of modeling phenotypic biological data instead of *in vitro* data might be also considered to identify, through *in silico* approaches, more efficacious drug candidates.

While many of these techniques have already been fully integrated in the field of anti-infective drug discovery of antibiotics, antiviral and antimalarial agents, their incorporation to the field of anti-infective discovery for neglected diseases is still meager.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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