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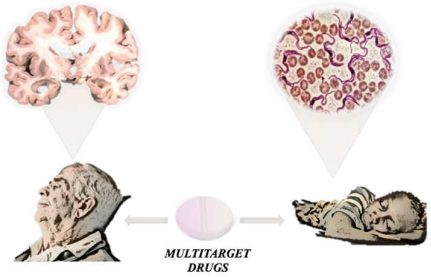
Multitarget drug discovery may represent a promising therapeutic approach to treat Alzheimer's and neglected tropical diseases.



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REVIEW

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Two diseases, one approach: multitarget drug discovery in Alzheimer's and neglected tropical diseases

F. Prati,[a, b] E. Uliassi[b] and M. L. Bolognesi[b]

In the past decade, scientific advances in network pharmacology have laid the foundations for a polypharmacological approach to discovering new drugs for complex diseases. There is now a comprehensive understanding that many incurable diseases are multifactorial in nature and, consequently, conventional drugs directed to a single molecular target are inadequate. To achieve a desired clinical outcome, a polypharmacological approach seeks to intervene in the diseased network using either combinations of multiple drugs or single small molecules modulating multiple targets. Both these approaches are equally feasible from a clinical standpoint. However, for various reasons which will be discussed in this review, the latter approach may be favoured for Alzheimer's disease (AD) and neglected tropical diseases (NTD). With each passing year, an increasing number of multitarget drugs and drug candidates are being identified, and several proof-of-concepts for treating these two diseases have emerged. Herein, with an awareness of the obstacles and challenges faced, we explore small molecules that seek to modulate multiple targets with the ultimate goal of harnessing network pharmacology for therapeutic applications in AD and NTD.

Introduction

The multitarget approach is fast becoming one of today's most fruitful drug discovery areas, particularly in developing medicines against major complex diseases.[1] For more than two decades, an ever-increasing number of papers and books[2, 3] have appeared in the literature, with multitarget drugs (MTDs) and drug candidates entering the fray.[4] This growth can be traced to an increased understanding of the biological complexity of major incurable diseases.[5] In the 1990s, the advent of systems biology illuminated drug discovery, revealing that pathologies such as neurological disorders, cancer, diabetes and infectious disease are unlikely to arise from a single gene/protein defect.[6, 7] Systems biology is a branch of biology whose holistic approach not only maps all the components (genes, proteins) in a biological system, but also reveals the functions of the components, such as their interrelationships. Indeed, proteins rarely function in isolation; rather, they operate as part of highly interconnected cellular networks known as *interactome networks*.[8] Building on systems biology, network pharmacology[9] has shown how complex diseases arise from alterations in multiple pathways of the *interactome networks*, which have evolved to be very robust and redundant.[10] Hence, they are relatively insensitive to perturbations, with modulation of individual components through single-targeted drugs having only a little functional consequence.[11] It thus follows that, to impact disease pathogenesis, interventions need to be multimodal, albeit highly selective too. In other words, the modulation of several drug targets through a well-concerted polypharmacological approach is necessary to achieve the desired therapeutic effect.[12]

After an initial predictable resistance, the pharmaceutical community has moved from a Mendelian perspective of certain diseases to steadily embracing a polypharmacology viewpoint.[13, 14] The *one-drug-one-target-one disease* paradigm was dominant in the post-genomic era, but has proven to be inadequate to address complex diseases. It has been appraised as a major cause of the current clinical failures and low productivity of the pharmaceutical industry.[15]

The ever-increasing acceptance of polypharmacological concepts can be seen in analysis recently performed by Lu et al.[16] They retrospectively and prospectively assessed network-based relationships between drugs approved by the USA Food and Drug Administration from January 2000 to December 2009 and their targets. They found that the average target number for each drug is 2.5. This is higher than the 1.8 reported by Yildirim[8] et al. in a similar study in 2006. Indeed, drugs acting on single targets appear to be the exception nowadays.[17]

1 Multitarget ligands – smart medicines for rich and poor

We have fostered the development of MTDs as the appropriate option for two diseases that conventional single-target drugs cannot cure: neurodegenerative diseases and neglected tropical diseases (NTDs). At first glance, drug discovery approaches for Alzheimer's disease (AD) and NTDs may appear antithetical. However, both AD and NTDs are major health problems and leading causes of death worldwide, with no drug available that can change the prognosis of these diseases or lead to a dramatic recovery. In this light, links in methodology and meaning become apparent.

As of 2013, there were an estimated 44.4 million people with dementia worldwide (46% in more developed regions), with a societal cost of US$604 billion annually (77% in more developed regions).[18,19] This will increase to an estimated 75.6 million in 2030, and 135.5 million in 2050.[19] In parallel with the rise in cases, drug research has accelerated noticeably. But despite huge investments and a strong commitment from industry and academia, no effective drug has emerged.[20] AD is currently one of the most frustrating areas of drug discovery. Since 2006, more than 200 AD drug candidates have failed in clinical trials.[21] Hence, the only drugs available for patients remain the four marketed acetylcholinesterase (AChE) inhibitors. These restore the cholinergic tone to achieve palliative effects during the initial stages of disease. They were joined in 2003 by the NMDA receptor antagonist memantine, whose neuroprotective activity is still questioned.[22]

NTDs are also a difficult area for drug discovery. In the new millennium, NTDs pose an even greater global problem than at the beginning of the last century. It is estimated that over one billion people are affected worldwide, primarily those living in the poorest and most remote areas of the planet.[23] Key challenges include many years of scarce resources and funding, as well as limitations peculiar to developing countries, such as stringent cost-of-good constraints, the lack of robust infrastructure to effectively deliver and administer drug therapy, and socioeconomic considerations in endemic areas.[24] Of the NTDs, sleeping sickness, Chagas disease, and visceral leishmaniasis are those with the highest rates of death.[25] For all three, vaccine research has long been a global priority. However, it has been impeded by some key technical hurdles.[26] As a consequence, chemotherapy seems to be the only therapeutic option for controlling infection, but none of the available drugs is effective. This is because they suffer from toxic side effects, lack of efficacy, and development of resistance. Novel strategies are desperately needed to defeat these diseases.[27]

Against this backdrop, we propose a polypharmacological approach. In particular, MTDs capable of modulating a network of AD- and NTD-related targets (Fig. 1)

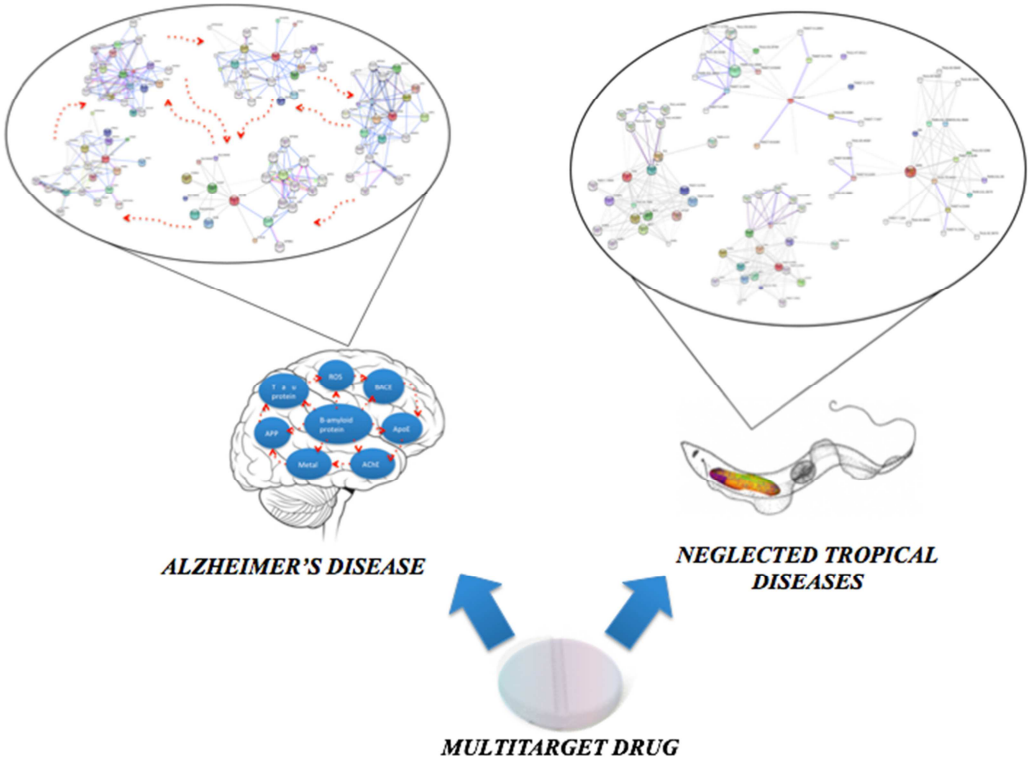

Fig. 1 Polypharmacological action of a multitarget drug. Such a molecule might be particularly suitable to modulate the *interactome networks* underlying Alzheimer's and neglected tropical diseases' pathogenesis. The interaction networks were obtained using the STRING database (http://string-db.org/).[28]

might be more efficacious, tolerable, and simpler than the single-target drugs available on the market.[29-32] Herein, we substantiate these concepts (at a conceptual and practical level) by providing selected examples taken from our recent research.

2 Polypharmacology: multitarget drugs vs drug combination

Polypharmacology can arise from one drug binding to multiple targets (i.e. MTDs) and from multiple drugs that bind to different targets (i.e. drug combinations).[33, 34] In principle, both approaches are equally feasible for reaching the desired outcome. Indeed, both have been pursued in academia and industry, and both are already used for the clinical treatment of cancer[35] and neurological diseases.[36-38] However, in a risk/benefit analysis, we believe MTDs may be superior to combinations, especially for AD and NTDs.[1]

An intrinsic risk of combinations is drug-drug interactions (DDIs). These are commonly caused by the inhibition or induction of the hepatic drug metabolism. Cytochrome P450-dependent monooxygenases are a large family of heme-containing enzymes that mediate the oxidative metabolism of endogenous and exogenous chemicals. Drugs that induce or inhibit CYP450 enzymes may decrease or increase, respectively, concentrations of co-administered drugs that are CYP450 substrates. Changes in drug concentrations resulting from DDIs can lead to treatment failures or toxicities. Combinations frequently cause concern to prescribers since DDIs in AD patients can be serious and even life-threatening.[39] Persons aged ≥65 use an estimated 4.5 prescription agents and 2 over-the-counter preparations per day,[40] and the number of concurrently used drugs is a significant predictor of adverse drug reactions.

Interactions are also known to occur in NTDs, although the pharmacovigilance data are quite scarce.[41] People suffering from these diseases are debilitated patients and young children, often with malnutrition or concomitant immunosuppressive diseases, thus particularly exposed to the risk of DDIs.[42]

Another inherent advantage of MTDs relates to the simplification of the therapeutic regimen. NTDs are most prominent in resource-poor settings, which require less staff-intensive therapies. Development of a more convenient treatment would greatly alleviate staff burden and, at the same time, will influence more patients to seek treatment. MTDs are also more manageable than combinations for forgetful AD patients and their caregivers.

Moving to drug discovery issues, the advantages of a standard approval process of an individual active pharmaceutical ingredient (API) compared to a combination cannot be ruled out. The prediction of pharmacodynamic and pharmacokinetic relationships should be substantially less complex when dealing with a single agent, rather than two or more. Similarly, manufacture and formulation should be easier with respect to a mixture. This may be the foremost advantage of MTDs in the field of poverty-related NTDs. It further reduces the potential for cost to be a barrier to access to therapy.[1]

Since the end of the 1990s, motivated by these considerations (outlined in Table 1), our medicinal chemistry efforts have sought to identify multitarget ligands for AD and NTDs. Working in academia, we exploited our insights and creativity to develop informative chemical probes. Although removed from real clinical application, we hope that our efforts will translate into innovative research, inspiring medicines that ultimately benefit patients.

Table 1 Pro and cons of drug combinations vs. multitarget drugs.

	Drug Combinations	vs.	Multitarget Drugs
Drug-drug interaction	✗		✔
Regimen simplification	✗		✔
Patient compliance	✗		✔
Pharmacodynamics and pharmacokinetics prediction	✗		✔

3 Multitarget approach vs. Alzheimer's disease

3.1 Bivalent ligand approach and dimebon-based bivalent compounds in AD

The bivalent ligand strategy is a viable approach for using purposely designed small molecules to effectively target a wide range of therapeutically relevant proteins.[43] It has emerged as particularly promising in an AD multitarget drug discovery context.[44, 45] Bivalent compounds - small molecules consisting of two identical pharmacophores joined via an appropriate spacer - have exhibited an improved biological profile with respect to the corresponding monovalent counterparts (Fig. 2).[43,46]

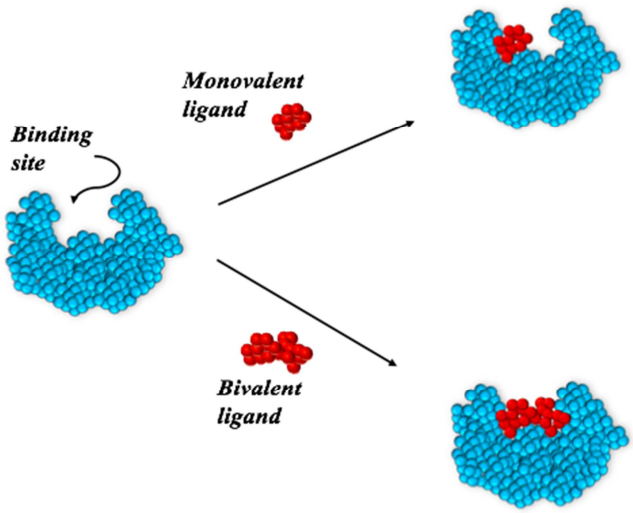

Fig. 2 Mechanism of interaction of a monovalent and a bivalent ligand.

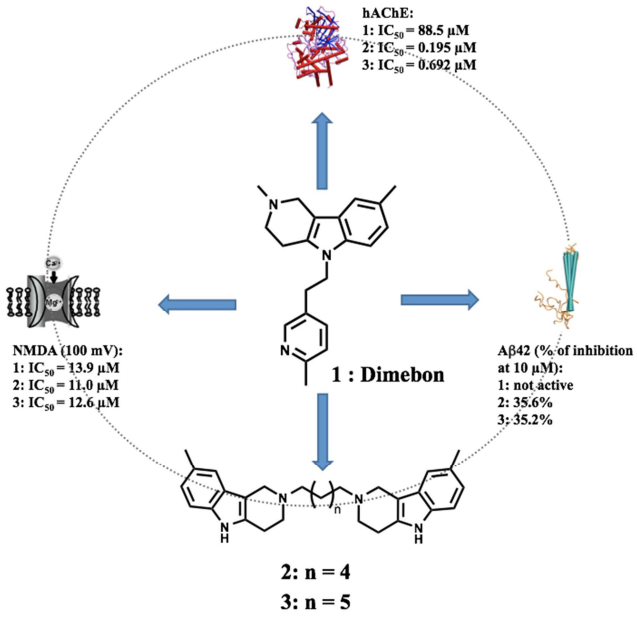

Fig. 3 Design strategy and multitarget activities of **1-3**.

Indeed, it is conceivable that molecular duplication could increase the binding affinity of monovalent ligands to a relevant target. Furthermore, the improved potency observed for such bivalent compounds is even higher than that of the sum of the two single pharmacophores, suggesting a synergic effect.[46]

In the AD field, this approach has inspired the design of numerous drug candidates over the past decade, motivated by the peculiarity of the molecular architecture of a prototypical AD target, the enzyme AChE.[47-50] The molecular similarity between the AChE catalytic (CAS) and peripheral anionic (PAS) sites has been exploited to develop bivalent AChE inhibitors that contact the two recognition sites simultaneously, resulting in inhibitors with markedly improved potency.[51] Furthermore, it has recently emerged that bivalent ligands may link independent recognition sites on other AD-validated targets, such as amyloid-β (Aβ) peptides.[52, 53] In fact, several amyloid-binding compounds share a common bivalent structure, and the concept of "bivalent tweezers" has been particularly fruitful in understanding the amyloid structure.[53] Considering the oligomeric and repetitive features of fibrillar aggregates, a bivalent molecule can interact simultaneously with two binding surfaces, achieving higher potency and significantly enhancing the recognition process, avidity, and selectivity.[43, 54, 55]

We recently exploited the bivalent ligand approach as a tool for improving the in vitro anti-AD multitarget profile of dimebon. Dimebon (latrepiridine) (**1** in Fig. 3) is an old antihistamine drug. In the last few years, it has been proposed for the treatment of neurodegenerative disorders such as AD and

Despite its conflicting story, dimebon may still be considered an interesting starting point in pursuing a multitarget design strategy. Accordingly, in 2013 a new series of dimebon-based bivalent derivatives was obtained by connecting the γ-carboline moieties of **1** with variable-length polymethylene spacers and heteroatom or aromatic linkers, in an attempt to enhance the Huntington's diseases, and more recently schizophrenia.[56] In 2008, it attracted considerable interest within the AD community, following the completion of a small six-month clinical trial in Russia, in which it showed impressive cognition-enhancing effects in patients suffering from mild to moderate AD.[57, 58] However, dimebon showed heterogeneous results in further replication trails,[57-59] and, although it seemed to generally improve cognitive scores, it failed to exert a significant beneficial effect. Initially, dimebon's mechanism of action was unknown, but it seemed to fulfil the promise of an effective multitarget drug for treating AD.[60] It has been proposed that it may modulate several crucial AD targets, including voltage-gate Ca^{+2} channels, mitochondrial permeability transition pore, and several neurotransmitter receptors and enzymes. In particular, early research suggested that the therapeutic benefits of dimebon resulted from the combination of cholinesterase inhibition with the modulation of both the NMDA receptor and mitochondrial permeability transition pore, producing a synergic effect of remarkable clinical efficacy.[61] However, this hypothesis has been potentially disproved by studies from Giorgetti et al., demonstrating that dimebon brain concentration, after acute oral administration to rats, is too low to significantly affect AChE or NMDA pathways (nanomolar vs. micromolar).[62] Although exposure levels of **1** in plasma and cerebrospinal fluids have not been reported, it is likely that the same situation could be replicated in humans.[63] cholinesterase and NMDAR-blocking activities.[64] The dimebon congeners resulted in new multitarget compounds with a markedly improved in vitro biological profile.

Derivatives **2** and **3** (Fig. 3), with sub-micromolar activity against AChE (IC_{50} = 0.19 and 0.69 μM, respectively), were 454- and 128-fold more potent than the parent compound, and

also more active than the marketed drugs galantamine (IC_{50} = 2.01 µm)[65] and rivastigmine (IC_{50} = 3.03 µM).[66] The molecular duplication increased inhibitory potency against AChE, but had no negative effect on molecular recognition at NMDARs, providing inhibitory profiles close to that of the reference drug memantine. More importantly, the bivalent strategy allowed **1** to be transformed from an ineffective compound against in vitro amyloid aggregation into a series of effective inhibitors. This reinforces the concept that bivalent ligands may be effective tools in amyloid recognition. These positive results support further evaluation of the bivalent ligand approach in multitarget drug discovery, and serve as the basis for developing new dimebon-based bivalent ligands that could yield more consistent clinical benefits.

3.2 Memoquin-like quinones bearing non-steroidal anti-inflammatory fragments in AD

Memoquin (**4** in Fig. 4), one of the first rationally designed multitarget drug candidates against AD,[67] presents a remarkable in vitro profile, including a free-radical scavenger action and inhibition of Aβ aggregation and AChE activity. It also acts in several AD mouse models as an effective cognitive enhancer.[68, 69]

Building on **4**, a new series of conjugates bearing memoquin-like quinones combined with non-steroidal anti-inflammatory fragments, have recently been reported as second-generation multitarget ligands for AD.[70]

The 2,5-diamino-benzoquinone scaffold of **4** has been deemed to have a crucial role in conferring the multiple activities, particularly inhibition of amyloidogenic protein aggregation (Fig. 5).[71-75] This hypothesis is further supported by the anti-aggregating capability displayed by several hybrid molecules featuring a 2,5-diamino-1,4-benzoquinone core connecting two aromatic appending moieties.[76-80]

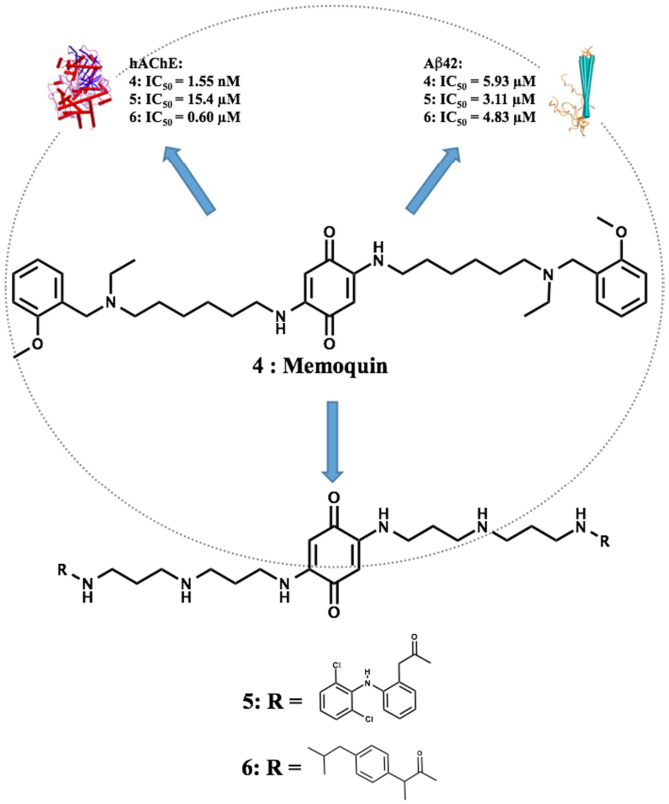

Fig. 4 Design strategy and multitarget activities of **4**-**6**.

The 2,5-diamino-1,4-benzoquinone fragment can thus be considered a truly privileged motif for interfering with protein-protein interactions and a useful starting point for designing novel multitarget ligands against AD. Accordingly, a new series of hybrids was designed and synthesized by connecting the 2,5-diamino-benzoquinone core with several non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen, sulindac, indomethacin, diclofenac, and flurbiprofen, through appropriate polyamine linkers. This specific NSAID subset was chosen because it directly inhibits Aβ fibril formation, destabilizes preformed Aβ fibrils,[81] and affects the production of Aβ.[82] Furthermore, epidemiological studies have indicated that NSAIDs may lower the risk of developing AD.[83]

The most striking result was that all quinone-NSAID conjugates inhibited Aβ aggregation in the low micromolar range. In agreement with the anti-aggregating properties reported for some NSAIDs,[81] the presence of the anti-inflammatory fragments led to an inhibitory potency similar to that of the parent compound **4**. Notably, the diclofenac hybrid **5**

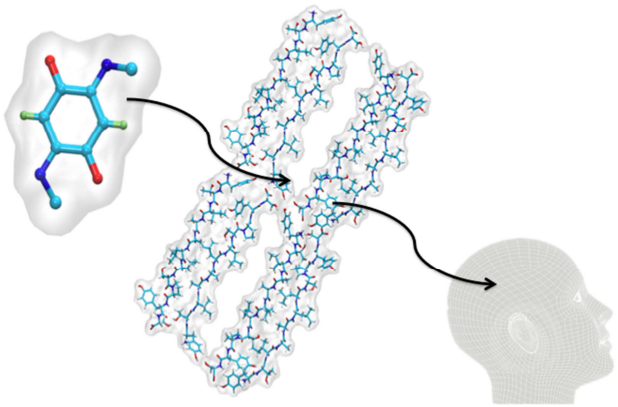

Fig. 5 Schematic interaction between Aβ42 and the 2,5-diaminobenzoquinone scaffold. Due to the possible hydrogen bond and π-stacking interactions, it can be considered an anti-aggregating privileged motif.

(Fig. 4), with a remarkable IC_{50} of 3.15 μM, was not only the most active compound of the current series, but also the top-ranked among all the **4** derivatives tested so far.[65, 76, 80, 84]
Additionally, the current series retained the cholinesterase inhibitory activity, which nicely complements the anti-amyloid one. The new hybrids are 3-fold less potent than **4**, but still retain good inhibitory potencies in the micromolar range against human AChE (hAChE) and human BuChE (hBuChE). Notably, the ibuprofen derivative **6** (IC_{50} = 0.60 μM) was the most active of the series, being more potent than the marketed drugs galantamine [65] and rivastigmine.[66]
Thanks to a balanced micromolar Aβ/cholinesterase profile, these quinone-NSAIDs hybrids could be a promising starting point in the search for new multitarget ligands against AD.

4 Multitarget approach vs. Neglected tropical diseases

4.1 Target-based and phenotypic approaches for NTDs

Nowadays, both target-based and phenotypic approaches are widely used in NTD drug discovery.[85]
The two approaches are not mutually exclusive, but should rather be viewed as complementary.[86] As reported below, the integrated combination of both phenotypic and target-based approaches was particularly productive when searching for multitarget ligands.[86]
To identify multitarget hit compounds, natural products are attractive for several reasons. First, it is recognised that natural product fragments offer evolutionarily selected and biologically pre-validated frameworks with high hit rates for compound collection development.[87, 88] Second, due to their mode of generation, natural products are intrinsically able to bind to multiple targets.[89] This is because they have a complex structure and their synthesis involves a range of enzymes, each of which has distinct architectures and molecule-binding cavities. The synthesised molecule must be able to interact with all of these.[89] Third, natural products serve plants and animals as potent defence chemicals with an intrinsic pleiotropic mechanism of action.[90]
In natural product chemical space, naphthoquinone and other related quinone derivatives have been reported as one of the major classes with significant activity against Trypanosoma and Leishmania parasites.[91-93] For instance, lapachol (2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone), obtained from *Tabebuia avellanedae*, exhibits a marked anti-trypanosomal activity, while displaying a good safety profile.[94] In view of the well-known biological properties of this compound class, it is highly conceivable that naphthoquinones exert their trypanocidal activity by means of a multitarget mechanism.
We have reported a focused library of 16 natural-inspired 1,4-naphthoquinone and 1,4-anthraquinone derivatives, showing a promising anti-trypanosomatid profile in a phenotypic screening.[95] The lead of this series, 2-phenoxy-1,4-naphthoquinone (**7**, B6 in Fig. 6), showed an ED_{50} of 80 nM against *Trypanosoma brucei rhodesiense*. Its selectivity index (ratio of the compound's ED_{50} values on mammalian cell lines and trypanosomes) was 74, which is very close to the specifications required by WHO/TDR to be considered an anti-trypanosomatid hit.[96] The putative molecular target(s) of B6, initially undisclosed, were subsequently fished out from trypanosomal cell lysates, by means of chemical proteomics.[97]
Two potential targets of B6 have been identified, namely glycosomal glycerol kinase (TbGK) and glycosomal glyceraldehyde-3-phosphate dehydrogenase (TbGAPDH). In biochemical experiments, B6 inhibited both enzymes with IC_{50} values in the micromolar range.[97] GK may be considered a sub-optimal drug target because it does not play an essential role in the trypanosome's metabolism under most physiological conditions. However, GAPDH is a vital parasitic enzyme and a well-validated molecular target for antiparasitic drug discovery.[98] Accordingly, evidence from several experiments suggests a GAPDH covalent inhibition, probably through a cysteine trapping mechanism, which is still to be confirmed by ongoing studies. To fully account for the nanomolar efficacy of B6, and considering that chemical proteomics is not suitable for identifying non-protein targets, other mechanisms needed to be considered.[97] Based on a vast literature reporting on the general properties of quinones and naphthoquinones to generate free radicals and interact with the mitochondrial respiratory chain,[99-102] we evaluated oxygen consumption in permeabilized trypanosomes and production of reactive oxygen species (ROS) in trypanosome mitochondrial cell fractions. We thus demonstrated that B6 also interfered with the respiratory chain by generating ROS, supporting the likelihood that B6 interacts with additional targets located in *T. brucei*'s mitochondrion. [97]
Although the phenotypic approach is useful and might limit the attrition rate in NTD drug discovery, we found that the subsequent target deconvolution step is not so straightforward. However, identifying the molecular targets involved is crucial

for understanding the underlying mechanisms and further optimizing the identified active compounds.[103]

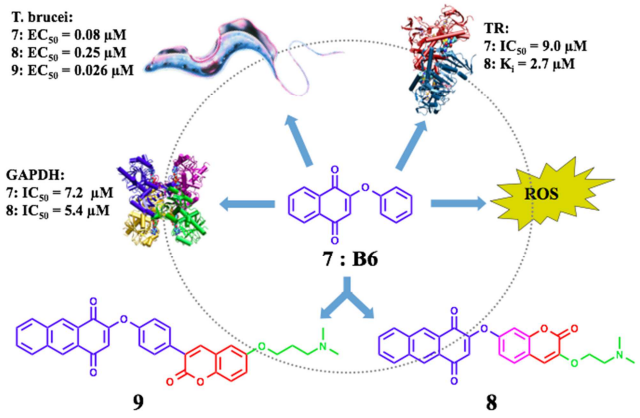

Fig. 6 Design strategy and multitarget activities of **7**-**9**.

Thus, in parallel to the phenotypic approach we also used hypothesis-driven target-based design to develop dual-targeted inhibitors of trypanosomatid enzymes.[104] Of particular interest were inhibitors directed towards GAPDH and trypanothione reductase (TR), another promising NTDs drug target.[105, 106] We took B6 as a suitable starting point because of its ability to inhibit GAPDH and TR in the micromolar range. For this, we used a framework combination approach, which is frequently exploited in multitarget drug design.[107, 108] The 2-phenoxyquinone structure of B6 was combined, through merging and fusing strategies,[109] with an aminoalkoxy coumarin scaffold. We selected the coumarin core, contained in chalepin, a natural product GAPDH inhibitor (IC_{50} 64 µM),[110] to potentially enhance the anti-GAPDH profile of B6, with the amino tail theoretically allowing selectivity for TR/hGR (the human counterpart, glutathione reductase). Thus, we sought to exploit the structural differences between the two enzymes.[111] Of the merged derivative, **8** (Fig. 6) showed an IC_{50} value of 5.4 µM against TbGAPDH and a concomitant Ki of 2.7 µM against TR. This molecule is the first derivative ever reported that shows a truly dual profile against enzymes from trypanosomatid pathways. However, when tested against the whole parasites, the in vivo data did not mirror the enzymatic inhibitory potencies. This suggests that other targets might be involved in the mechanism of action. Despite this, compound **9** (Fig. 6) belonging to the fused series displayed a remarkable EC_{50} value for *T. brucei* parasites (0.026 µM) combined with a very low cytotoxicity towards mammalian L6 cells (7.95 µM). This promising low toxicity might be because it does not interfere with hGR and has a low propensity to act as a subversive substrate against this enzyme. Collectively, these results point to multitarget drug discovery being an extremely complex task. However, the potentially high rewards of MTDs may make their development a risk worth taking.

Conclusions

If the positive features of MTDs are over-emphasised, they may be seen as a panacea. This would be rather naive and overoptimistic, given the various challenges and failures already experienced in MTD development.

Although seductive, there is much about the MTD concept that is problematic, and the rush to use 'MTD ideas' has run ahead of many fundamental conceptual, theoretical, and practical questions.

From a systems biology perspective, the major drawback is that we only partially know the pathways/mechanisms of many diseases at the molecular level. It is exceedingly difficult to derive a full polypharmacological network without the complete data.[14]

From a medicinal chemistry perspective, this area still represents a major challenge, despite the tremendous recognized potential.[112] Lead identification and optimization are a central and mainly unsolved problem in MTDD. It is unclear how to optimize a compound series for multiple targets. In most cases, we do not even know how much potency at each single target is required and what the desired balance is between the potencies. We have only just begun to develop the concepts and methodologies that can address the varied nature of these problems.[108, 113]

How to overcome the challenges that lie ahead and avoid the risk of stagnation in MTDD?

One proven route to the discovery of new drugs is innovation through synergistic industrial-academic collaborations.[114] With increased interaction, mutual understanding, and respect for the respective priorities, it should be possible to further bridge the gap and translate the large public investment in basic sciences into drugs that can effectively cure these and other devastating diseases.

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Notes and references

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