Chapter 2

Literature Review

2.1 The cholinesterase enzymes

Cholinergic neurotransmission plays a key role in impaired cognitive function in AD and adult-onset dementia disorders. Treatments to counter amyloid- accumulation, tau hyperphosphorylation, and immunotherapy have failed to provide effects. Cholinesterase inhibitors (ChE-Is) are the first drugs approved for symptomatic treatment of AD. The drugs are classified as nonspecific when these inhibit AChE, BChE, and other cholinesterases and specific when they inhibit AChE only [29, 30].

AChE enzyme comprises of three amino acid residues of Ser200, His440, and Glu327 which make up the catalytic triad, housed at the bottom of a narrow and deep gorge (approximately 20 Å long and 4.5 Å narrow), lined by 14 aromatic residues (such as decamethonium) [31]. In addition, the active site features a subsite (the "anionic subsite") near the bottom of the cavity that includes Trp84 as a crucial residue for the contact with the quaternary ammonium group of the substrate acetylcholine and other ligands via cation-pi interaction. This interaction also involves the conserved aromatic residue Phe330[32].

ChE-Is activity is characterized by the inhibition of AChE, allowing to prolong the action of the deficient neurotransmitter in the brain. Three compounds belong to this therapeutic class, includes donepezil, rivastigmine, and galantamine[33]. The benefits of ChE-I therapy are modest, with a mean effect size of 1.08, 1.0, and 1.10 points on the MMSE. DOMINO-AD trial found that discontinuation increased the probability of nursing home placement within the first year. AChE-I donepezil maintains efficacy in severe dementia[34]. Studies have found a small but significant benefit in favor of AChE-I therapy, but there is no evidence that ChE-Is have a clinically meaningful disease modifying activity[29]. Further, combining ChE-Is with other classes of drugs may represent a renewed interest in the treatment of adult-onset dementia disorders.

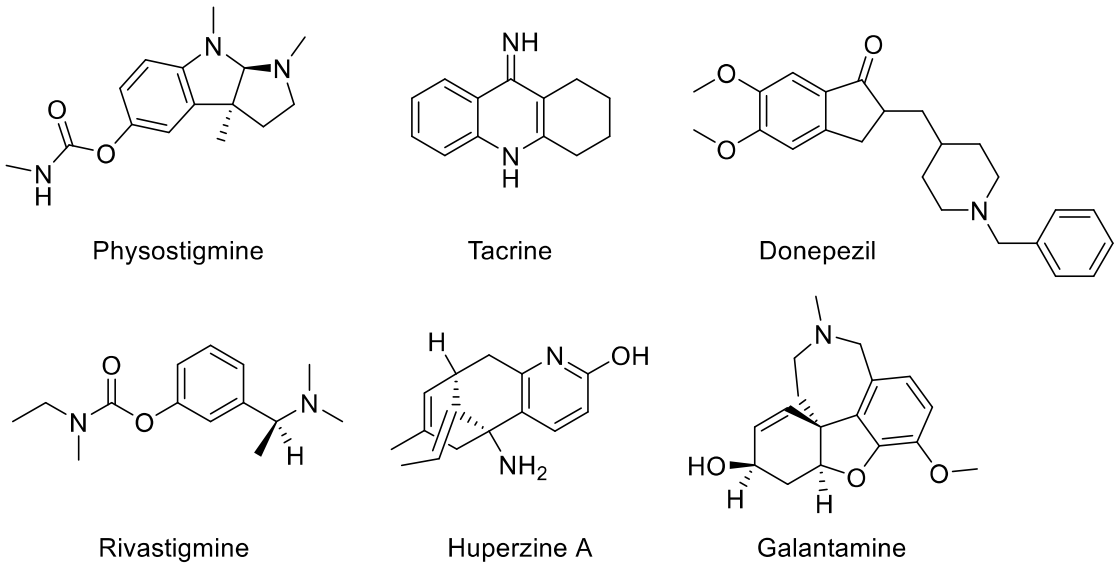


Figure 2.1 Popular drugs from the Cholinesterase inhibitor class

2.2 Matrix Metalloproteinases

Structurally related calcium and zinc-dependent endopeptidases are known as matrix metalloproteinases. These are also sometimes referred as metzincins, because they interact with the Zinc ion of the conserved methionine (Met) residue in the active site[35]. MMPs participate in intercellular and extracellular activities as well as the usual physiological processes and are essential for cell signalling pathways in a variety of conditions, including inflammation, angiogenesis, bone resorption, angiogenesis, apoptosis, and wound healing. The breakdown of extracellular matrix is facilitated by at least 25 MMPs, which include membrane type-MMPs (MT-MMPs), gelatinases, stromelysins, collagenases, and others. The proinflammatory cytokines TNF-, IFN-, IL-17, chemokines, etc. can all trigger MMPs, like many other proteases[36].

MMP-2 and MMP-9 show high order of structural and functional similarities among all the MMPs. The structures of most of the MMP family members consist of a signal peptide, propeptide domain, a catalytic domain, hinge region and PEX domain. Some of the MMPs also contain a transmembrane or FN domain. MMP-9 or gelatinase B has 707 amino acids (AAs)

and gets activated in two sequential steps. In the first step, protease disrupts the thiol interaction of Cys99 with Zn+2 active site and thus opening the second site at Arg106/Phe107 in the subsequent steps[37].

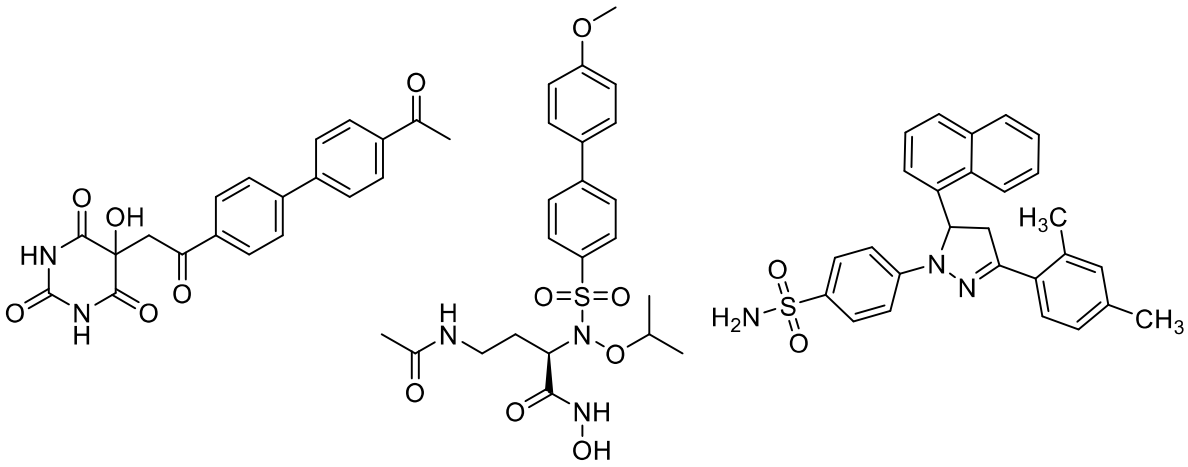
MMP inhibitors (MMPIs) are mostly nonselective due to the high structural similarities among the members. Therefore, designing of specific MMP-9 inhibitors is very challenging and requires detailed study of substrate interactions. Although, MMP-2 and MMP-9 have structural similarities, selectivity can still be achieved via targeting the S2 subsite of MMP-9 that consists of Asp, whereas, MMP-2 has Glu in the region. Many of the angiotensin-converting enzyme (ACE) inhibitors also have the ability to inhibit MMP-9[38].

Most of the MMPIs are nonselective and therefore, suffer with various side effects to overcome this. The specific substrate interactions are required to be studied at length. Initially, most MMPIs were designed by mimicking the MMP substrate structure with a Zn chelating group. Later on, various nonpeptidic molecules were designed and evaluated for their potential to inhibit MMPs. Most of the MMPIs share the common feature of having a Zn-binding group (ZBG). Significant efforts have been made to design inhibitors having specific interactions with S1′ pocket and with loop connecting the outside wall of S1′ pocket along with the ZBGs, as S1′ pocket confers selectivity among different MMPs[39]. The S1′ pocket is most prominent, less solvent exposed and thus becomes very attractive site for drug targeting. The unprimed site (S) has not been extensively explored due to its less segregation and more solvent exposed properties. It has been shown that MMP-2 and MMP-9 have structural similarities but selectivity can be achieved by targeting the S2 subsite, as MMP-9 has Asp, while MMP-2 has Glu in this region [40].

Some of the common MMP inhibitors include hydroxamate acid based retro hydroxamate, carboxylic acid bases, phosphorus based, thiol-based, thiirane based, 6-H 1,3,4 thiadiazine scaffold and 5,5-disubstitutedpyrimidine-2,4,6-triones.

Nicolotti et al., synthesized 5-hydroxy, 5-substituted pyrimidine-2, 4, 6 triones and evaluated their MMP-2 and MMP-9 inhibiting activities. The biphenyl part of the molecule appended at the fifth position via ketomethylene linker, showed favorable interaction with S1′ pocket of the enzyme subsite. In particular, biphenyl derivative bearing OCF3 (**Compound 7**) substituents at the para position inhibited MMP-2 and MMP-9, with IC_{50} values as low as 143, 21 nM and 30, 170 nM, respectively [41].

The N-O-isopropyl sulfonamido-based hydroxamates showed improved activity toward MMP-2, MMP-9 and MMP-14 with **Compound 8** displayed subnanomolar inhibitory activity against MMP-2[42]. Furthur, a set of compounds were designed by incorporating dihydropyrazole and sulphonamide with **Compound 9** showing potent inhibitory activity due to its ability to make two H-bonds with Leu387, one H-bond with Leu418 and coordination of N atom with Zn [14].



7	**8**	**9**
IC_{50} = 30 ± 3 nm for MMP-2; 170 ± 30 nm for MMP-9	IC_{50} = 0.13 nm for MMP-2; 2 nm for MMP-9	IC_{50} = 0.21 um for MMP-2; 1.87um for MMP-9

Figure 2.2 MMP-2 Inhibitors with activities in terms of IC50

2.3 BACE-1

The β-site amyloid-precursor-protein-cleaving enzyme 1 (BACE-1), an aspartic protease that cleaves the amyloid precursor protein (APP) at the β-secretase site, is a preferred Aβ based

target of AD. The three most recent BACE-1 inhibitors moving through the trial pipeline are JNJ-54861911, CNP520, and LY3202626, whereas verubecestat (MK-8931) is the most advanced BACE-1 inhibitor in clinical development.

BACE-1 is a monomeric protein with a bilobal structure, which accommodates the catalytic aspartic dyad (Asp 32 and Asp 228) between the N- and C-terminal domains. A flap, also known as β-hairpin loop, which makes up a sizable and flexible component of the binding pocket, protects the rather large active site. The intial BACE-1 inhibitors to be reported were peptidomimetics. These displayed low pharmacokinetic (PK) properties despite having high *in vitro* potency, which often rendered them ineffective for treating AD[43].

The identification of 2-amino heterocycles that interact with both Asp moieties in the BACE-1 active site was a significant development in the design of non-peptide BACE-1 inhibitors. The ligands enabled a superior physical-chemical profile, increased brain penetrance, and improved *in vivo* efficacy, when compared to the acylguanidine inhibitors. In this vein, Merck developed the aminohydantoin BACE-1 inhibitor which was effective in the enzymatic assay (Ki = 22 nM), yet Pgp efflux caused a significant cell shift when it was evaluated in the whole-cell experiment (cell IC_{50} = 258 nM). Additionally, it had a limited oral bioavailability in CRND8 mice and decreased plasma but not cerebral A40 levels[44].

A promising investigation by Costanzo et al. using a number of donepezil compounds as dual AChE/BACE-1 inhibitors was reported. The scientists examined the novel compounds for selectivity against AChE (against BChE) in addition to reporting a sustainable multistep methodology for the synthesis of a number of donepezil derivatives. The group also examined these compounds for BACE-1 inhibition because donepezil only has weak anti-BACE-1 action. **Compound 10** had a promising inhibitory profile against BACE-1 (less than 20% of residual enzymatic activity at 1 nM dose), although being significantly less effective than the parent

molecule against AChE (Ki = 29 nM versus 10 nM of donepezil). In fact, the substance might be a potential option for creating AD-treating AChE/BACE-1 multitarget inhibitors [45].

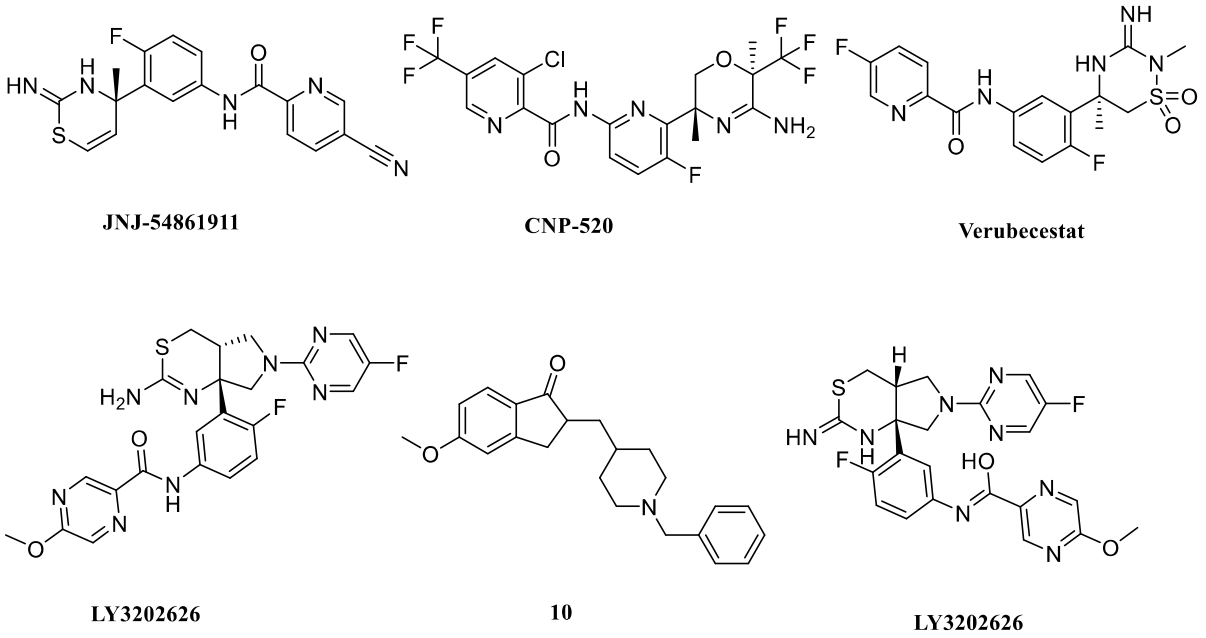


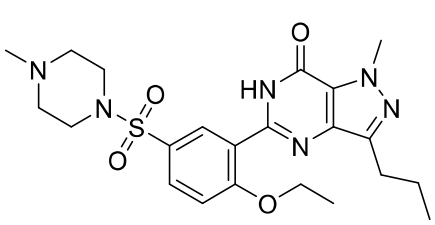
Figure 2.3 Popular BACE-1 inhibitors in the drug discovery pipeline

2.4 PDE-9A

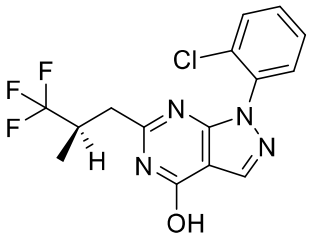
The superenzyme family known as phosphodiesterases are involved in the hydrolysis of the two second messengers, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). There are 11 distinct subtypes within the PDE superfamily, which is encoded by 21 identified genes (PDE1–11). Among these subtypes, PDEs 4, 7, and 8 specifically hydrolyze cAMP, whereas PDEs 5, 6, and 9 specifically hydrolyze cGMP[46].

PDE inhibitors are used to regulate biological processes by increasing cellular levels of cAMP and cGMP. Sildenafil, a PDE5 inhibitor is a successful example of this drug class, used for the treatment of male erectile dysfunction (Viagra) and pulmonary hypertension (Revatio). Impaired CREB phosphorylation plays an important role in neurodegenerative disorders, especially AD [47, 48].

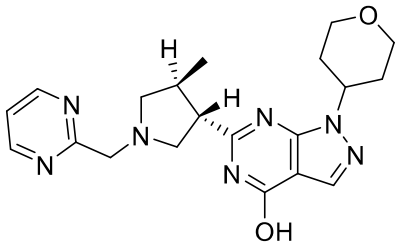
PDE9 subfamily hydrolyzes cGMP, with highest levels in the CNS and bladder. Bayer's BAY 73-6691 is a selective PDE9 inhibitor with an IC_{50} of 55 nM and moderate selectivity over PDE1. It has been used to investigate the underlying mechanism of PDE9 inhibition in AD. In APP transgenic Tg2576 mice, the compound restored A_{42} oligomer-induced LTP and improved memory performance [49]. Similarly, PF-04447943 is a highly selective and brain penetrant PDE9A inhibitor that reverses scopolamine-induced deficits, improves cognitive performance, and regulates dendritic spine density of hippocampal neurons [50].



Sildenafil



BAY 73-6691



PF-04447943

Figure 2.4 Popular PDE inhibitor drugs

2.5 Multi-target directed ligands (MTDLs)

It is increasingly being observed that superior therapeutic efficacy and side effect profile as compared to that of a selective ligand, could be achieved through a balanced modulation of various targets. Several ligands that span a wide range of targets and target classes have been invented through the utilization of rational approaches incorporating structural features of the selective ligands. Finding a balanced activity at each target of interest while also obtaining a

broader selectivity and an appropriate pharmacokinetic profile is a major bottleneck in the design of such MTDLs [51].

Treatment of complex disorders like AD seems to benefit the most from such an approach. Caproctamine, an antagonist of the presynaptic muscarinic acetylcholine M2 autoreceptors and an AChE inhibitor was one of the earliest examples of purposefully designed MTDL displaying synergistic cholinergic activity against AD [52]. The strategy has seen gradual yet tremendous application in AD drug disovery research during the past two decades [53].

2.5.1 Development of MDTLs

Either additive or synergistic therapeutic effects are the desired outcomes of a successful MDTL design. This is achieved through the combined targeting of individual, intricately selected therapeutic targets in a simultaneous fashion. Target selection is, by far, the most crucial aspect in the drug design and development of MTDLs.

2.5.2 Rational combination of multiple targets for MTDLS

Designing multitarget ligands for closely similar targets within the same superfamily is typically simpler. Targets must have comparable or even identical endogenous ligands if these are members of separate superfamilies of targets (often a monoamine or an eicosanoid). If so, it is more likely that the binding sites of several targets will accept a common ligand frame[54].

A very good example of MTDLs designed according to the clinical observations based target combination include antipsychotic drugs which target antiserotonergic 5-HT_{2A} in addition to their original targets i.e., Dopamine D_2-like receptors, whereby these displayed improved efficacy in terms of both potency and reduced adverse effects [55].

MTDLs designed according to the target combination based on phenotypic screening apply a different approach whereby cellular, tissue, and animal models are utilized for screening large sets of compound combinations to arrive at possible synergistic combinations. Since the high

throughput approach employs large animal sizes it was deemed feasible to utilize genetic knockdown or knockout of one target to reduce animal usage. This is exemplified in the study which used FAAH (−/−) in an inflammatory mice model to screen for synergistic combinations with the COX inhibitor diclofenac.

A third approach is the target combination based on *in silico* technique where methods such as machine learning and network pharmacology are utilized for screening suitable target combinations. Subsequent wet-lab validation is always essential to establish the biological feasibility of the approach viz. in case of target combination of IGF1R(insulin-like growth factor 1 receptor) with that of CDK4 (cyclin-dependent kinase 4) for the treatment of dedifferentiated liposarcoma (DDL), validated for synergistic activity in DDL-derived cells[54].

2.5.3 MTDL lead generation

MTDL structure is said to have been designed using a "framework combination", when targets are chosen utilising the "knowledge-based design". In this compound is designed by combining two (or more) pharmacophoric structural scaffolds, each of which is known to be active against a particular target. The MTDL structure may be split into three subgroups based on their design, where the two domains are either linked together (linked), directly coupled (fused), or have more or less overlapping structures (merged)[56].

- **Knowledge-Based Approach**

Also known as the pharmacophore-based approach, predominant approach towards MTDL generation. Selective ligand pharmacophores of multiple targets are combined into a single compound through this approach. There are several methods to achieve this i.e. linked, fused and merged integrations.

a. ***Linked Pharmacophores*** : The pharamcophoric moieties in this type of MTDL are separated by a linker which isn't native to either of the original ligands. The composition, position, and length of the linkers can be varied according to the expected pharmacophoric activity. Steric effect and other molecular interactions are taken into consideration during the design of compounds. Further, cleavable or non-cleavable type of linkers can be design based on the bioavailability requirements. Example of the cleavable linked MTDL is the **Compound 3**, a new anti-inflammatory analgesic candidate which combines the pharmacophores of cinnamic acid (LOX inhibitor) and paracetamol 2 (NSAID) via an ester linker, showing 91% improvement in the analgesic activity[57].

b. ***Fused Pharmacophores*****:** Partial overlap of pharmacophoric features can be seen in this type of MTDLs. Example of this category include **Compound 9**, formed by the fusion of the phenyl ring of Rivastigmine to that of the dihydroindene ring of rasagiline thereby exhibiting simultaneous activity against both AChE as well as monoamine oxidases (MAOs)[58].

c. ***Merged Pharmacophores*** **:** Highest level of pharmacophore overlap via identification of the so called " tolerant region" for each receptor along with the common pharmacophoric features of the ligands is accomplished to design highly optimized small molecular weight compounds. HLA20 was integrated with two of the FDA approved drugs i.e., Rivastigmine and Donepezil resulting in a merged scaffold showing cytotoxicity lower than that of HLA 20 and efficient inhibition of BChE and AChE [54].

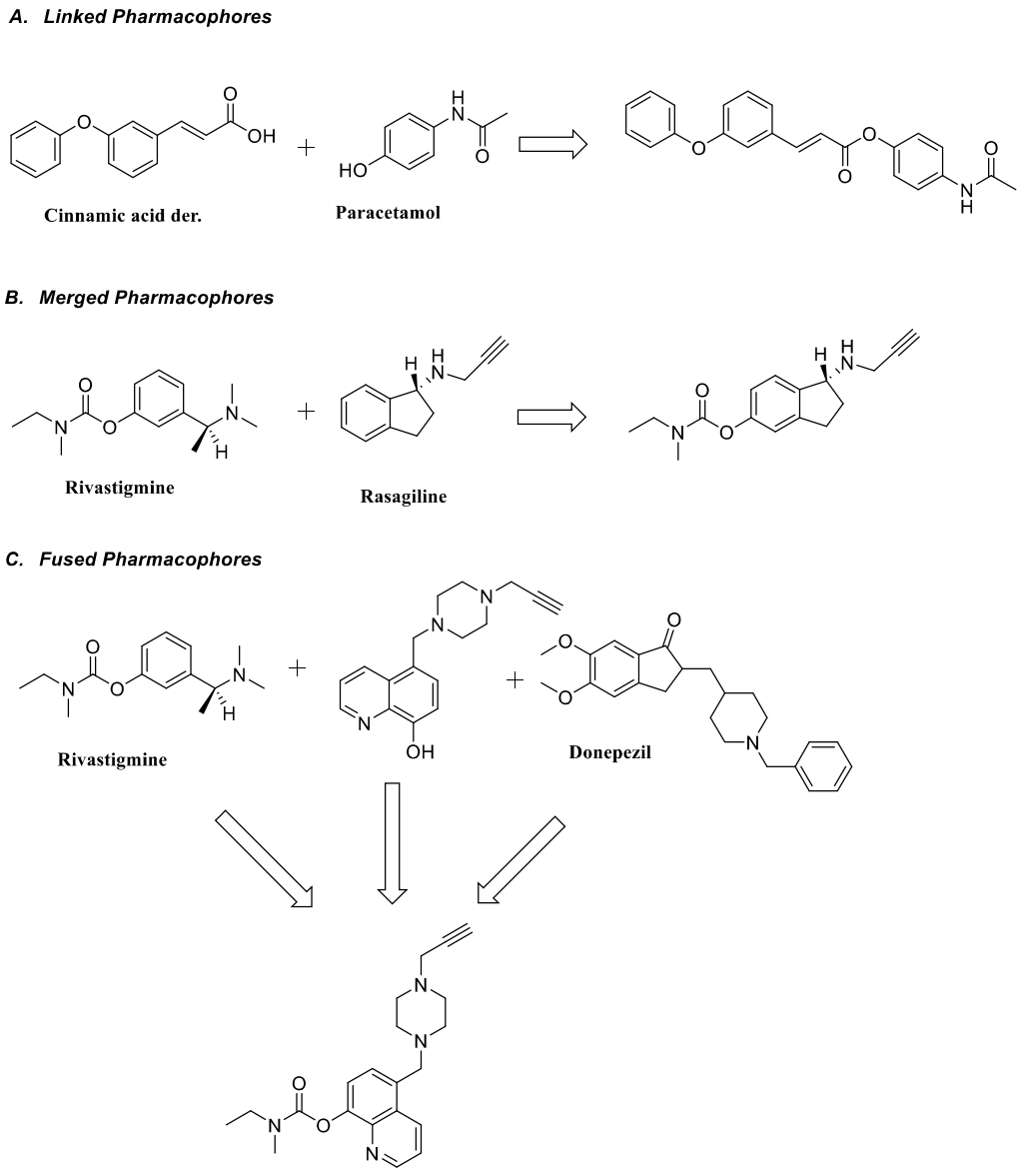


Figure 2.5 Various approaches towards design of MTDLs

- **Screening Approach**

Focussed screening rather than high throughput screening (HTS) is increasingly becoming the preferred screening approach for MTDL design. Typically, focused screening involves screening for additional targets using compound classes that have previously been found to be effective against one of the targets of interest. MTDLs thus generated may be active against both targets. However, it is extremely improbable that the generated MTDLs will have balanced affinities to various targets; as a result, the affinities would need to be optimized

accordingly. Moreover, the lead could engage in activities towards unwanted targets, which must be "designed-out" during optimisation.

2.5.4 MTDL lead optimization

The lead ligand designed through the various aprocahes must be optimised for balanced activity and superior physicochemical characteristics post the lead production step. “Design-in” and “Design-out” are the two main approaches in the lead optimization phase.

- **Design-in approach**

According to the molecular architecture and functional groups necessary for interacting with the biological targets, the pharmacophore of one ligand is incorporated into the pharmacophore of the other. For this approach, the two selective lead compounds should be sufficiently similar to one another structurally. Maintaining affinity for one target while increasing affection for the other remains the limitation of this strategy.

The feasibility and practicality of the scaffold hopping hybrid can be determined on the basis of the information provided by the SAR data of the selective ligands. The rational generation of merged pharmacophores approach could be combined with the design-in approach down the line for better results.

- **Design-out approach**

Typically, the approach begins by designing a compound that acts on all the desirable as well as undesirable targets at once in the first step. Selectivity to the desired targets will then be increased by lowering affinity to undesirable targets. This strategy may gain more from X-ray structural analysis than design-in and merged pharmacophore approaches, since it begins with a single ligand that already interacts with all the intended targets. Cocrystal structure analysis can be used to determine the binding mode of the lead compound towards undesirable targets. The disruption of the efficacy on the "off-target(s)" is made possible by the determination of

variations in binding modes between desired and undesirable targets. The problems in the design-in method might rise exponentially with each additional target, therefore it is still appealing if there are more than two targets of interest, however owing to the scarcity of leads with multitarget activities, this method is less popular.