RATIONALE, OBJECTIVES AND PLAN OF WORK

3.1. Rationale

The socioeconomic burden of multi-factorial pathologies such as NDDs is enormous worldwide. The discovery of novel drug candidates for the treatment of NDDs through tailor-made attempts appears to be hindered because of the subsequent causes:

(i) Poor understanding of underlying molecular mechanisms of onset and progression of numerous, cellular physiological events that are caused by genetic, environmental and endogenous pathogenic factors.

(ii) Non-availability or limited availability of explicit drug targets for therapeutic intervention – a major obstacle in the development of drug candidates for the multifaceted brain disorders including, Alzheimer's disease and Parkinson's disease, epilepsy, depression, anxiety, etc.

3.1.1. Drug discovery strategies for NDDs

3.1.1.1. Classical approach

The one-drug-one-target paradigm has been the dominating drug discovery approach since the early 1990s which attempts to recognize a single chemical entity that binds to a single target [Yang *et al.* 2008; Bolognesi *et al.* 2009]. This paradigm has led to the development of many successful drugs, and it will probably remain as a milestone for the years to come. Drug discovery and development against the neurodegenerative diseases has followed the same trend. As a matter of fact, memantine, an N-methyl-D-aspartate (NMDA) receptor (NMDAR) antagonist, was developed for the treatment of moderate to severe Alzheimer's disease in 2003 [Witt *et al.* 2004]. However, owing to disappointing clinical results, the success rate of this paradigm has been interrogated. This may be because the ligand is unable to recognize the target in-vivo, or the ligand is unable to reach the site of action, or the interaction of ligand with the respective target does not have enough impact on the diseased system to restore it effectively. Recent studies have established considerable redundancy in proteinaceous drug targets, signifying that drugs directed toward single pathophysiological mechanisms may have more limitations than multifunctional drugs [Van der Schyf and Youdim 2009; Noorbakhsh *et al.* 2009]

3.1.1.2. Multi-target-directed ligand (MTDL) approach

More recently, a new paradigm that addresses disease etiological complexity using a multi-target-directed ligand approach has gained increasing acceptance. Novel compounds are specifically designed to target the multiple mechanisms underlying the etiology of a specific disease, and these have shown superior efficacy and safety profiles. These agents offer the advantages of preventing, arresting, or slowing decline through disease modification. Thus, the major drug discovery paradigm is shifting from a one-drug-one-target strategy to a one-drug-multiple-targets strategy.

Advantages of MTDL design:

(i) Simplification of administration regimen

(ii) Improvement of the patient compliance

(iii) Decreased risk of adverse drug-drug interactions

(iv) Easy pharmacokinetic and pharmacodynamic profiling of drug

3.1.2. Pharmacophore and its role in drug design

Drugs can be designed computationally by using two strategies [Oprea and Matter 2004; Seifert *et al.* 2003]:

a) Structure or target based drug designing (SBDD)

b) Analogue or ligand based drug designing (LBDD)

In the method (a), the structure of the receptor or the target is known. From the knowledge of the active sites and the site points in the receptor, ligands are designed. Whereas in case of method (b), there is no knowledge of the receptor or the target, drugs can be designed in this case with the help of geometry of already known ligands. This is particularly useful in the case of protein targets which are not crystallisable and for which the 3D structure is unknown.

The word “pharmacophore” comes into picture in case of LBDD. As active sites to the receptors, the pharmacophore defines the functional part of the ligand. Drugs are known to be ligands or small molecules in both cases. The knowledge of pharmacophore will result in the generation of several leads.

3.1.2.1. Introduction to pharmacophore

The earliest use of the pharmacophore (PCP) concept dates back to the early 1900s when Paul Ehrlich devised a way to develop dyes through chromophores (*i.e.*, the part of a molecule responsible for imparting color). Around 1903, Ehrlich started to test these dyes

for their ability to kill trypanosomes, a microorganism that causes African sleeping sickness [Guner 2002]. For all practical purposes, this may be the first use of the pharmacophore concept in drug discovery and design. Despite this early discovery, effective use of pharmacophores only became possible more than half a century later, with the development of computational models of pharmacophores. The earliest papers published on computational pharmacophores were in the late 1970's, again by Peter Gund [Gund 2000; 1977] and Garland Marshall *et al*. [Marshall *et al*. 1979].

3.1.2.2. Definition

There are number of definitions for the pharmacophore but all implies the similar essential features only. When first introduced in the early 1900s by Paul Ehrlich, a "pharmacophore" was referred to as a "molecular framework that carries (*phoros*) the essential features responsible for a drug's (*pharmacon*) biological activity" [Ehrlich, 1909]. With the increased knowledge of three-dimensional molecular structures, this concept expanded to also include the requirement of spatial arrangement of essential molecular "features", e.g. steric and electrostatic characteristics or hydrogen-bonding capabilities [Loew *et al.* 1993].

A pharmacophore may be defined as the essential geometric arrangement of atoms or functional groups necessary to produce a given biological response [Guner 2002; Gund 2000].

The strict IUPAC definition of pharmacophore [Wermuth *et al*. 1998] is: "*A pharmacophore is the ensemble of steric and electronic features that is necessary to ensure the optimal supramolecular interactions with a specific biological target structure and to trigger (or to block) its biological response.*"

As a consequence:

(i) The pharmacophore describes the essential, steric and electronic, function-determining points necessary for an optimal interaction with a relevant pharmacological target.

(ii) The pharmacophore does not represent a real molecule or a real association of functional groups, but a purely abstract concept that accounts for the common molecular interaction capacities of a group of compounds towards their target structure.

(iii) Pharmacophores are not specific functional groups (e.g. sulphonamides) or "pieces of molecules" (e.g. dihydropyridines, arylpiperazines).

3.1.2.3. Role of PCP in drug design

(i) It can be used as a query to screen databases of commercially available compounds or, alternatively, to guide chemists in the synthesis of new compounds during the hit-to-lead optimization process.

(ii) It can also be used to align molecules based on the 3D arrangement of chemical features or to develop predictive 3D QSAR models [Khedkar *et al.* 2007].

(iii) Recently, a new application of pharmacophore modeling, namely parallel screening, has been developed and successfully applied [Steindl *et al.* 2006]

3.1.3. Development of pharmacophore for MAO inhibitors (MAOIs)

Medvedev *et al.*, 1996, proposed a pharmacophore model for MAO inhibitors consisting of three features [Medvedev *et al.* 1996]:

a) Two hydrophobic rings (A) (One or more hydrophobic ring(s))

b) One acceptor site (B) (At least one hydrogen bond acceptor)

c) One donor atom (C) (At least one hydrogen bond donor)

Interestingly, these pharmacophoric features have been identified in most of the reference MAO inhibitors as shown in **Figure 3.1.**

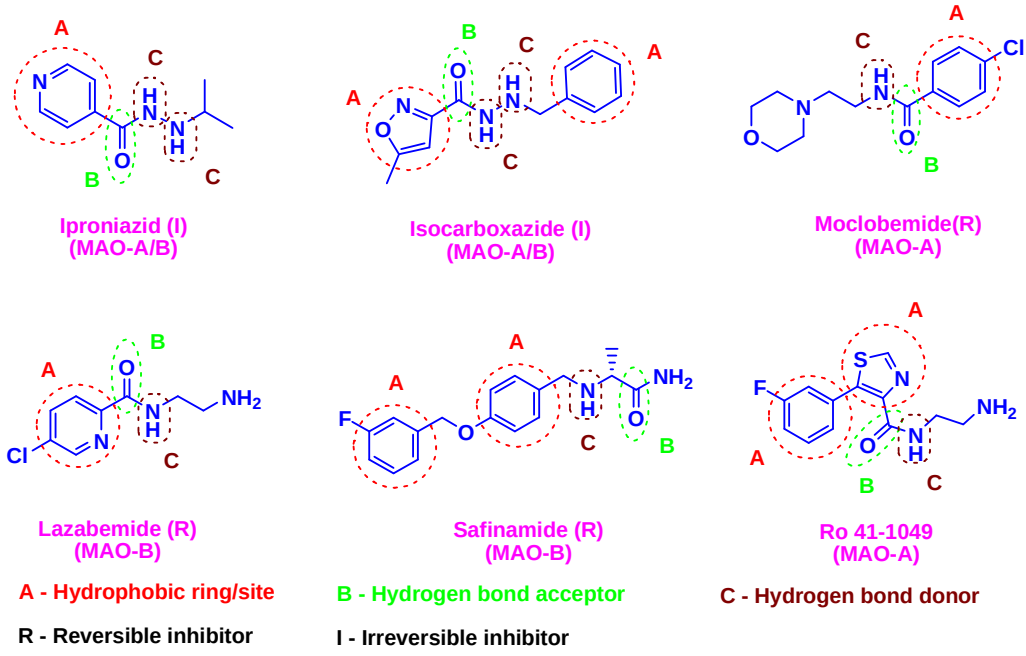
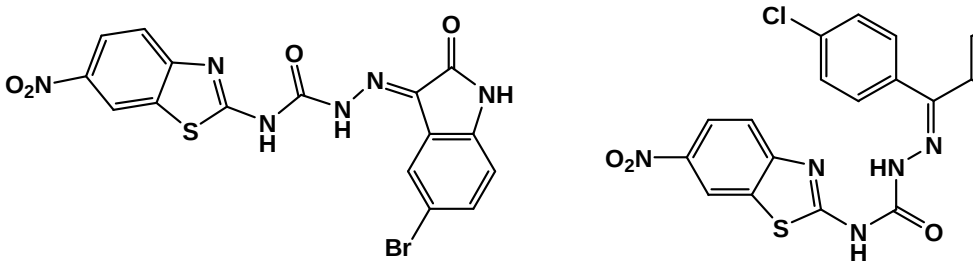


Figure 3.1. Structure of reference MAOIs showing the shared pharmacophoric features

Working in this mainstream, in our laboratory, a library of about 26 compounds of 2-amino-6-nitrobenzothiazole derived semicarbazones was synthesized and investigated for their ability to inhibit MAO-A and MAO-B isozymes *in-vitro*. 1-(5-Bromo-2-oxoindolin-3-ylidene)-4-(6-nitrobenzothiazol-2-yl) semicarbazide (**SBTZ-11**) and 1-[(4-chlorophenyl)(phenyl)methylene]-4-(6-nitrobenzothiazol-2-yl)semicarbazide (**SBTZ-18**) emerged as the lead MAO-A and MAO-B inhibitors respectively in both *in-vitro* and molecular docking studies (**Figure 3.2.**) [Tripathi *et al.* 2013].



SBTZ-11
MAO-A lead
IC_{50} = 0.002 ± 0.0006 µM
K_i = 0.903 µM

SBTZ-18
MAO-B lead
IC_{50} = 0.004 ± 0.001 µM
K_i = 1.08 µM

Figure 3.2. Structures of semicarbazone based lead MAO inhibitors identified in our laboratory

On the basis of MAO inhibition data of potent compounds and their structure-activity relationship studies, we identified the essential pharmacophoric features necessary for the design of potent MAO-B inhibitors.

Accordingly, the pharmacophoric features consisted of:

a) A large hydrophobic heterocyclic ring for binding with the substrate cavity of MAO-B active site.
b) Another hydrophobic aryl ring for effective binding and stabilization in the entrance cavity space of MAO-B.
c) A flexible linker possessing hydrogen bonding regions for guiding the optimal orientation of both hydrophobic aryl residues in their respective binding pockets within the active site of MAO-B.

However, the most active compounds were found to possess moderate to high toxicity in i.p neurotoxicity screen which prompted us to perform further optimization studies for the want of reducing the toxicity profile.

The above results encouraged us to further carry out some modifications in semicarbazone template. Further studies on more structural analogs were required to establish the SAR, potency and future prospective of their development as novel neurotherapeutic agents for the treatment of neurological disorders.

3.1.4. Development of pharmacophore for AChE inhibitors

The proposed pharmacophore model for AChE inhibitors (AChEIs) [Bag *et al.* 2013] consisted of four features which include

a) One aromatic ring (A)
b) One hydrogen acceptor motif (B)
c) One hydrogen donor motif (C)
d) One positively charged group (D)

These pharmacophoric features have been identified in the reference AChE inhibitors as shown in **Figure 3.3.**

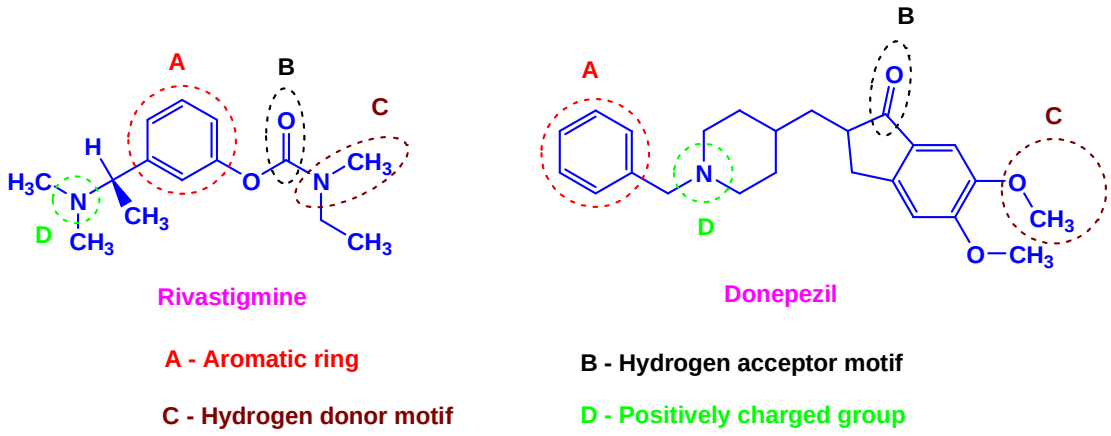


Figure 3.3. Structure of reference AChE inhibitors showing the shared pharmacophoric features

3.1.5. Hybrid PCP features in MTDLs targeting dual MAO and AChE inhibition

Based on the PCP features of MAOIs and AChEIs, it can be concluded that for the development of MTDLs targeting dual MAO and AChE inhibition, the ligands should possess at least five pharmacophoric features:

a) At least one hydrophobic motif and an aromatic ring (A)
b) One hydrogen bond acceptor motif (B)
c) One hydrogen bond donor motif (C)
d) One positively charged group or an electron donor group (D)

Interestingly, these pharmacophoric features have been identified in the reference MTDLs targeting both MAO and AChE inhibitions. (**Figure 3.4.**)

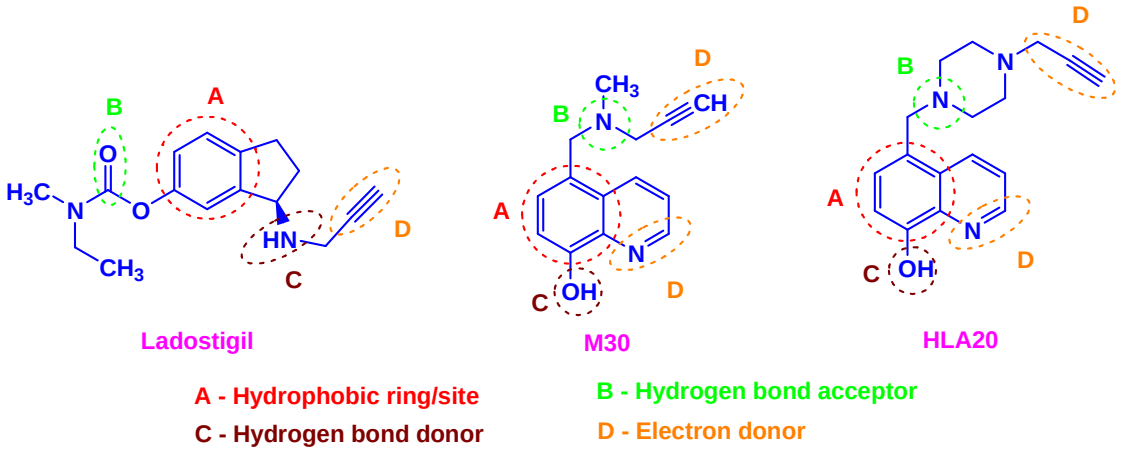


Figure 3.4. Structure of reference MTDLs showing the shared pharmacophoric features for both MAO and AChE inhibitions

3.2. Objectives of the present study

The specific objectives of design of each series of compounds are presented in the following section.

1) To develop a standard protocol incorporating modern drug design tools (pharmacophore/ligand based analog design/multi-target directed ligand design) for discovering novel MAO inhibitors (MAOIs) and MTDLs with dual MAO and AChE inhibitory properties in addition to exhibiting antioxidant properties.
2) To design and synthesize some new chemical library of compounds bearing hydrazone, semicarbazone and isatin scaffolds.
3) To evaluate the bioactive potential of designed compounds (MAOIs and AChEIs) in virtual and physical environments through virtual molecular docking and physical enzymatic screening assays respectively.
4) To establish the structure-activity relationship (SAR) of the designed extended hydrazones, semicarbazones and 3-hydroxy-3-substituted oxindole analogues with respect to both MAO and AChE inhibitory properties.
5) To identify the pharmacophore model with a well-defined geometry and distance constraints based on the resulting MAOI and AChEI lead compound(s).

3.3. Design of chemical library

Based on the previously reported PCP models for MAO and AChE inhibition, we identified five PCP features from well known inhibitors of MAO-A, MAO-B and AChE and tried to incorporate such pharmacophores into MTDLs. These MTDLs are expected to interact with both CAS and PAS sites of AChE enzyme and also with the active site cavities of both MAO-A/B isozymes. The most common strategy to rationally design MTDLs has been mainly based on linking two appropriately selected pharmacophores endowed with different pharmacological activity through a linker (spacer) against two different molecular targets, MAO and AChE.

Keeping in mind all the necessary pharmacophoric requirements for dual inhibition targeting MAO and AChE, we designed a library of ligands (MTDLs) presenting multifunctional activity against MAO-A (ligand binding to substrate cavity), MAO-B (ligand binding to both entrance and substrate cavity) and AChE (ligand binding to CAS and PAS simultaneously) by incorporating the following features:

(i) Hydrophobic domain resulting in hydrophobic interactions formed by aryl/heteroaryl moiety (A)
(ii) At least one hydrogen bond acceptor motif (B)
(iii) At least one hydrogen bond donor motif (C)
(iv) An electron donor group (D)
(v) An aromatic ring featuring additional increased interaction at PAS (E)

Based on the above rationale and objective, a chemical library of compounds *viz.* extended hydrazones (**BTA** series) and semicarbazones (**NTA** series and **MDA** series) and have been designed and the general structure along with molecular modifications done are shown in **Figure 3.5**.

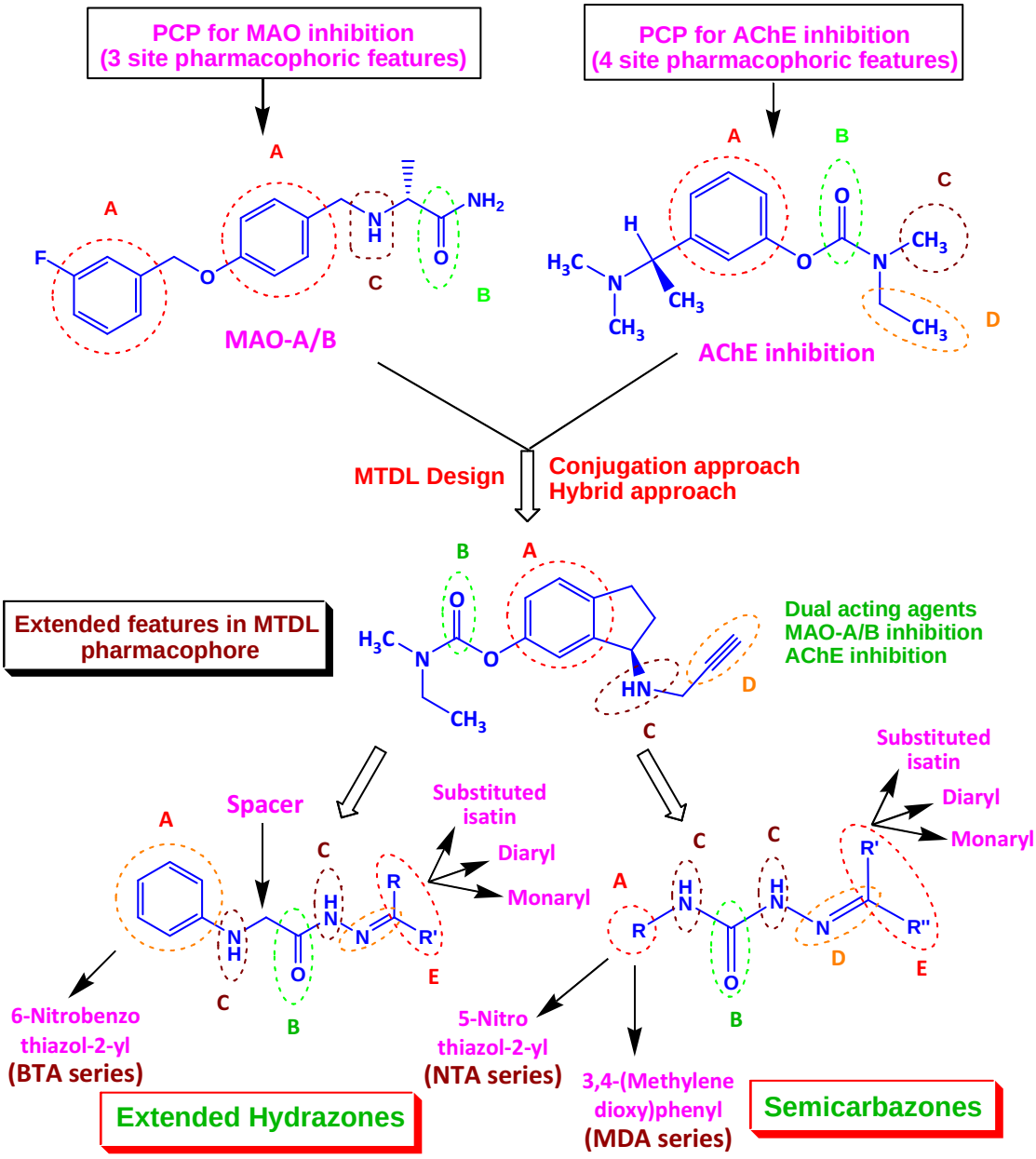


Figure 3.5. Designing strategy for MTDLs targeting dual inhibition

3.3.1. Design of 2-amino-6-nitrobenzothiazole derived extended hydrazones (BTA series)

A series of novel 2-amino-6-nitrobenzothiazole derived hydrazones was designed;

a) To study the effect of this class of compounds against multiple targets (MAO-A/-B and AChE)

b) To improve the selectivity and potency of compounds w.r.t above targets by various structural modifications –

(i) Substitution with a sterically bulky substituent by incorporating a *heteroaryl ring* (*6-nitrobenzothiazole*) instead of the aryl ring and various monoaryl/diaryl/isatin ring systems, respectively.

(ii) Effect of *electron-withdrawing* and *electron-donating* substituents on the aryl/heteroaryl ring and *variations in the ring size*.

(iii)Effect of $–CH_2–$ spacer on the *stability* and *affinity*.

The design approach and the designed compounds of BTA library are presented in **Figure 3.6.** and **Figure 3.7.** respectively.

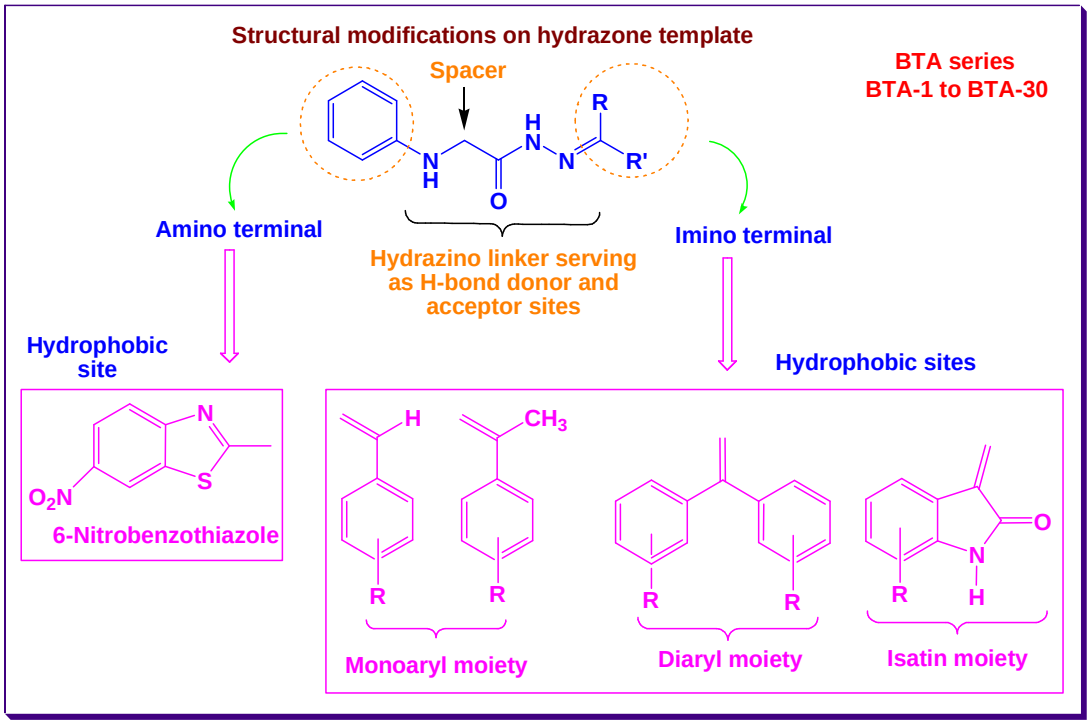
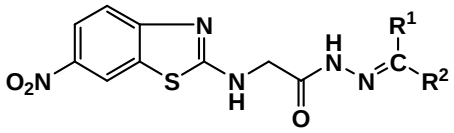
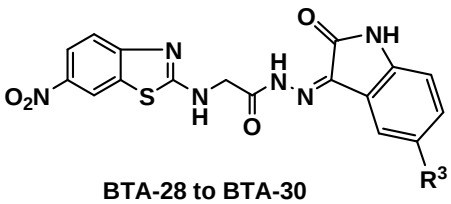


Figure 3.6. Design approach of extended hydrazones incorporating 6-nitrobenzothiazole moiety



BTA-1 to BTA-27

BTA-1 : $R^1 = CH_3$, $R^2 = CH_3$
BTA-2 : $R^1 = CH_3$, $R^2 = C_6H_5$
BTA-3 : $R^1 = CH_3$, R^2 = 4-Br C_6H_5
BTA-4 : $R^1 = CH_3$, R^2 = 4-Cl C_6H_5
BTA-5 : $R^1 = CH_3$, R^2 = 4-F C_6H_5
BTA-6 : $R^1 = CH_3$, R^2 = 4-OH C_6H_5
BTA-7 : $R^1 = CH_3$, R^2 = 4-NO_2 C_6H_5
BTA-8 : R^1 = H, $R^2 = C_6H_5$
BTA-9 : R^1 = H, R^2 = 2-OH C_6H_5
BTA-10 : R^1 = H, R^2 = 4-Br C_6H_5
BTA-11 : R^1 = H, R^2 = 4-$N(CH_3)_2$ C_6H_5
BTA-12 : R^1 = H, R^2 = 4-OH C_6H_5
BTA-13 : R^1 = H, R^2 = 4-OCH_3 C_6H_5
BTA-14 : R^1 = H, R^2 = 4-NO_2 C_6H_5
BTA-15 : R^1 = H, R^2 = 2,3-Cl_2 C_6H_5
BTA-16 : R^1 = H, R^2 = 2,4-Cl_2 C_6H_5
BTA-17 : R^1 = H, R^2 = 2,6-Cl_2 C_6H_5
BTA-18 : R^1 = H, R^2 = 2,5-$(OCH_3)_2$ C_6H_5
BTA-19 : R^1 = H, R^2 = 3,4-$(OCH_3)_2$ C_6H_5
BTA-20 : R^1 = H, R^2 = 3,4,5-$(OCH_3)_2$ C_6H_5
BTA-21 : R^1 = H, R^2 = 4-Cl-C_6H_4-CH_2-O- C_6H_5
BTA-22 : $R^1 = C_6H_5$, $R^2 = C_6H_5$
BTA-23 : $R^1 = C_6H_5$, R^2 = 4-Cl C_6H_5
BTA-24 : $R^1 = C_6H_5$, R^2 = 4-OH C_6H_5
BTA-25 : R^1 = 4-Cl C_6H_5, R^2 = 4-Cl C_6H_5
BTA-26 : $R^1 = CH_2Br$, R^2 = 4-Br C_6H_5
BTA-27 : R^1, R^2 = Cyclohexyl



BTA-28 to BTA-30

BTA-28 : R^3 = H
BTA-29 : R^3 = Cl
BTA-30 : $R^3 = NO_2$

Figure 3.7. Designed compounds for BTA library (**BTA-1** to **BTA-30**)

3.3.2. Design of 2-amino-5-nitrothiazole derived semicarbazones (NTA series)

A series of novel 2-amino-5-nitrothiazole derived semicarbazones was designed;

a) To study the effect of this class of compounds against multiple targets (MAO-A/-B and AChE)
b) To improve the selectivity and potency of compounds w.r.t above targets by various structural modifications –
    (i) Substitution with a sterically bulky substituent by incorporating a *heteroaryl ring* (*5-nitrothiazole*) instead of the aryl ring and various monoaryl/diaryl/isatin ring systems, respectively.
    (ii) Effect of *electron-withdrawing* and *electron-donating* substituents on the distal aryl ring.
    (iii) Effect of –NH–CO–NH–N=C linker on the *stability* and *affinity*.

The design approach and the designed compounds of NTA library are presented in **Figure 3.8.** and **Figure 3.9.** respectively.

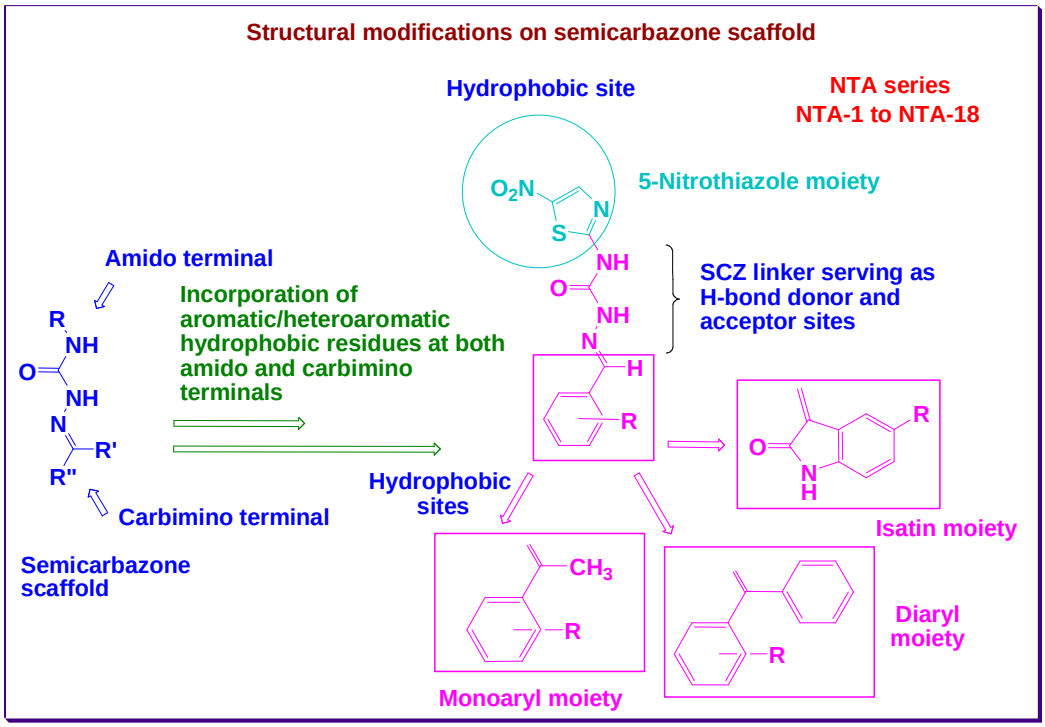
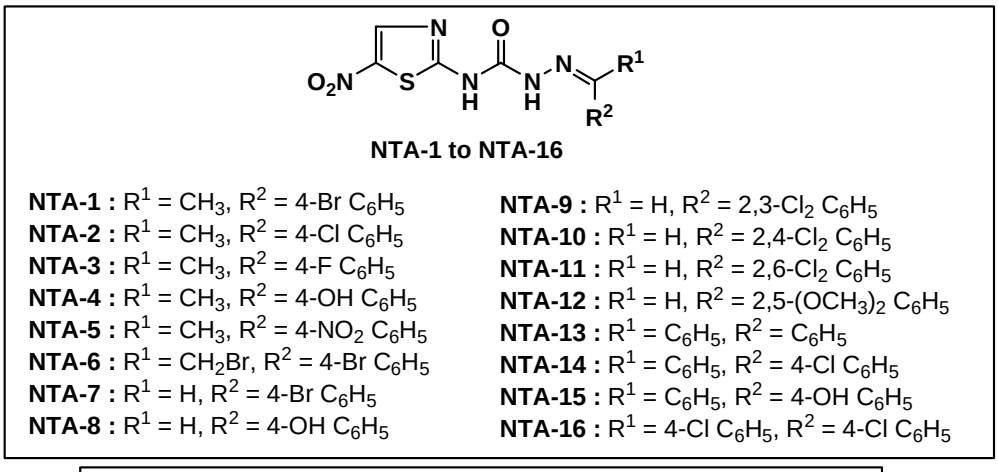
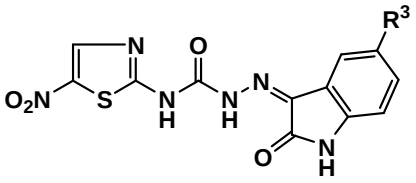


Figure 3.8. Design approach of semicarbazones bearing 5-nitrothiazole moiety



NTA-1 to NTA-16

NTA-1 : R^1 = CH_3, R^2 = 4-Br C_6H_5
NTA-2 : R^1 = CH_3, R^2 = 4-Cl C_6H_5
NTA-3 : R^1 = CH_3, R^2 = 4-F C_6H_5
NTA-4 : R^1 = CH_3, R^2 = 4-OH C_6H_5
NTA-5 : R^1 = CH_3, R^2 = 4-NO_2 C_6H_5
NTA-6 : R^1 = CH_2Br, R^2 = 4-Br C_6H_5
NTA-7 : R^1 = H, R^2 = 4-Br C_6H_5
NTA-8 : R^1 = H, R^2 = 4-OH C_6H_5
NTA-9 : R^1 = H, R^2 = 2,3-Cl_2 C_6H_5
NTA-10 : R^1 = H, R^2 = 2,4-Cl_2 C_6H_5
NTA-11 : R^1 = H, R^2 = 2,6-Cl_2 C_6H_5
NTA-12 : R^1 = H, R^2 = 2,5-$(OCH_3)_2$ C_6H_5
NTA-13 : R^1 = C_6H_5, R^2 = C_6H_5
NTA-14 : R^1 = C_6H_5, R^2 = 4-Cl C_6H_5
NTA-15 : R^1 = C_6H_5, R^2 = 4-OH C_6H_5
NTA-16 : R^1 = 4-Cl C_6H_5, R^2 = 4-Cl C_6H_5



NTA-17 to NTA-18

NTA-17 : R^3 = H
NTA-18 : R^3 = Br

Figure 3.9. Designed compounds for NTA library (**NTA-1** to **NTA-18**)

3.3.3. Design of 3,4-(methylenedioxy)aniline derived semicarbazones (MDA series)

A series of novel 3,4-(methylenedioxy)aniline derived semicarbazones was designed;

a) To study the effect of this class of compounds against multiple targets (MAO-A/-B and AChE)

b) To improve the selectivity and potency of compounds w.r.t above targets by various structural modifications –

(i) Substitution with a sterically bulky substituent by incorporating a *heteroaryl ring* (*benzo[1,3]dioxole*) instead of the aryl ring and various monoaryl/diaryl/isatin ring systems, respectively.

(ii) Effect of *electron-withdrawing* and *electron-donating* substituents on the distal aryl ring.

(iii)Effect of –NH–CO–NH–N= linker on the *stability* and *affinity*.

The design approach and the designed compounds of MDA library are presented in **Figure 3.10.** and **Figure 3.11.** respectively.

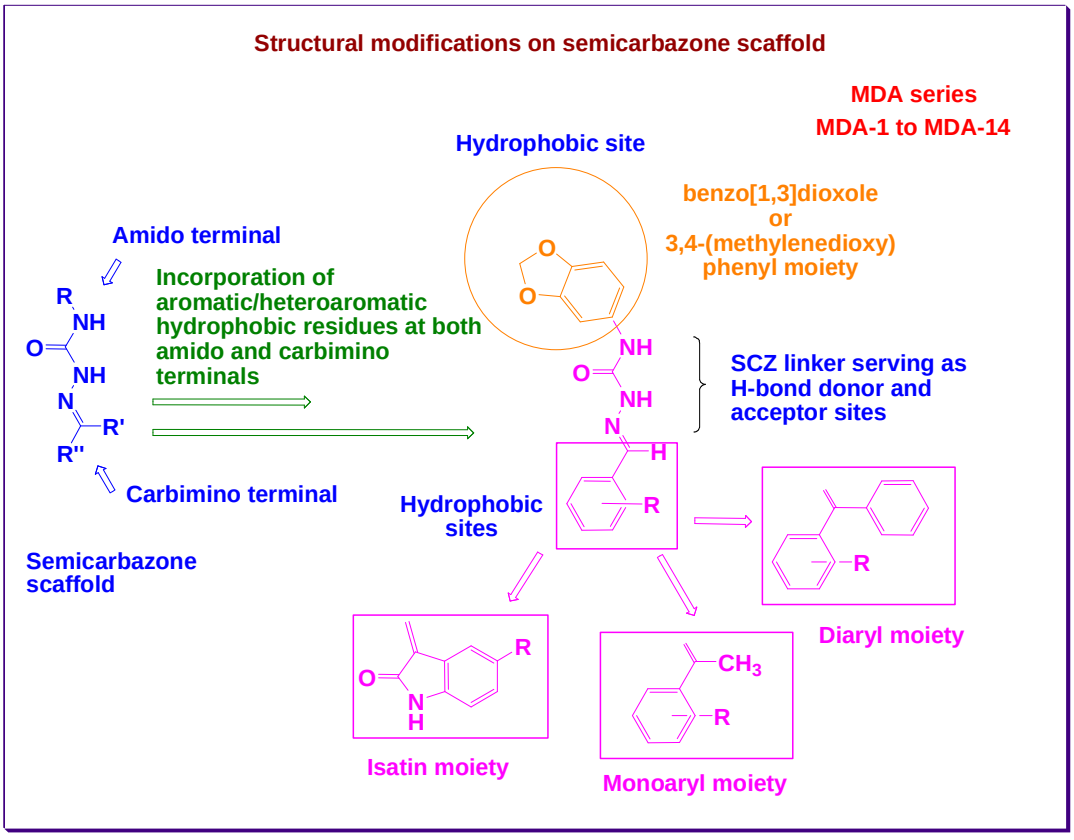


Figure 3.10. Design approach of semicarbazones bearing 3,4-(methylenedioxy)phenyl moiety

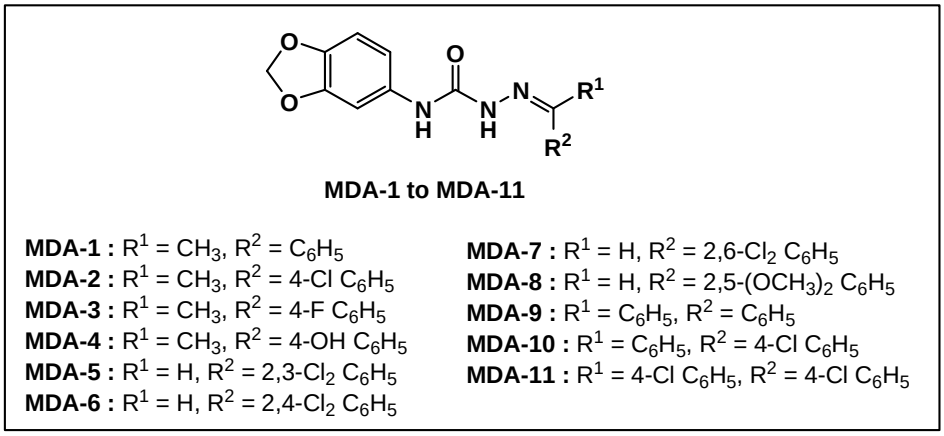


Figure 3.11. Designed compounds of MDA library (**MDA-1** to **MDA-14**)

3.3.4. Design of 3-hydroxy-3-substituted oxindole analogues of isatin (HPO series)

Based on the promising activity displayed by derivatives possessing isatin scaffold, a series of novel rigid 3-hydroxy-3-substituted oxindole analogues of isatin and substituted isatin was designed;

a) To study the effect of this class of compounds against multiple targets (MAO-A/-B and AChE)
b) To improve the selectivity and potency of compounds w.r.t above targets by various structural modifications –
    (i) Substitution with a sterically bulky substituent by incorporating a *heteroaryl ring* (*isatin* and *5-bromoisatin*).
    (ii) Effect of *electron-withdrawing* and *electron-donating* substituents on the phenacyl side chain.
c) To study the effect of *3-OH group* and *absence of flexible linker* (in comparison to other series) on the stability and affinity.

The design approach and the designed compounds of HPO library are presented in **Figure 3.12.** and **Figure 3.13.** respectively.

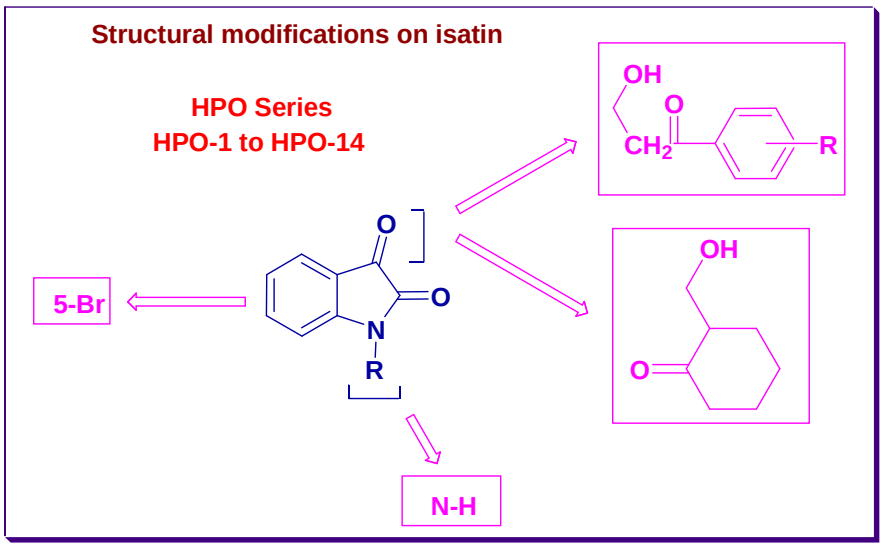


Figure 3.12. Design approach of 3-hydroxy-3-substituted oxindole analogues of isatin

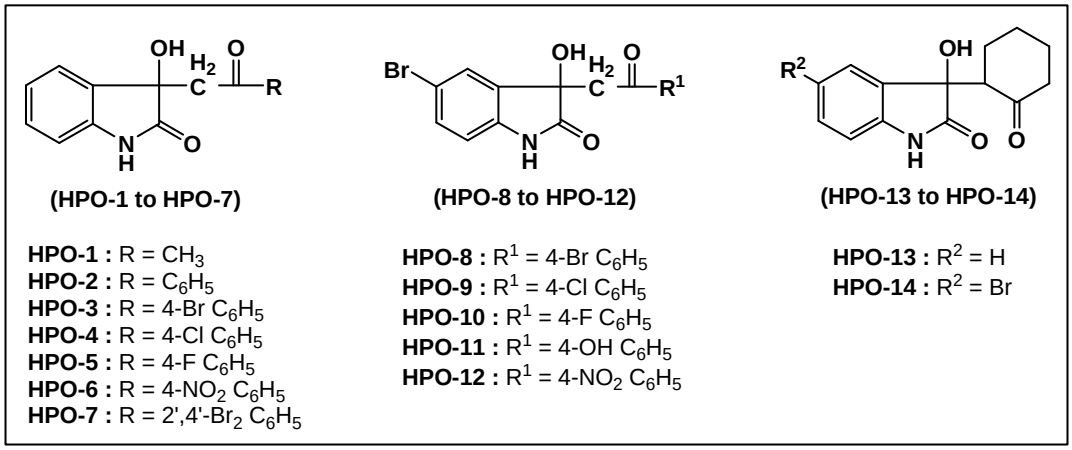


Figure 3.13. Designed compounds of HPO library (**HPO-1** to **HPO-14**)

3.4. Plan of work

The detailed plan of the research work includes:

- Design of ligand library
  - ✓ 2-Amino-6-nitrobenzothiazole derived extended hydrazones (BTA series)
  - ✓ 2-Amino-5-nitrothiazole derived semicarbazones (NTA series)
  - ✓ 3,4-(Methylenedioxy)aniline derived semicarbazones (MDA series)
  - ✓ 3-Hydroxy-3-substituted oxindole analogues of isatin (HPO series)
- Synthesis of the designed compounds
- Characterization of synthesized compounds

- ✓ Physicochemical characterization by MP, TLC, LogP, solubility, etc.
- ✓ Spectral characterization by UV-VIS, FT-IR, ^{1}H-NMR, ^{13}C-NMR, mass spectrometry (MS), elemental (CHN) analysis and X-ray powder diffraction (XR-PD)

- ➢ Evaluation of MAO inhibitory activity of synthesized compounds
  - ✓ *In-vitro* MAO-A/B inhibition assay
  - ✓ Kinetic studies of lead compound(s)
  - ✓ Reversibility and Irreversibility studies
  - ✓ Molecular modeling (docking) studies
- ➢ Evaluation of AChE inhibitory activity of synthesized compounds
  - ✓ *In-vitro* AChE inhibition assay
  - ✓ Kinetic studies of lead compound(s)
  - ✓ Reversibility and Irreversibility studies
  - ✓ Molecular modeling (docking) studies
- ➢ Behavioural studies
  - ✓ Antidepressant activity
    - Forced swim test (FST)
  - ✓ Anxiolytic activity
    - Elevated plus maze (EPM) test
  - ✓ Sedative-Hypnotic activity
    - Pentobarbitone potentiation test
- ➢ Neurotoxicity screening studies
  - ✓ Rotarod test
- ➢ Antioxidant activity
  - ✓ DPPH radical scavenging assay
- ➢ Assessment of liver function
  - ✓ Liver toxicity tests
  - ✓ Liver histopathological studies
- ➢ *In-silico* molecular property analysis and ADMET prediction studies