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Recent advances in multitarget-directed ligands targeting G-protein-coupled receptors

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Abstract

Mounting evidence indicates that single-target drugs might be inadequate to achieve satisfactory therapeutic effects on complex diseases. Recently, increasing attention has been paid to developing drugs that can manipulate multiple targets to generate beneficial effects through potential synergy. G-protein-coupled receptors (GPCRs) become desirable targets for developing multitarget-directed ligands (MTDLs) because of their crucial roles in the pathophysiology of various human diseases and the accessibility of druggable sites at the cell surface. Herein, we review the most recent advances in the development of GPCR-targeted MTDLs in treating complex diseases, and discuss their potential therapeutic strategies to reveal current trends and shed insights into the utility of GPCR-targeted MTDLs for future drug design and development.

Introduction

Since the lock-and-key model proposed by Erlich in 1894 [1], drug discovery has mainly focused on developing selective molecules that target a single mechanism in a single disease, namely the ‘one drug, one target’ approach [2]. Following this doctrine, numerous selective drugs have been developed, demonstrating their pivotal roles in treating certain diseases [3]. However, complex diseases, also known as multifactorial diseases, are posing enormous challenges to the medicinal chemistry community because they involve multiple target proteins and/or signaling pathways associated with diseases such as cancers [4], neurodegenerative diseases [5], pain disorders [6], and infection and inflammation [7]. For these complex diseases, acting on a single target might be inadequate to achieve satisfactory therapeutic effects [8,9]. Hence, there is an emerging trend to develop MTDLs that are capable of manipulating multiple targets simultaneously, with the aim to enhance efficacy and improve safety.

GPCRs are the most intensively studied target proteins in drug design and development [10,11]. For decades, the design of bivalent and bitopic molecules has evolved as intriguing approaches to obtain highly selective ligands targeting GPCRs with desirable efficacies and/or signaling bias. As first proposed by Phil Portoghese in 1982 [12], a bivalent ligand refers to a molecule that has two pharmacophores on both ends of an appropriate linker, aiming to target the orthosteric binding site (OBS) of two adjacent or dimerized receptors to

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improve affinity and/or potency. Based on the identity of pharmacophores, bivalent ligands can be further branched into homobivalent and heterobivalent ones. By contrast, a bitopic ligand is proposed to improve GPCR subtype selectivity and functionality. Typically, a bitopic ligand comprises two distinct pharmacophores connected by a linker, thus conferring concomitant binding to the OBS and an allosteric binding site (ABS) of the same receptor monomer [13]. This results in favorable pharmacological properties of the molecule, through receptor selectivity, functional selectivity, and/or allostery [14]. Currently, a new avenue for GPCR drug discovery has emerged by modulating more than one receptor at once to generate beneficial effects synergistically [15], and the resulting ligands have been termed ‘GPCR-targeted MTDLs’.

Although many publications have reported promising results, there is a paucity of review literature focusing on GPCR-targeted MTDLs in treating complex diseases. Meanwhile, Class A GPCRs, the largest and most widely characterized family, has triggered the interest of medicinal chemists both in academia and pharmaceutical companies because of their roles in various diseases and their relatively facile accessibilities. Thus, we review here recent research into Class A GPCR-targeted MTDLs, and discuss their potential therapeutic strategies to reveal current trends in the field and provide insights into the utility of GPCR-targeted MTDLs for future drug design and development.

Rational combination of targets for MTDLs

The identification and validation of target combinations is pivotal to obtain an MTDL to treat complex diseases efficaciously. Generally, targets that belong to the same superfamily are easier to utilize in the design of multitarget ligands [16]. By contrast, for targets from different superfamilies, it would be realistic to perform comprehensive structure–activity relationship (SAR) studies for each of the components to exploit the relevant positions and subsequently combine pharmacophores. Three primary approaches of target combination have been applied to MTDL design and discovery [17]. First, clinical observations, especially for combination therapies, such as drug cocktails, provide increasing insight into how to improve efficacy and reduce adverse effects when more targets are involved. Target combination based on clinical observations has been typically utilized in the design of antipsychotics, several of which have been approved for marketing [18]. Second, phenotypic screening is another feasible approach for target combination;it provides visual effects of compound combinations for synergies at the cell or organism level. Moreover, the rapid development of genetic technology, such as genetic knockdown, makes it possible to validate the synergistic effect of the involved targets [19]. Third, *in silico* techniques are a relevantly recent approach to screen appropriate target combinations. By using machine learning, the analysis of biological target networks can be conducted to predict efficacious and safe target combinations. However, such hypothetic results require laboratorial verification to confirm their biological foundation and feasibility [20].

Strategies for designing MTDLs

There are three approaches to multitarget therapeutics utilized in clinical practice [21]: the first is multiple-medication therapy (MMT), also referred to as drug cocktails, which

comprise two or three different single active drugs that combine different therapeutic mechanisms; the second approach is multi-compound medication (MCM), also known as single-pill drug combinations, which incorporates different drugs into the same formulation; the third approach is MTDLs, by which one active drug might have multiple biological properties by modulating multiple targets simultaneously [22,23]. Despite some advantages of the former two approaches, such as dose flexibility and lower treatment cost, they usually have adverse effects, such a drug–drug interactions and poor patient incompliance [24].

As defined by Morphy *et al.*, MTDLs are compounds that are designed to treat complex diseases by acting at multiple targets associated with pathogenesis [21]. This strategy has inherent advantages over MMT or MCM [5], and also obviates redundant mechanisms caused by single target drugs. There are two well-accepted approaches to generate MTDLs: knowledge-based approaches and screening approaches [25]. Following these strategies, MTDLs have become effective therapeutics for treating complex diseases and several have advanced into clinical trials and to market [26].

Knowledge-based approach

The knowledge-based approach, also known as the pharmacophore-based approach, is the main approach used to generate MTDLs [27]. Pharmacophores from selective ligands are incorporated into a single molecule to integrate the biological activities of the ligands. Based on the degree of integration between pharmacophores, the MTDLs are categorized as linked, fused, or merged (Fig. 1a) [21].

Linked pharmacophores—The molecular framework of a linked MTDL is typically separated by a distinct linker group that does not belong to either of the selective ligands, and can be cleavable or noncleavable [21,25]. Although this strategy appears straightforward, it is vital to select the attachment point, length, and chemical composition of linkers carefully to maintain the biological activities of the template scaffolds [28]. In our opinion, there are two approaches that can be used to determine the best positions on two pharmacophores to attach the linker. The first is the application of traditional medicinal chemistry, that is, an understanding of the SAR of each monomeric counterpart as well as consideration of the synthetic feasibility would be beneficial to identify the attachment point on each pharmacophore. In addition, the rapid development of structural biology as well as increasing molecular modeling efforts provide structure-based approaches to select appropriate attachment points on pharmacophores. Most reported linked MTDLs use noncleavable linkers, which are likely stable *in vivo*, whereas the linkers in cleavable MTDLs can be degraded by specific enzymes to release ligands targeting multiple proteins [25]. An example of noncleavable MTDLs is shown in Fig. 1b, where compound **3** was found to be a multifunctional ligand with well-balanced potencies against the 5-HT_6 receptor (5-HT_6R) and cholinesterases (ChEs). It was designed by linking a 5-HT_6R antagonist pharmacophore **1** with a cholinesterase inhibitor pharmacophore **2** via a polymethylene linker. In addition to its ability to penetrate the blood–brain barrier (BBB), compound **3** also exhibited central cholinomimetic activity in a scopolamine-induced hyperlocomotion model, suggesting its potential application in treating Alzheimer's disease (AD) [29]. By comparison, as a cleavable MTDLs, compound **6** was reported as a novel

nonsteroidal anti-inflammatory drug (NSAIDs) with potent anti-inflammatory and antiaggregatory activities, which integrated ibuprofen **4** and a nitric oxide (NO)-releasing group **5** through an ester linker that can be cleaved by plasma esterases (Fig. 1b) [30].

Fused pharmacophores—Once the pharmacophores are adjacent to each other, these MTDLs can be classified as fused. This strategy is particularly advantageous when handling highly dissimilar targets, because it might be impossible to achieve multiple activities in a compact molecule in which the pharmacophores would have to be highly overlapped. One feasible option in such a situation will be to fuse pharmacophores together and explore tolerant positions in each component [21]. For example, it was suggested that introducing substitutions around the C3′ tolyl group in compound **7** would be tolerated and, thus, a histamine H_2 pharmacophore **8** was fused onto the C3′-methyl group of **7** to generate novel dual-functional compound **9**, which showed balanced activity at both the H_2 and gastrin receptors (Fig. 1d) [31].

Merged pharmacophores—With the highest level of overlapping pharmacophores, merged MTDLs, featuring lower molecular weights and simpler structures, take advantage of commonalities in the structures of the original ligands. As exemplified by a successful case detailed in Fig. 1e, by merging the aminopropoxyphenyl moiety from both compounds **10** and **11** (SKF-64346) and additionally fusing with deferiprone **12**, compound **13** was designed, synthesized, and biologically characterized [32]. Its excellent H_3R affinity and portent amyloid-β (Aβ) aggregation inhibition, as well as metal ion chelation, demonstrated its potential as a multifunctional candidate to treat AD.

Screening approach—The screening approach is another important strategy in the development of MTDLs [33]. This approach can be further divided into diversity-based and focused screening [21]. Generally, in diversity-based screening, a high-throughput screening (HTS) is applied to diversified compound collections for one target protein, followed by further screening for the second target. Compared with diversity-based screening, focused screening is more commonly used. In this method, compounds are screened for another target once they have already been validated to be effective at one of the targets of interest [17]. The resulting MTDLs might show decent activity against selected targets. However, a well-balanced affinity profile towards all targets of interest is rarely obtained, while the affinity for at least one target is usually subject to further optimization. If the resulting MTDLs are nonselective over many targets, then a ‘design-out’ approach is applied, in which structural modifications are pursued to reduce the affinity for undesired targets to improve the selectivity towards the those of interest.

Recent progress in GPCR-targeted MTDLs in drug design and development

Comprehensive coverage of recently discovered GPCR-targeted MTDLs in treating all complex diseases is beyond the scope of this review. Therefore, we focus here on the application of MTDLs targeting GPCRs for several most common complex disease groups: central nervous system (CNS) diseases, cardiovascular diseases, and diabetes.

GPCR-targeted MTDLs to treat CNS diseases

MTDLs targeting serotonin and dopamine receptors to treat schizophrenia—

Schizophrenia is a chronic mental disease affecting ~1% of the global population [34]. Although typical antipsychotics targeting the dopamine D_2 receptor (D_2R) show potency to control some positive symptoms, they can exacerbate negative symptoms and cognitive dysfunction, as well as causing extrapyramidal symptoms (EPSs) [35]. It is believed that additional interaction with serotonin 5-HT receptors (5-HTRs) could improve their therapeutic profile and reduce adverse effects in treating schizophrenia [21,36]. In this regard, Cao *et al.* [37] reported a series of compounds using the pharmacophore-linking approach, in which a D**2**R privileged structure (phenylpiperazine) and a fused tricyclic heterocycle from a reported multitarget lead compound reported by Pfizer [38] were connected by an aliphatic linker. Further SAR studies suggested that tricyclic heterocycle, as shown in compound **14** (Fig. 2), was crucial to maintain balanced affinities for both the 5-HTRs and D_2R. Moreover, a four-carbon length of linker was optimal for binding at both receptors. Further *in vivo* studies indicated that compound **14** was efficacious in different animal models of psychoses with fewer adverse effects. Additionally, a favorable pharmacokinetics profile with oral bioavailability of 58.8% in rats was also observed.

By utilizing a similar pharmacophore linking strategy, another lead compound was discovered recently (**15;**Fig. 2) [39], which showed potent antagonistic activities over the D_2R (IC_{50} = 3.0 nM) and 5-HT2AR (IC_{50} = 15.1 nM). In addition, compound **15** showed reasonable *in vivo* metabolic stability with a half-life of 2 h in Institute of Cancer Research (ICR) mice and an acceptable ability to penetrate BBB with Kp value of 4.03. The incorporation of 6-fluoro-1,2-benzisoxazolyl piperidine moiety in both compounds **14** and **15** demonstrated the promising role of such a pharmacophore in the design of multitarget antipsychotics.

By adopting a D_2R homology model, Kaczor *et al.* conducted a structure-based virtual screening from a library of 6.5 million compounds, which resulted in the discovery of another D_2R/5-$HT_{2A}R$ antagonist (**16**; D2AAK4; Fig. 2) [40,41]. *In vitro* studies showed that compound **16** had a balanced D_2R and 5-HTR binding affinity profile. Moreover, *in vivo* studies suggested that it decreased amphetamine-induced hyperactivity and improved memory consolidation in passive avoidance tests. As a modification of another experimentally confirmed virtual screening hit D2AAK1 [40], a series of indole derivatives was synthesized and pharmacologically studied. SAR studies revealed that a thiopheneylmethyl group attaching to the tetrahydropyridine fragment contributed favorably towards increased 5-$HT_{2A}R$ and D_2R affinities, whereas other types of substitution on the indole ring did not significantly influence potency on either receptor. Eventually, compound **17** was found to be a dual antagonist of both 5-$HT_{2A}R$ and D_2R with affinities in the nanomolar range (Fig. 2). Further behavioral studies revealed that it exhibited antipsychotic, procognitive, and antidepressant activities in chimney tests and forced swim tests (FST) in mice [42].

To obtain novel multitarget analogs with improved D_2R affinity and high affinity for 5-HTRs, Del Bello *et al.* performed extensive SAR studies on a 6,6-diphenyl-1,4-dioxane

scaffold, which lead to the discovery of compound **18**. *In vitro* results indicated that, in addition to desirable binding affinity at target receptors, compound **18** showed a multitarget combination of 5-HT_{1A}/D_4 agonism and D_2/D_3/5-HT_{2A} antagonism, which made it a good starting point to develop new pharmacological tools potentially useful in treating schizophrenia [43]. These results suggested that ligands that simultaneously modulate the function of several monoaminergic receptors provide beneficial therapeutic effects for patients with schizophrenia.

MTDLs targeting serotonin and dopamine receptors to treat depression— Depression remains a major burden on societies worldwide [44]. The pharmacological profile of the molecules with high affinities for 5-HT_{1A}R and D_2R is thought to improve the treatment of depression [45]. As a versatile drug template with high affinities for these two receptors, a long-chain arylpiperazine (LCAP) was linked with another tricyclic heterocycle to give compound **19**, which showed a balanced affinity profile over 5-HT_{1A}R and D_2R (5-HT_{1A}R, K_i = 19 nM; D_2R, K_i = 2 nM) (Fig. 2) [46]. Further molecular docking studies showed that compound **19** orientated towards the second transmembrane helix rather than the sixth one, compared with other analogs in the D_2R-binding pocket. Such a unique binding conformation conferred the largest number of hydrophobic interactions, which could explain its excellent affinity at the D_2R.

Given that combining selective serotonin reuptake inhibitors (SSRIs) with agonist/antagonist activity at various 5-HTRs could significantly increase the effectiveness of depression therapy [47], Wrobel *et al.* [48] recently reported the discovery of compound **20** (Fig. 2), in which a 3-piperidin-3-yl-1*H*-indole moiety, conferring high affinity for the 5-HT_{1A}R and serotonin transporters (SERTs), was tethered with a cyclic imide moiety through an appropriate linker. Further studies indicated that compound **20** exhibited balanced affinities for the 5-HT_{1A}R/5-HT_6R/5-HT_7R/SERT/D_2R and showed antidepressant-like activity in the FST model. The metabolic stability of **20** was similar to those of compounds without the fluorine substitution on the aromatic ring. In light of *in silico* predictions of the site of metabolism, one explanation might be that cytochrome P450 (CYP)-mediated oxidations of two-carbon atoms in the alkyl linker lead to *N*-dealkylation and, thus, the degradation of the molecule into two smaller parts.

After replacing the 3-piperidin-3-yl-1*H*-indole moiety with different LCAP moieties, a series of novel MTDLs was generated. Further SAR studies revealed that halogen substituents in the phenylpiperazine ring potently improved the binding affinity for 5-HT_{1A}R, which could be attributed to the formation of specific halogen bonds between the ligand and the receptor. In addition, substitutions at the *para*-position of phenylpiperazine increased the affinity for the SERT, whereas *ortho*-position substitution was beneficial for 5-HT_{1A}R. Among these ligands, compound **21** emerged with promising mixed receptor profiles for 5-HT_{1A}R, D_2R, and the serotonin transporter (K_i = 1.3 nM, 182 nM, and 64 nM, respectively) (Fig. 2) [49].

Stimulating both the 5-HT_{1A}R and D_4R simultaneously could be a promising strategy to ameliorate anxiety [50]. However, no specific drugs with selective dual agonism for both receptors have been reported. Recently, Sumitomo Dainippon Pharma Co. Ltd patented

compound **22** with dual agonism for 5-HT_{1A}R and D_4R by utilizing the pharmacophore-linking strategy (Fig. 2). Besides its excellent binding affinity profile (5-HT_{1A}R, K_i = 0.4 nM; D_4R, K_i = 11 nM) and relatively long half-life in cynomolgus monkey ($t_{1/2}$ = 11 h), as well as acceptable hERG inhibition activity (IC_{50} = 2.5 μM), compound **22** exhibited a 82.5% decrease in the cataleptic freezing reaction in a contextual fear conditioning test and a 74.7% decrease in the immobility time in a FST model [51]. This evidence demonstrates the therapeutic potential of multitarget ligands with high affinities for 5-HTRs and D_2Rs in combatting depression.

MTDLs targeting serotonin and other GPCRs to treat depression—The noradrenaline (NE) and 5-HT systems have reciprocal interactions and modulate each other's actions in the brain [52]. It has been suggested that novel molecules acting at specific 5-HT and NE receptors would achieve a higher efficacy with fewer adverse effects in treating anxiety and depression. Compound **23** (ACH-000029, Fig. 2) is a newly released compound that acts at a range of serotonergic (5-HT_{1A} partial agonism and 5-HT_{2A} antagonism) and α-adrenergic (α-1A, 1B and 1D antagonism) receptors (α_1Rs). It also exhibited high permeability in Caco-2 cells and low CYP inhibition as well as acute anxiolytic effects in the marble-burying (MB) and light–dark box (LDB) models [53]. These properties, together with its low toxicity, supported the further development of ACH-000029 as a drug candidate for the treatment of psychiatric disorders.

Balanced modulation of 5-HTRs and α_1Rs could be beneficial for the treatment of several psychiatric disorders [54]. Through ring expansion/opening and molecular elongation/simplification of a versatile 1,3-dioxolane scaffold, a series of novel compounds was obtained recently in the search for novel antidepressants. Preliminary SAR studies suggested that insertion of a methylene in the lateral chain slightly improved binding affinities for all the α_1R subtypes, while leaving an unchanged affinity for 5-HT_{1A}R. Similar results were observed when adding a methylene into the 1,3-dioxolane scaffold. The most promising compound showed potent agonism at 5-HT_{1A}R and high binding affinities for α_1Rs (**24**; Fig. 2). In addition, compound **24** has a high biodistribution in brain compartments when orally administered. Further open-field tests demonstrated the anxiolytic and antidepressant effects of **24**, suggesting its potential as an antidepressant [55]. The above examples demonstrate that targeting 5-HTRs and another specific GPCR simultaneously could be a valuable approach to generate novel MTDLs with desirable therapeutic effects for combatting depression.

MTDLs targeting MAO-B and other GPCRs to treat Parkinson's disease—Parkinson's disease (PD) is one of the most common CNS disorders, with a significant impact on the aging human population. The progress of this disease is typically complicated and multifactorial, leading to degeneration of neurons and inflammatory processes in the brain [56]. As an emerging strategy, MTDLs have gained significant attention in the treatment of PD. One of the proposed strategies is the combination of monoamine oxidase B (MAO-B) inhibition and adenosine A_{2A} receptor (A_{2A}AR) blockade [57]. Following this strategy, Kuder *et al.* [58] reported a series of novel annelated xanthine derivatives bearing a *N*9-substituted benzyl group targeting both proteins. Follow-up SAR studies suggested that

fused rings adjacent to the xanthine scaffold were tolerated towards both MAO-B and A_{2A}AR, whereas the heterocyclic aromatic substitutions at the *N*9 position were detrimental to affinities. The attached benzyl group appeared to be favorable and further halogen substitutions increased the potency toward both targets. After *in vitro* screenings, compound **25** (A_{2A}AR, K_i = 264 nM; MAO-B, IC_{50} = 243 nM) was advanced into pharmacological evaluations. Unfortunately, moderate hepatotoxicity of **25** was observed when used with the HepG2 cell line. Meanwhile, a similar phenomenon was also found in other analogs, indicating that such a tricyclic scaffold presents a risk of hepatotoxicity.

Two series of phenyl xanthine (PX) derivatives were synthesized and biologically evaluated to explore the speculation that PX derivatives have antiparkinsonian properties [59]. Among these new PX derivatives, compound **26** acted as a potent A_{2A}AR antagonist with K_i value of 330 nM and a MAO-B inhibitor with a IC_{50} value of 290 nM (Fig. 3). It did not show noticeable cytotoxicity against human neuroblastoma SH-SY5Y cells at 10 μM concentration. Additionally, it also displayed significant anticatalepsy ability at a dose of 50 mg/kg in haloperidol-induced catalepsy studies. These favorable properties suggested compound **26** as a promising candidate for treating PD.

The design of MAO-B/H_3R dual-targeting ligands is a potentially innovative approach to modulate the dopamine imbalance in patients with PD [60]. Three series of compounds were generated by merging a H_3R pharmacophore with indanone-related MAO-B motifs [61]. The derivatives with substitutions at the C6 and C5 positions of indanone did not show the desired H_3R affinity, although they did show moderate MAO-B inhibitions, and similar results were found in more lipophilic 2-benzylidene-1-indanone derivatives. After introducing a basic moiety with a polymethylene linker to the C4′ position of the benzylidene, the resulting compounds showed favorable potencies at both targets. Of these, compound **27** showed desirable binding affinity for H_3R (K_i = 32 nM) and inhibition of MAO-B (IC_{50} = 1455 nM) (Fig. 3). Given that a reversible MAO-B inhibitor might have fewer adverse effects than an irreversible one, following dilution studies in excess of substrates revealed a reversible inhibition of compound **27** against MAO-B, suggesting it as a lead for further design of novel therapeutics against PD.

By merging structural elements of the antioxidant ASS234 and the H_3R antagonist ciproxifan, a series of novel indole derivatives was designed, synthesized, and biologically evaluated [62]. SAR studies suggested that the length of the spacer between the cyclic aliphatic amines and indole core was crucial to the binding to MAO-A/B, with a two-carbon atom spacer being optimal for piperidine derivatives. From this small series, compound **28** (contilisant) was found to have inhibitory activities towards MAO-A/B and H_3R as well as ChEs (Fig. 3). From dilution studies in substrate excess, contilisant was found to have improved reversible inhibition of MAO-B compared with ASS234. Additional *in vitro* studies showed that contilisant offered significant neuroprotection against the toxic insults assayed at 0.3 μM, which was comparable to that offered by melatonin. Further *in vivo* studies indicated that contilisant (at 1 mg/kg intraperitoneally) also significantly improved lipopolysaccharide (LPS)-induced cognitive deficits.

In the light of the above studies, targeting both MAO-B and relevant GPCRs appears to be a promising approach to identify novel therapeutics with high potency and desirable druggability to confront the multifactorial nature of PD neurodegeneration.

MTDLs targeting H3 receptor and other targets to treat Alzheimer's disease— AD is a progressive neurological disorder with multifactorial etiology, and is particularly prevalent in older people [63]. Mounting evidence indicates that combining ChE inhibitory activity with H**3**R antagonism could provide new tools to combat the cognitive symptoms of AD [64]. By applying virtual screening from 200 structures of pitolisant and ciproxifan analogs, Bajda *et al.* obtained 26 hits with high ChemScore values targeting ChEs. Of these, 15 compounds were synthesized and biologically evaluated. According to preliminary SAR study results, homopiperidine with a five- or six-carbon atom linker was preferable for H_3R affinity, whereas an eight-carbon atom linker was beneficial for ChE inhibition. After further *in vitro* screenings, compound **29** stood out and showed potent inhibitory activities toward ChEs (AChE, IC_{50} = 0.48 μM; BuChE, IC_{50} = 0.44 μM) as well as potency against H_3R with K_i value of 159.8 nM [65]. Further *in silico* ADMET prediction by SwissADME demonstrated that **29** had properties consistent with the rule proposed by Veber [rotatable bonds ≤10 and topological polar surface area (TPSA) ≤140] [66]. These findings suggested that compound **29** could serve as a lead for further optimization and development of more potent MTDLs with more accurate pharmacokinetics profiles.

Through multicomponent synthesis, Malek *et al.* incorporated a typical H_3R antagonist motif cycloalkylamine into 1,4-dihydropyridine (1,4-DHP) via a convenient linker to generate several new tri-target small molecules. SAR studies revealed that, when a three-carbon atom linker was attached, desirable H_3R activities were always observed for synthesized compounds, whereas a longer linker appeared more favorable for BuChE inhibition. As a promising lead, compound **30** showed potent affinity for H_3R (K_i = 565 nM) and moderate BuChE inhibition (IC_{50} = 7.83 μM) as well as a reasonable Ca^{2+} channel blockade activity (IC_{50} = 21 μM) (Fig. 3). Further *in vivo* studies demonstrated that compound **30** was capable of restoring cognitive impairment induced by LPS at 10 mg/kg, which was comparable to the effect of donepezil at 1 mg/kg [67].

Ghamari *et al.* [68] recently tested a computationally identified lead compound **31** (Fig. 3). Biological evaluations revealed that it acted as a potent H_3R antagonist (EC_{50} = 31 nM) and a dual inhibitor against AChE and BuChE with IC_{50} values of 8.40 and 4.93 μM, respectively. Moreover, MTT assays suggested that compound **31** did not exhibit significant cytotoxicity in H_3R-expressing CHO-K1 cells (GI_{50}≈100 nM). Such a ligand with dual inhibitory activity on H_3R and ChEs could be utilized for lead optimization to achieve higher potency and efficacy. These aforementioned observations further validate the hypothesis that hitting the multiple targets implicated in AD etiology may provide novel therapeutics with desirable pharmacological profiles.

MTDLs targeting CB2 receptor and ChE to treat Alzheimer's disease—CBR subtype 2 (CB_2R) agonists exert beneficial effect in neurodegenerative disorders [69]. By contrast, ChEs inhibition has been a well-established approach to achieve symptomatic cognition improvement in patients with AD [70]. Dolles *et al.* [71] merged pharmacophores

from a BuChE inhibitor and a CB_2R agonist into benzimidazole-based small molecules. For these novel ligands, different chains at position 5 of the benzimidazole core had varying effects on CB_2R affinity, whereas it appeared that bulky and nonpolar substituents were tolerated. Among them, compound **32** was identified as a dual-acting ligand with a balanced affinity–inhibitory activity and excellent selectivity over both CB_1R and AChE (Fig. 3). *In vivo* studies suggested that compound **32** was active with sustained prevention of $A\beta_{25–35}$-induced learning deficits in passive avoidance tests at 1 mg/kg. These data showed the possibility of combining a ligand with selective and balanced GPCR activity and/or enzyme inhibition to achieve *in vivo* potency for AD treatment.

On the basis of these observations, the Dolles research group further adopted the pharmacophore-linking strategy and synthesized a series of novel compounds in which a ChE inhibitor tacrine and a benzimidazole-based CB_2R agonist were linked by an appropriate spacer [72]. In this study, the types of linker, chain length, and connection point were extensively investigated. SAR studies indicated that if tacrine was attached to position 1 of the benzimidazole core, the affinities for both ChEs and CB_2R increased with the length of the spacer. However, when switched to position 5, experimental data did not support such a correlation. Additionally, the introduction of a 2-PEG-linker or a disulfide linker had no significant effect on either targets. Among them, compound **33** emerged as a potent AChE and BuChE dual inhibitor (AChE, pIC_{50} = 7.10; BuChE, pIC_{50} = 8.7) and a partial agonist at the CB_2R (K_i = 4.5 μM;EC_{50} = 3.05 μM; E_{max} = 51%) (Fig. 3). In addition to the inhibition of AChE-mediated and self-induced Aβ aggregation, compound **33** did not show any neurotoxicity at the doses tested (max. 10 μM). Further *in vivo* studies indicated that compound **33** significantly prevented the development of learning deficits and completely prevented $A\beta_{25–35}$ impairments at a dose of 0.3 mg/kg. Given the promising *in vitro* and *in vivo* study results and its low hepatotoxicity, compound **33** could be a potential clinical candidate for treating AD. As an emerging approach, MTDLs that have a balanced affinity and desirable selectivity profile on CB_2R and ChEs could be a solution for the treatment of both cognitive and pathophysiological impairments in AD.

GPCR-targeted MTDLs to treat cardiovascular diseases

Hypertension is a key risk factor for cardiovascular diseases, which directly or indirectly cause deaths of at least 9 million people globally every year [73]. Among various approaches to control hypertension, angiotensin II type 1 receptor (AT_1R) antagonists with additional binding affinity and activation at peroxisome proliferator activated receptor-γ (PPARγ) might be beneficial in controlling blood pressure and lipid metabolism [74]. Choung *et al.* [75] applied structural modification on the basis of a Korean Food and Drug Administration (FDA)-approved PPARγ partial agonist, fimasartan. According to the docking mode of fimasartan with the AT_1R crystal structure, the pyrimidinone core was maintained because it can form hydrophobic and hydrogen bonding interactions with key amino acid residues in the binding pocket. Meanwhile, rational modifications were conducted, including bioisosteric replacement of the tetrazole moiety in fimasartan and side-chain variation on the pyrimidinone core. Eventually, lead compound **34** was observed to have excellent AT_1R antagonism (Ca^{2+} mobilization IC_{50} = 2.2 nM) and PPARγ agonism (EC_{50} = 3.1 μM) (Fig. 3). Additionally, *in vivo* studies indicated that compound **34** exhibited

suppressive effects on blood pressure increase at a dose of 30 mpk in an angiotensin II-induced hypertension rat model and significantly suppressed blood glucose increase at a dose of 60 mpk in leptin-deficient db/db mouse diabetes model.

Dual inhibition of the renin angiotensin system (RAS) and the cardiac natriuretic peptide system (CNPS) could provide superior reductions in blood pressure [76]. To this end, McKinnell *et al.* designed and synthesized a series of MTDLs by merging the structures of an AT_1 antagonist losartan and a neutral endopeptidase (NEP) inhibitor thiorphan (Fig. 3) [77]. SAR studies showed that introduction of an *S*-isobutyl group into the thiorphan moiety maintained the best balance of potency at both targets. It was notable that adding a fluorine atom *ortho*- to the head group maintained NEP activity and significantly boosted AT_1 potency, whereas alternative fluorine substitution patterns compromised the potency at both targets. Based on these findings, compound **35** (TD-0212), a dual AT_1 antagonist/NEP inhibitor, was chosen for *in vivo* studies. It was suggested that compound **35** resulted in blood pressure reductions similar to omapatrilat in models of renin-dependent and -independent hypertension. Additionally, it did not increase tracheal plasma extravasation (TPE) at antihypertensive doses, indicating its low risk of angioedema. These aforementioned observations highlight the importance of the approach by targeting AT_1R and another target involved in hypertension pathological processes in the development of novel antihypertension agents.

GPCR-targeted MTDLs for diabetes

Type 2 diabetes mellitus is a metabolic disease with insulin resistance and/or insulin deficiency. The important role of the free fatty acid receptor 1 (FFAR1 or GPR40) in stimulating incretin secretion and mediating glucose-stimulated insulin secretion makes it a desirable target for antidiabetic drugs [78]. As mentioned earlier, PPARs have a pivotal role in metabolic regulation and are highly associated with glucose control and lipid metabolism [79]. Taken together, the multifunctional FFAR1/PPARs agonists could be a potential strategy to improve insulin secretion and sensibility. To test this hypothesis, Li *et al.* designed and synthesized several compounds by fusing the common scaffold of PPARs and FFAR1 agonists. Further biological assays demonstrated their multifunctional profiles targeting both the PPARs and FFAR1. First, SAR studies indicated that the linker between two pharmacophores had a strict requirement for the length and conformational flexibility, with a five-carbon atom one appeared to be preferred for the potency of both targets. Moreover, the carboxylate moiety was crucial for activities as well as for maintaining balance between FFAR1, PPARδ, and PPARγ. Among them, compound **36** had relatively balanced activities between FFAR1 and PPARs (Fig. 3) and also improved the glucose tolerance of leptin-deficient ob/ob mice in a dose-dependent manner [80].

By inserting a thiazole-based fragment into compound **36**, another novel MTDL compound, **37**, was generated [81]. In addition to the enhancement of tolerance of ob/ob mice for glucose loading, the introduction of a thiazole-type heterocyclic ring also conferred reasonable pharmacokinetics profiles with high plasma concentrations, a sustained half-life, and low clearance. Given the important role of the thiazole ring in optimizing pharmacological profiles of compound **37**, Li *et al.* further conducted structural modification

by incorporating bioisosteres to replace the thiazole ring [82]. *In vitro* study results revealed that most of the thiazole bioisosteric derivatives showed lower activities on FFAR1 and PPARδ compared with the parent compound **37**, whereas thiadiazole and oxadiazole derivatives appeared to be more potent. Moreover, the introduction of fused heterocycles had varying effects on potency towards both targets. Among them, compound **38** had an improved balance between FFAR1 and PPARδ and also exerted better glucose-lowering effects and insulin sensitivity compared with compound **37**. These desirable effects of multifunctional FFAR1/PPARs ligands reveal their potential applications in the development of novel antidiabetics.

Concluding remarks and future perspectives

From the above-discussed examples, it appears that a bioactive compound carrying therapeutic effects on the modulation of a single target might not be an optimal drug candidate for treating complex diseases [21]. In an effort to tackle this issue, the development of MTDLs has become a promising approach in drug design and development campaigns. Meanwhile, the pivotal roles in the pathophysiology of various human diseases and accessibilities of druggable sites at the cell surface make GPCRs desirable targets for developing MTDLs. Recent advances in MTDLs targeting GPCRs have mainly focused on treating CNS disorders, with very few for cancer treatment. However, several GPCRs have been suggested as potential cancer targets based on mRNA expression analyses of tumors [83]. Moreover, accumulating data suggest that crosstalk between GPCRs and other well-established anticancer targets [e.g., epidermal growth factor receptor (EGFR)] occur in various cancers. This reveals a promising strategy of developing novel MTDLs targeting both the GPCRs and EGFR for cancer therapy [84]. Although GPCRs are associated with nearly every aspect of human pathophysiology [15], many of them, namely untapped GPCRs, have not yet been targeted. The exploitation of these targets will further open new possibilities in GPCR-targeted MTDL discovery. Furthermore, owing to recent breakthroughs in GPCR crystallography, at least 44 distinct GPCR structures and 205 ligand–receptor complexes are now resolved, which has not only expedited the development of GPCR-targeted MTDLs [85], but also provided more opportunities to refine pharmacophores and further guide the design of multitarget ligands.

Meanwhile, drug repositioning/repurposing, referring to the exploration of new indications for marketed drugs, has gained interest over the past decade because it can reduce the time and cost of bringing a therapeutic to the market [86]. Of the approved GPCR-targeted drugs, 156 (33%) have more than one indication and the overall average is 1.5 indications per drug [15], showing the potential utility of bioactive compounds targeting GPCRs for several indications. In this sense, GPCR-targeted MTDLs could be identified as natural candidates by retargeting those off-target drugs; thus, numerous so-called ‘dirty drugs’ showing multifunctional profiles in their early stage of research could be reconsidered for new purposes [36]. Moreover, for some selective GPCR-targeted drugs already at the later stages of development, other targets outside their main therapeutic targets might be identified, which will further facilitate the development of GPCR-targeted MTDLs. With increasing understanding of the pathogenesis of complex diseases as well as new breakthroughs in the structural biology and pharmacology of GPCRs, the search for novel GPCR-targeted

MTDLs with an appropriate balance of biological activities will generate major impacts in drug design, discovery, and development.

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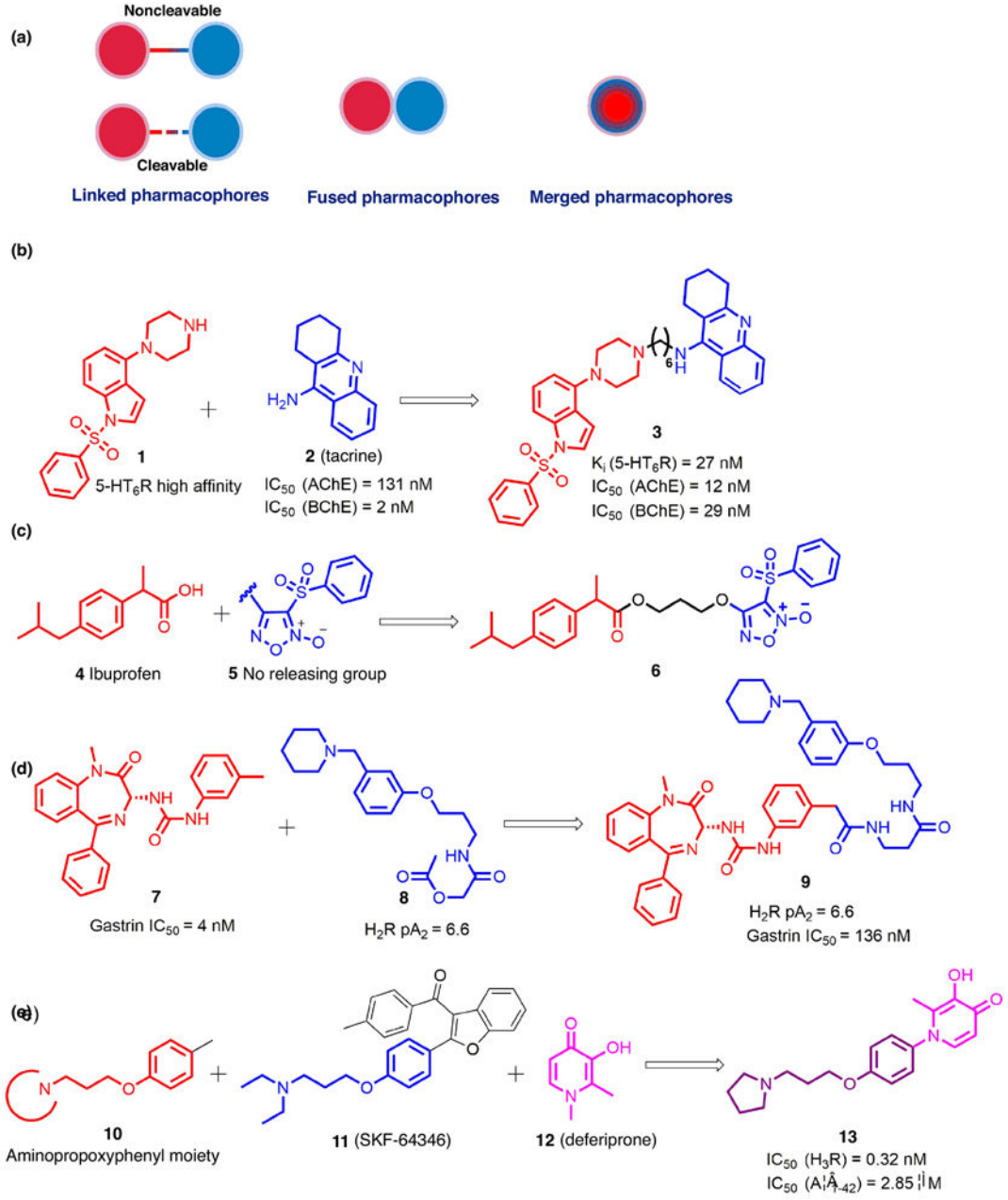

FIGURE 1.

(a) Multitarget drug design strategies based on pharmacophore integration levels; (b) A representative example of noncleavable multitarget-directed ligands (MTDLs); (c) A representative example of cleavable MTDLs; (d) A representative example of fused pharmacophores; (e) A representative example of merged pharmacophores. The parts of structures colored red, blue, and pink represent different pharmacophores conferring corresponding biological activities; the parts in black and purple represent the linker and the merged scaffold between the two pharmacophores, respectively.

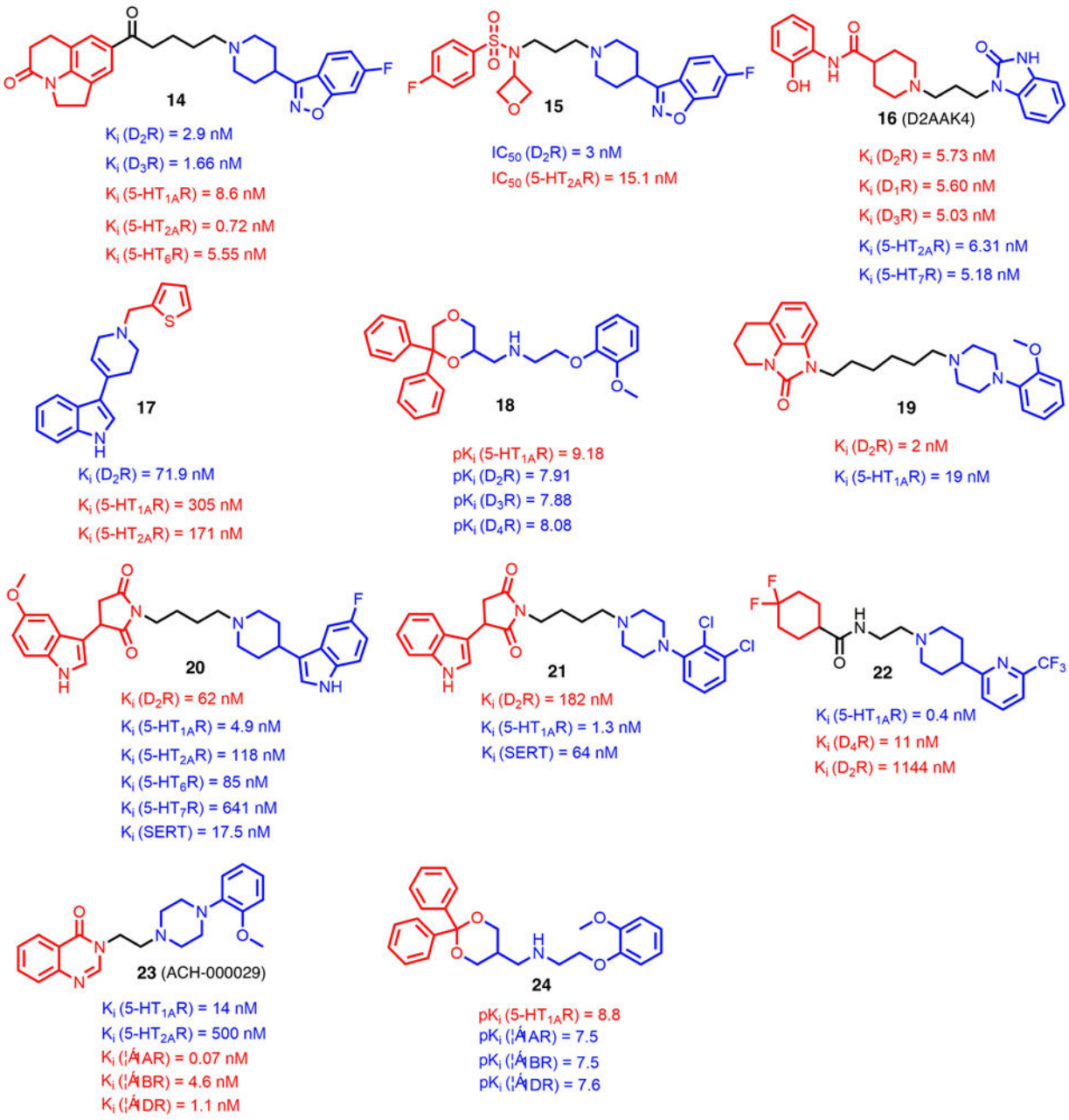

FIGURE 2.

Chemical structures of recently reported G-protein-targeted receptor (GPCR)-targeted multitarget-directed ligands (MTDLs) (compounds 14–24). The parts of structures colored red and blue represent the two pharmacophores conferring corresponding biological activities, and the parts colored black represent the linkers between the two pharmacophores.

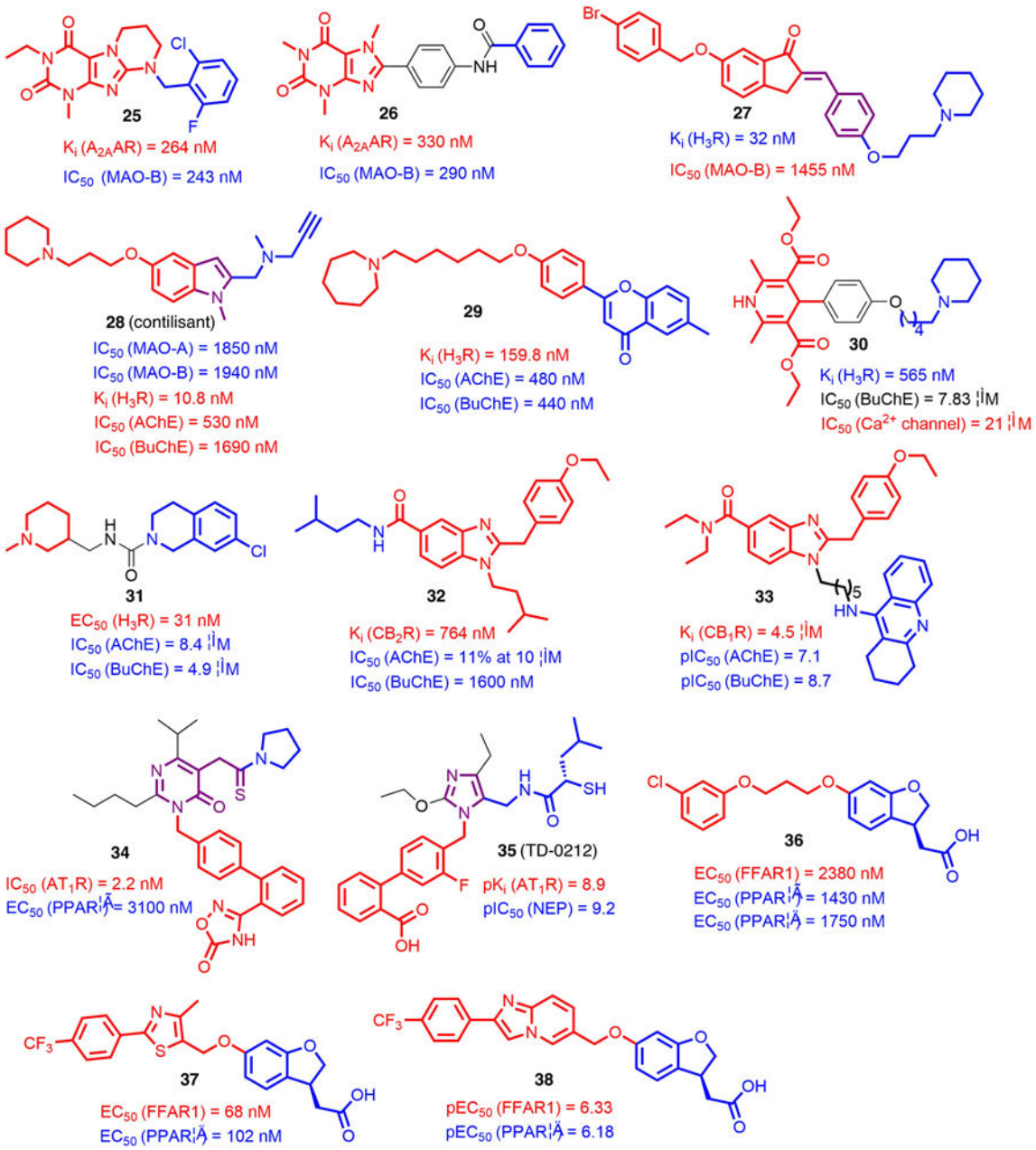

FIGURE 3.

Chemical structures of recently reported G-protein-targeted receptor (GPCR)-targeted multitarget-directed ligands (MTDLs) (compounds 25–38). The parts of structures colored red and blue represent the two pharmacophores conferring corresponding biological activities, and the parts colored black and purple represent the linkers and the merged scaffolds between the two pharmacophores, respectively.