Polypharmacology Analysis of Anti-osteoporosis Agents and Cannabinoid 2 Receptor Inverse Agonists for Osteoporosis Drug Research

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Osteoporosis (OP) is a complex bone metabolic disease indicated by excess bone resorption over bone formation. Some cannabinoid receptor 2 (CB2) inverse agonists have shown dual antiresoptive and anabolic effects, and thus are potential anti-OP agents. However, the underlying mechanism, especially for their anabolic effects, is not very clear. To fully understand the pharmaceutical roles of CB2 inverse agonists, we implement polypharmacology analysis by using two off-target prediction tools (HTDocking and TargetHunter). First, we construct osteoporosis domain specific knowledgebase (OP-KB) that contains the data of anti-OP targets, related proteins, drugs, and chemicals collected from a variety of available databases. Five approved anti-OP drugs undergo HTDocking to screen all OP-related proteins in OP-KB for the detection of potential off-targets. This prediction validates the reliability of HTDocking method in OP-KB based on the concordance of actual and predicted targets. Similarly, we analyze polypharmacological effects of six CB2 inverse agonists including two in-house compounds (Xie95-1042, Xie95-1171). Potential targets are predicted and ranked based on the degree of connectivity to drugs. To validate our prediction and reveal possible interaction modes, we perform molecular docking between two in-house compounds and AM630 with six top predicted targets that are all responsible for bone formation. We observe similar binding interactions between these three compounds with similar nearby residues in comparison to initial

crystal structures. Hence, we hypothesize that CB2 inverse agonists may act on other anti-osteoporosis targets such as NOS3, DHI1, VDR, ALDH2, TRFL and ESR1 to achieve their anabolic effect for bone formation. In addition, TargetHunter is used to predict off-targets beyond OP-related protein list. Based on the prediction of two in-house compounds, aldehyde dehydrogenase 1A1 (ALDH1A1), a key protein involved in cancer development discovered recently, is probably another target for those two CB2 inverse agonists. This finding may imply new mechanism of the anti-cancer effect of CB2 inverse agonists, and also facilitate relevant drug repurposing. In summary, our approach provide a paradigm for polypharmacology study on a specific disease domain, which we believe can be widely applied to other complex diseases study to accelerate drug discovery.

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1.0 INTRODUCTION

1.1 OSTEOPOROSIS

Osteoporosis has been a global public health problem that affects over 200 million people worldwide.[1] The high risk population includes men aged over 50 and menopausal women. Recent reports show in the United States alone, 52 million people are suffering from osteoporosis or are at risk of developing this disease.[2] Fracture is the most common complication of osteoporosis that brings costly consequences in both household and socioeconomic terms[3]—it is estimated that 40% of women and 15-30% of men with osteoporosis have a fracture during their lifetime with a related cost of 19 billion dollars each year in US.[4, 5] In addition, osteoporosis is responsible for more hospital days than diabetes, breast cancer or myocardial infarction.[6]

Osteoporosis is associated with abnormal bone remodeling with increased ratio of bone resorption over bone formation. Bone remodeling is a continuous lifetime process where old bone cells are removed by the osteoclasts and new bone cells are deposited by the osteoblasts.[7] In the healthy human body, the balanced bone remodeling cycle facilitates repair of microdamage and provides calcium from bone tissue for cellular functions.[8] However, bone remodeling is accelerated in postmenopausal women and the aging population, leading to excess

bone resorption over bone formation, acute or sustained loss of bone mass, disrupted bone architecture, and finally the occurrence of osteoporosis.[9] The detailed mechanism of bone remodeling in osteoporosis is shown in **Figure 1**.

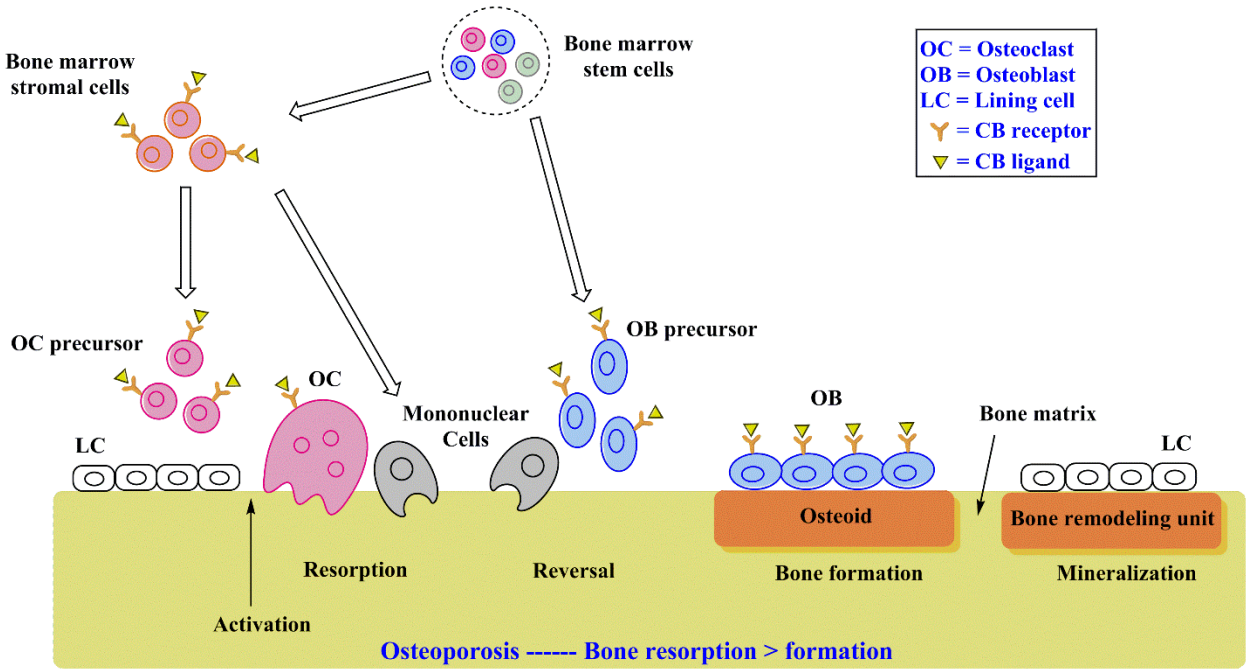


Figure 1. Regulation of bone remodeling in osteoporosis by the endocannabinoid system. 1)Remodeling starts when osteoclasts are activated, resorb bone, and create bone cavities. Osteoblast activation follows, as well as formation of osteoid, which fills in the bone cavities. Once the bone martix synthesis is done, osteoblasts become embedded in the matrix and function as osteocytes. However, in osteoporosis, bone remodeling occurs with excess bone resorption, resulting in unreversable bone loss. 2) The endocannabinoid system is mainly composed of cannabinoid receptors (CB) and cannabinoid receptor ligands (CBL). They are widely found in skeleton, including osteoclasts, osteoblasts, their precursors, and bone marrow stromal cells. They work together to control bone cell proliferation, differentiation and function.

Currently, there are two therapeutic strategies for osteoporosis: one is antiresorptive therapy, which inhibits bone resorption; the other is anabolic therapy that is perceived to augment bone formation. However, all current marketed anti-OP drugs have shortcomings because of limited potency and potential for toxicities.[10] For instance, bisphosphonates, the first-line antiresorptive agents, eventually cause a decrease of osteoblast function in long-term use that is attributed to atypical bone fractures.[11] Another antiresorptive agent, Calcitonin, is recently withdrawn from the market in Europe due to its increased risk of cancer with long-term use.[12] On the other hand, the use of the anabolic agent, Teriaratide, is restricted in two years' use due to increasing risk of osteosarcoma.[13] Taking the increasing health concern of osteoporosis and safety issue of currently available drugs together, there is great interest and demand in exploring new-generation drugs with novel mechanisms for the treatment of osteoporosis. The focus of current and future perspective would be better understanding of the biology of osteoclasts and osteoblasts and development of new drugs that can hold synergistic effect for both osteoclast and osteoblast regulation.

1.2 CANNABINOID RECEPTOR 2 AND ITS ROLE IN OSTEOPOROSIS

1.2.1 The skeletal endocannabinoid system

The endocannabinoid system is composed of two cannabinoid receptors (cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2)), their endogenous ligands (Anandamide and 2-Arachidonylglycerol (2-AG)), and ligand metabolizing enzymes.[14] The endocannabinoid system has been recognized to play an important role in regulating a variety of physiological

processes and are involved in pharmacological effects against several diseases and disorders, such as pain,[15] immune and neurodegenerative disorders,[16, 17] cancer,[18] drug abuse,[19] cardiovascular and gastrointestinal disorders,[20] stroke and inflammation,[21, 22] etc.(**Figure 2**). Hence, developing synthetic cannabinoids targeting the endocannabinoid system shows promise in therapies for these diseases.

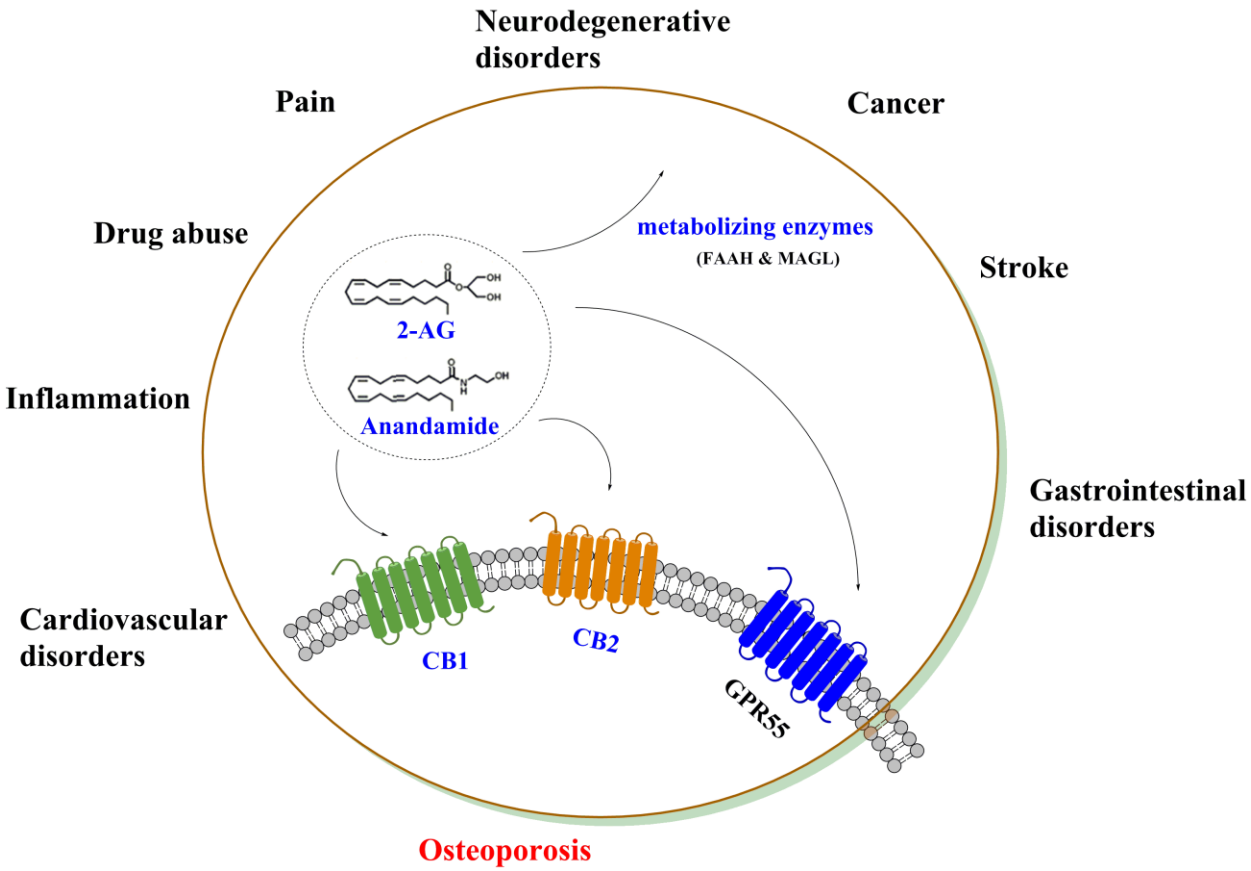


Figure 2. The endocannabinoid system and its role in diseases. Abbreviations are listed in Appendix A.

In recent years, the skeletal endocannabinoid system and its therapeutic potential in osteoporosis have drawn extensive attention.[23-26] Several studies demonstrate its key role in adjusting the bone remodeling process by claiming that cannabinoid receptors, endocannabinoids

and their metabolizing enzymes are expressed in the skeleton (**Figure 1**), where they collaborate with each other to control bone cell proliferation, differentiation and function.[27] Moreover, the endocannabinoids are produced by osteoclasts and osteoblasts with similar level to those found in other organs.[27, 28] Also, the treatment with endocannabinoids is effective for ovariectomy-induced bone loss in preclinical studies.[29, 30]

Together with endocannabinoids, a growing number of synthetic cannabinoid receptor binding ligands are reported to influence bone cell proliferation and activity both in vivo and in vitro (**Table 1.**).[29-35] They includes agonists such as CP55490, WIN55212, HU308, etc., and inverse agonists such as AM630, SR144528, and Rimonabant, which upon binding to CB receptor can not only block agonist-induced receptor activation, but also reverse subsequent activity thereby modulating downstream signaling pathways in the opposite direction to those of classical agonists.[24] From **Table 1**, we can see that overall, the CB agonists can increase the number and activity of both osteoclasts and osteoblasts, which may be served as anabolic agents in osteoporosis therapy. On the other hand, CB inverse agonists, which behave as antiresorptive agents, can reduce the number and activity of both osteoblasts and osteoclasts. However, some inconsistent data arise between different groups. For example, HU308, a selective CB2 agonist, shows a stimulatory effect on osteoclast formation in the study of Dr. Idris' group.[31] But in complete contrast, Orr Ofek and colleagues claim that HU308 can inhibit osteoclast formation.[30] For another case, AM630, a CB2-selective inverse agonist, inhibits osteoclast differentiation (IC_{50} = 0.33 μM) and suppressed osteoclast activity (IC_{50} = 7.2 μM) *in vitro*.[36] But at high concentration, AM630 can stimulate human osteoclast formation instead.[34] These conflicting data may result from complex pharmacological and off-target effects of certain CB

ligands, as well as unclear mechanisms of the influence that the CB receptor has on bone cell activity.

Table 1. The role of cannabinoid receptor ligands in the regulation of osteoclast and osteoblast proliferation and activity in vitro and in vivo.

	Ligands	Receptor	Osteoclast number	Osteoclast activity	Osteoblast number	Reference
CB Agonists	Anadamide	CB1/CB2	↑	↑	↑	[29]
	2-AG	CB1/CB2	↑	↑	↑	[29]
	CP55490	CB1/CB2	↑	↑	↑	[30, 31]
	WIN55212	CB1	NT*	NT	↑	[30, 31]
	JWH133	CB2	↑↓	NT	↑	[31, 32]
	JWH015	CB2	NT	↑	↑	[31, 32]
	HU308	CB2	↑↓	NT	↑	[30, 31]
CB Inverse agonists	AM630	CB2	↓↑	↓	↓	[34, 35]
	SR144528	CB2	↓	↓	↓	[34, 35]
	AM251	CB1	↓↑	↓	↓	[33]
	Rimonabant	CB1	↓	↓	↓	[33]

* Not tested. This table is modified based on the reference [23]

1.2.2 Cannabinoid receptor 2 inverse agonist as treatment of osteoporosis

Although both CB1 and CB2 ligands exhibit therapeutic potential in osteoporosis, CB2 ligands have one inherent advantage. Unlike CB1 that is expressed predominantly in brain,[37] CB2 is primarily expressed in peripheral cells and tissues,[38] which provides an approach to avoiding psychotropic side effects caused by targeting CB1.[39] In particular for osteoporosis therapy, CB2 ligands hold more potential over CB1 ligands according to human genetic studies that indicated polymorphisms in CNR2 (the gene encoding CB2) were associated with postmenopausal osteoporosis.[40, 41] Moreover, osteoblasts, osteoclasts and osteocytes express CB2 receptors at a significantly higher level than that reported for CB1.[31, 34, 42] Taken together, selective targeting of CB2 over CB1 might shed light on the treatment of osteoporosis.

Our lab is focusing on the design and discovery of CB2 inverse agonists in the treatment of osteoporosis. A series of promising compounds has been discovered to show potent and selective CB2 binding and good osteoclast inhibition.[43, 44] Importantly, one of our compounds, Xie95-1042, shows anabolic effects by enhancing osteoblast activity at the concentration of 10^{-12} M[45] (Some data is not published). In agreement with this observation, the phytocannabinoid Tetrahydrocannabivarin, known as a CB2/CB1 inverse agonist with assumed inhibitory effect on bone formation, is reported to promote bone module formation.[33, 46] Another reason for focusing on CB2 inverse agonist study is attributable to the aforementioned genetic study.[32,33] The CB2 gene is validated to be associated with postmenopausal osteoporosis, and CB2 deficient mice are protected from ovariectomy-induced bone loss, which can be reproduced using CB2 inverse agonists.[24]

In this thesis, we are exploring the mechanism of dual anti-OP functions of CB2 inverse agonists. One explanation could be the pharmacologic complexity of CB2 inverse agonists. They may act on diverse osteoporosis therapeutic targets or signaling pathways to accomplish a synergetic effect on both osteoclasts and osteoblasts.

1.3 POLYPHARMACOLOGY AND DRUG DISCOVERY

Polypharmacology describes the interaction of multiple drugs with multiple targets, which may be involved in a single or multiple disease pathways.[47] In recent years, conventional “one drug — one target” paradigm in drug discovery has been challenged by

"multiple drugs — multiple targets" mode advocated by the polypharmacology concept.[48, 49] Polypharmacology has gained prominent success in drug discovery for some complex diseases such as cancer[50] and psychiatric disorders,[51] in which most marketed drugs can interact with multiple disease-relevant targets to maximum therapeutic efficacy. On the other hand, polypharmacology is believed to prevent drug resistance due to mutations or expression changes.[52] Furthermore, polypharmacological drugs could be safer because of reduced on-target adverse effects.[53] However, polypharmacology also brings about unintended antitarget-caused adverse effects that must be avoided in drug development.[54]

Given the complex nature of osteoporosis, polypharmacology study is designed to understand the cross-talk between therapeutic targets, signaling pathways-related proteins, and their binding drugs. This can not only help completely unravel the mechanism of action for existing drugs, but also predict possible off-target therapeutic/adverse effects and guide multiple drug combination therapy. A polypharmacology study for CB2 inverse agonists, in particular, may elucidate the conflicted outcomes of some compounds in modulating bone cell activity. Moreover, new therapeutic targets of osteoporosis may be predicted for CB2 inverse agonists for the use of drug repurposing. On the basis of other potential targets that CB2 inverse agonists may act on, multiple-target CB2 inverse agonists can be designed with anticipated greater efficacy and lower toxicity for the treatment of osteoporosis.

2.0 MATERIAL AND METHOD

2.1 OSTEOPOROSIS DOMAIN SPECIFIC KNOWLEDGEBASE (OP-KB)

To provide a comprehensive platform for anti-OP drug discovery and particularly for a polypharmacological study, we data-mine the information of osteoporosis-related proteins, signaling pathways, therapeutic targets and their associated drugs from various public databases, and compiled them into our in-house osteoporosis domain specific knowledgebase (OP-KB) together with a cloud computing server: http://cbligand.org/OP. In fact, the disease domain specific knowledgebase was not a newborn concept in our group, which has gain success in building a drug abuse knowledgebase (DA-KB)[55] and an Alzheimer's disease knowledgebase (ALzPlatform: http://www.cbligand.org/AD).[56] The current version of OP-KB contains the following features:

1) Database infrastructure and web interface

OP-KB is designed with a MySQL database (http://www.mysql.com) and an apache web server (http://www.apache.org) with OpenBabel[57] at the backend as the search engine for chemical structures. The web interface is written in PHP language (http://www.php.net).

2) Data collection and content

The validated target data for osteoporosis therapy is collected according to approved drugs, clinical trials drugs, and discontinued drugs from a variety of public databases including Metacore,[58] DrugBank,[59] ClinicalTrials.gov,[60] BindingDB,[61] SuperTarget,[62] and ChEMBL database.[63] The information for drugs with unknown targets is also included. We further gather OP-related proteins information and corresponding signaling pathways from the KEGG pathway database.[64] Finally, all the chemical structures, bioactivity values, together with data for pathways, bioassays, and references are imported into OP-KB.

3) Chemoinformatics Tools

For facilitating *in silico* drug design for osteoporosis treatment, the OP-KB cloud computing server also integrate diverse chemoinformatics tools based on state-of-the-art machine learning algorithms developed by our group or from public resources. For example, this server employ the properties explorer (http://www.cbligand.org/OP/Property_Explorer.php) and the blood-brain barrier (BBB) predictor (http://www.cbligand.org/BBB) to analyze the drug-likeness of small molecules. It also applies the PAINS predictor (http://www.cbligand.org/PAINS) and the toxicity predictor (http://www.cbligand.org/Tox) which allow exclusion of molecules with potential safety issue in the early phase of drug discovery. To our interest of study, two of our developed polypharmacology analysis tools, HTDocking and TargetHunter, are imbedded as core functions in OP-KB. Detailed description can be found in section 2.2.

2.2 POLYPHARMACOLOGY ANALYSIS TOOLS

2.2.1 HTDocking

We have established a high-throughput docking program (HTDocking, http://cbligand.org/OP/docking_search.php), which is a web-interface computing tool that automates docking procedures to search for protein targets and explore ligand-protein interactions. In the current version of OP-KB, crystal structures of osteoporosis-related proteins have been collected from Protein Data Bank (http://www.rcsb.org/pdb)[65] to build an OP domain-specific subset by searching with a sequence from our database. Water molecules and ligands are removed if needed, hydrogen atoms are added, and the active site(s) of each protein are defined by the residues around the co-crystalized ligands or predicted using our published method.[66] Docking scores are used to assess and rank potential target proteins for a queried compound.

2.2.2 TargetHunter

Complementary to structure-based HTDocking tool, TargetHunter, a ligand-basedtool (http://www.cbligand.org/TargetHunter), has also been employed to predict potential off-targets of compounds. The detailed description of TargetHunter algorithm is elaborated in our recently published paper[67] based on a well-known medicinal chemistry principle: structurally similar compounds have similar physiochemical properties that may result in similar pharmaceutical profiles. TargetHunter is a powerful cloud computing tool with attractive features: (i) ease of use; (ii) query data retrieval function; (iii) user choices of desired fingerprints and databases; (iv)

high accuracy; and (v) Bioassay Finder function implemented in BioassayGeoMap program to facilitate users to find the laboratories who have published bioassay(s) for experimental validation. The combination of HTDocking and TargetHunter will assist researchers in conducting polypharmacology studies for off-target prediction and thereby develop bioactive compounds for osteoporosis treatment.

3.0 RESULT

3.1 OSTEOPOROSIS RELATED TARGETS AND DRUGS

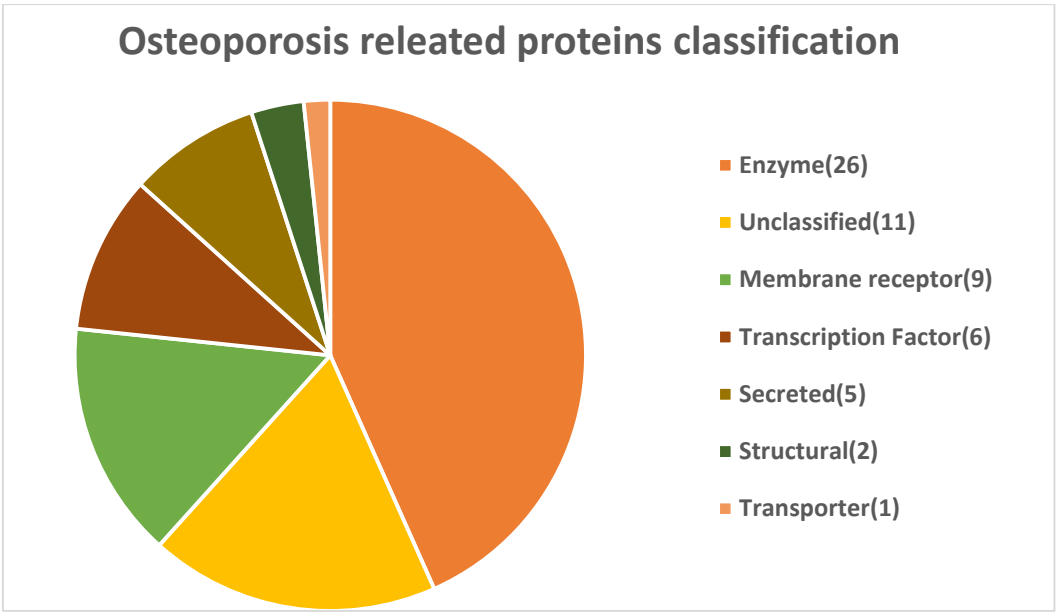
In the current version of OP-KB platform (http://cbligand.org/OP/), we archive 60 osteoporosis related proteins including 34 validated osteoporosis therapeutic targets with 37 approved drugs, 33 drugs in clinical trials, 3 discontinued drugs, and 120,371chemicals associated with 175,600 records of reported bioactivities from 5,255 corresponding bioassays and 1,928 references.

Detailed information of OP-KB is demonstrated in **Figure 3**. Most of the osteoporosis-related proteins are enzymes (26/60), including alcohol dehydrogenase 1B, steroid 17-alpha-hydroxylase/17, 20 lyase, corticosteroid 11-beta-dehydrogenase isozyme 1, insulin-like growth factor 1 receptor, mitogen-activated protein kinase 1 and endothelial nitric oxide synthaseand cytochrome P450 19A1. There are also 9 membrane proteins, such as the calcitonin receptor, the extracellular calcium-sensing receptor, the cannabinoid receptor 1, the cannabinoid receptor 2, and the parathyroid hormone-related peptide receptor in addition to 6 proteins belonging to transcription factors such as the androgen receptor, the estrogen receptor, the estrogen receptor beta, the progesterone receptor and vitamin D3 receptor. (**Figure 3A**).

We next plot anti-OP drugs in different stages of development with projection on their therapeutic targets (**Figure 3B**). As shown, estrogen receptor (ESR1) is considered as the most common and successful target for osteoporosis treatment because it has 10 approved drugs, and 2 drugs entering Phase III clinical trial as well. Farnesyl pyrophosphate synthase (FPPS) and androgen receptor (ANDR) seem to be also good targets: both of them have 4 approved drugs. However, FPPS has specific bias to bisphosphonates targeting,[68] which may infer that this target can't accomendate other chemotypes easily. Also, we find that 6 cathepsin K (CATK) inhibitors are in clinical trials with equal number in phase I, II and III study, indicating cathepsin K is a promising drug target for osteoporosis treatment. In addition, vitamin D3 receptor (VDR) is also top-ranked with 2 drugs approved and 2 drugs under clinical investigation. But VDR, on the other hand, should be paid more careful attention to because it has one discontinued drug, Falecalcitriol, that may cause hypercalcemia and bradycardia as adverse effcts.[69] To our surprise, cytochrome P450 19A1 (CP19A), a well-known metabolizing enzyme, also functions as an anti-OP target for the drug Letrozole, which prevents aromatase from producing estrogens by specific binding to the heme of its cytochrome P450 unit.[70] Finally, the OP-related targets-drugs associations are in connection with involed signaling pathways (**Figure 3C**). We can see that five osteoporosis and bone metabolism related pathways show up in the list (highlighted in yellow), including osteoclast differentiation (KEGGID: hsa04380), rheumatoid arthritis (hsa05323), estrogen signaling pathway (KEGGID: hsa04915), endocrine and other factor-regulated calcium reabsorption (KEGGID: hsa04961) and mineral absorption (KEGGID: hsa04978). However, some cancer related pathways are also enriched in the list (highlighted in green), such as pathways in cancer (hsa05200), proteoglycans in cancer (hsa05205) and prostate

cancer (hsa05215). This could imply that osteoporosis shares some common pathways with cancer, which means some anti-OP drugs may also act as anti-cancer agents.

A



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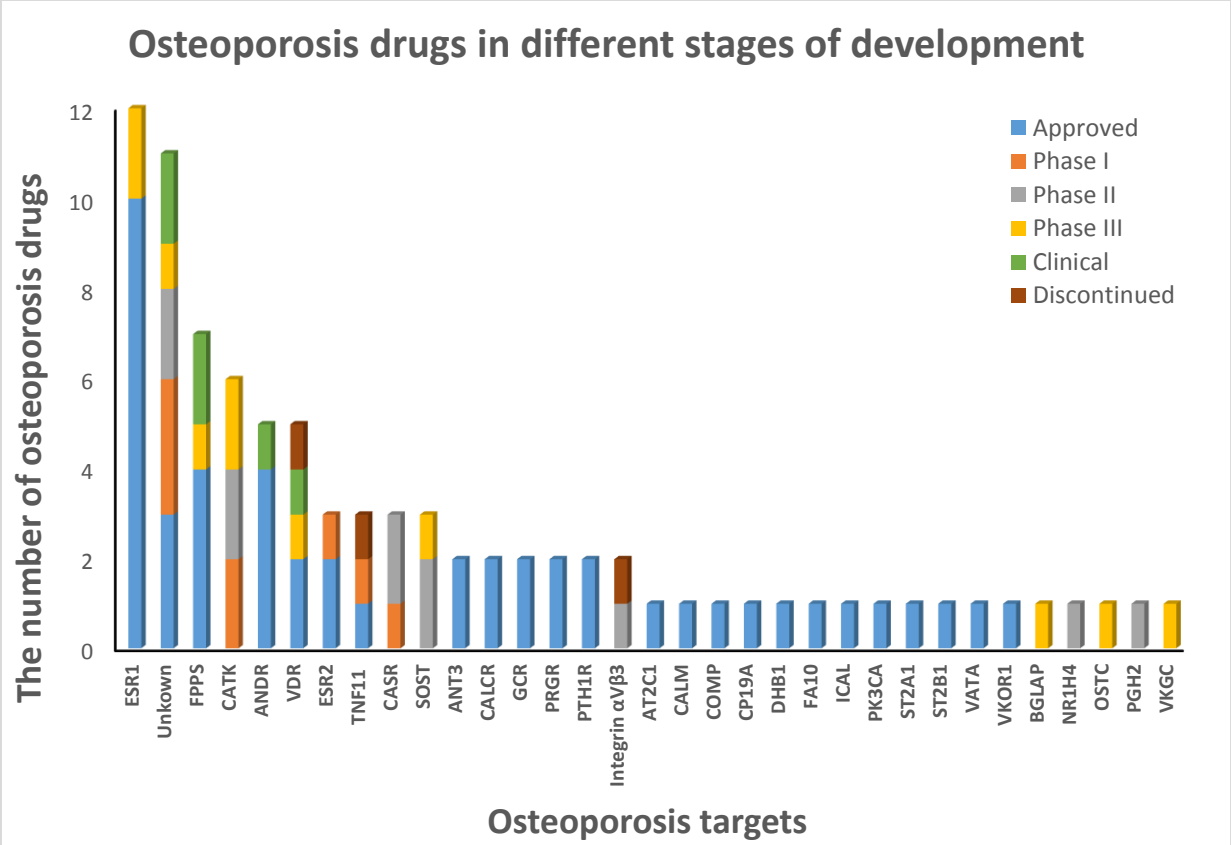

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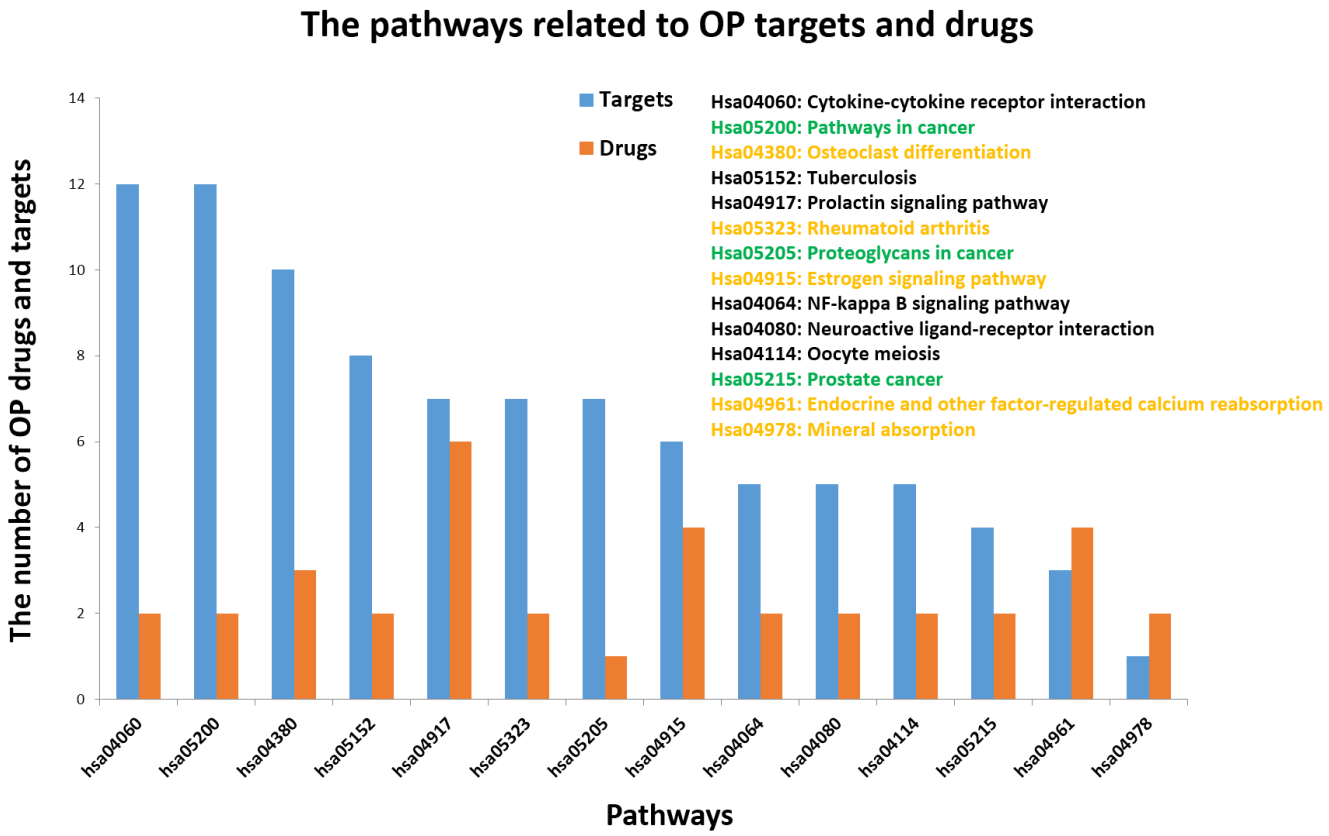


Figure 3. The overview of OP-KB data. (A) Summary of OP-related proteins. (B) OP drugs in different development phases associated with their targets. (C) The associations of OP drugs, their corresponding targets, and involved pathways. Only pathways that are assoicated with drugs were included and ranked based on the number of invovled targets. In addition, full names of pathways were given in the right-upper corner, with those related to opsteoporosis labeled in yellow and those related to cancer labeled in green. All abbreviations are listed in Appendix A.

3.2 POLYPHARMACOLOGY ANALYSIS OF ANTI-OSTEOPOROSIS DRUGS AND THEIR TARGETS

To explore potential polypharmacological effects for available osteoporosis drugs, we utilize the established HTDocking tool to screen all osteoporosis related proteins in our OP-KB database. In this study, we select five representative approved drugs that have different initial therapeutic targets. Queried with these five drug structures, a list of potential targets is displayed and ranked by docking scores. The five drugs and their top candidate targets (docking score ($-pK_d$) ≥ 6) are extracted to build an interacting network **(Figure 4)**. Predicted targets are displayed as green nodes (known OP targets) and pink nodes (predicted off-targets) with different sizes based on the degree of connectivity to drugs. As shown in this figure, all drugs can successfully find their known targets in this prediction. For example, Calcitriol is predicted to target VDR, and Lasofoxifene is likely to bind with both ESR1 and ESR2. In fact, those predicted targets were just primary therapeutic targets for each drug. As such, Calcitriol is a VDR antagonist with K_i = 0.1 nM,[71] and Lasofoxifene acts as a dual ESR1 and ESR2 modulator with IC_{50} equal to 1.8 nM and 1.3 nM, respectively,[72] which validates the reliability and robustness of our database and HTDocking method.

For our group's research interest on the role of CB2 in osteoporosis therapy, we intend to explore if some available drugs also act on CB2 or not. Due to the lack of crystal structure of CB2, we take advantage of our built computational 3D CB2 structure model (from Dr. Zhiwei Feng, manuscript submitted), predict the potential ligand-binding pocket, and perform molecular docking for 5 anti-OP drugs. The method and parameters for pocket prediction and docking are the same as HTDocking method. The docking scores are shown in **Table 2**, in which only

Lasofoxifene exhibits high binding potential with CB2 (Docking score = 8.9063). In fact, this prediction has been validated by recent studies of Prof. Zhao-Hui Song's group. They have identified Lasofoxifene, together with two other estrogen receptor modulators (Raloxifene and Bazedoxifene) as novel CB2 inverse agonists.[73, 74] In order to elucidate the binding mode between Lasofoxifene and CB2, we depict the docking result and detailed interaction (**Figure 5**). As shown in **Figure 5A**, the potential binding pocket of CB2 was mainly formed by helices II, III, V, VI, and VII. Lasofoxifene can access this pocket by forming hydrophobic interactions (**Figure 5B**) with nearby residues including Phe94 (not shown), Val105 (now shown), Phe106, Ile110, Leu182 (not shown), Trp194, Phe197, Trp258 and Phe281. Among these amino acids, Phe87 and Trp258 and can form π-π interactions with two benzene rings in Lasofoxifene, respectively. In particular, the N atom in pyrrolidine ring may be protonated to induce electrostatic interaction with Asp101 (shown in blue dash), as well as possible π-cation interaction with Phe106, both of which help stabilize the binding of Lasofoxifene with CB2. In fact, most interacting residues we describe above are supported by published mutational data and docking data. Recent docking reports show Phe87, Phe94 and Ile110 may contribute to the binding of CB2 ligands, as well as Asp101 which forms hydrogen bonding with CB2 ligands.[75-77] However, in our case, Asp101 even plays greater roles in forming electrostatic interaction. In addition, Leu182 is validated to be unique for CB2 other than CB1 by receptor chimera studies.[78] Also, Trp194 is reported to be critical in CB2 binding and corresponding adenylyl cyclase activity.[79] Moreover, the replacement of Phe197 with the corresponding Val in CB1 causes a 14-fold decrease of Win55212-2 (a CB2 agonist) binding affinity with CB2.[80] Lastly, Trp258 is highly conserved for most GPCRs due to its rotameric state involved in GPCR

activation.[81] Taken together, almost all residues predicted in the interaction of Lasofoxifene with CB2 has been validated to be key residues in the ligand recognition of CB2.

In addition, if we look at this prediction as a whole picture, the targets with relatively higher degree of connectivity (the nodes with bigger size) could be more promising in the treatment of osteoporosis. The possible explanations are proposed: first, these targets are capable of keeping diverse drug molecules staying in their active pockets, which demonstrates the druggability of the targets in some degree, and also allows medicinal chemists to design structure-diverse chemicals for these targets; second, drugs that act on multiple-ligand binding targets are more likely to bind with other targets to achieve polypharmacological effect. In our case, the promising osteoporosis therapeutic targets (the degree of connectivity ≥ 3) includes: mitochondrial aldehyde dehydrogenase (ALDH2), corticosteroid 11-beta-dehydrogenase isozyme 1 (DHI1), sex hormone-binding globulin protein (SHBG), endothelial nitric oxide synthase (NOS3) and estrogen receptor (ESR1).

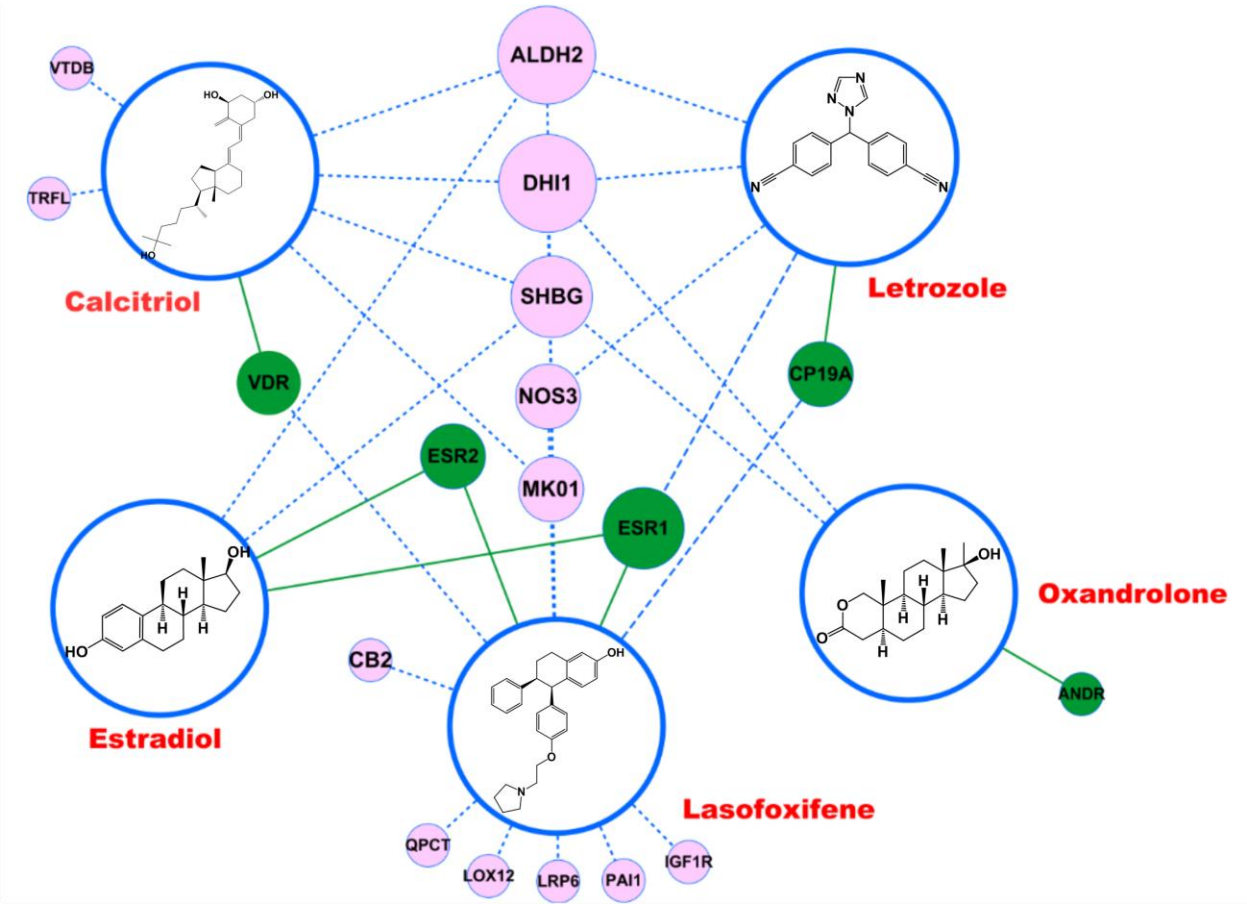


Figure 4. Polypharmacology network for OP drugs and predicted OP targets. The large circles (blue) represents approved OP drugs (Calcitriol, Letrozole, Estradiol, Lasofoxifene, Oxandrolone) that are linked to individual predicted targets. Among them, the green nodes and edges denoted known drugs targets and associations, and the pink nodes indicated novel potential off-targets connected with drugs by blue dashed edges. The sizes of target nodes varied with the degree of connectivity. All abbreviations are listed in Appendix A.

Table 2. Docking score of five OP drugs with CB2 receptor

Drugs	Initial Targets	CB2 Docking Score
Lasofoxifene	ESR1, ESR2	8.9063
Calcitriol	VDR	5.6306
Letrozole	CP19A	5.472
Oxandrolone	ANDR	4.9811
Estradiol	ESR1, ESR2	4.3952

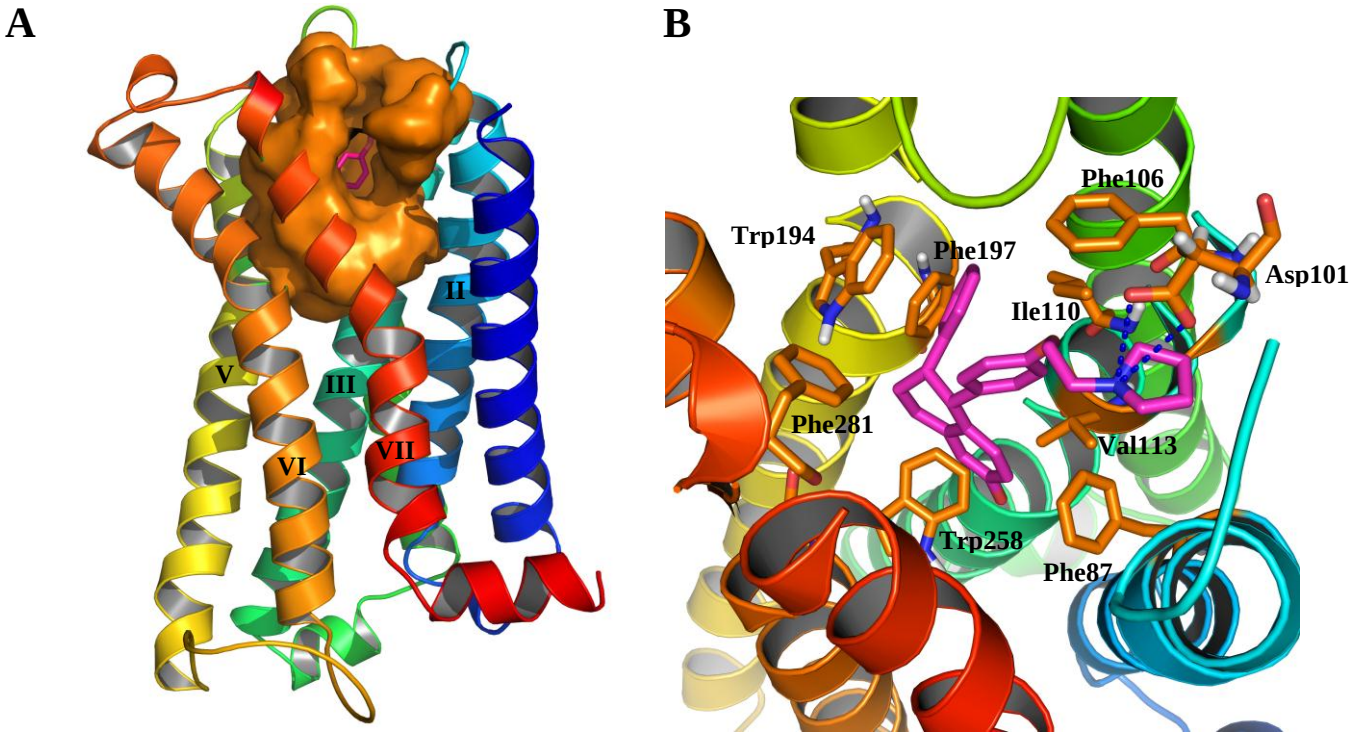


Figure 5. Interaction mode of CB2with Lasofoxifene via molecular docking. (A) Docking result of Lasofoxifene with CB2. Seven trans-membrane domains were indicated in different colors; the backbone of Lasofoxifene is shown in purple stick; the predicted binding pocket is shown in yellow shape. (B) Detailed interaction of CB2 and Lasofoxifene. The electrostatic interaction is indicated by blue dash. (This CB2 model is contributed by Dr. Zhiwei Feng)

3.3 POLYPHARMACOLOGY ANALYSIS OF CANNABINOID RECEPTOR 2 INVERSE AGONISTS

3.3.1 HTDocking for CB2 inverse agonists

As stated earlier in section 1.2.2, CB2 inverse agonists have exhibited therapeutic potential in the treatment of osteoporosis but with unclear mechanisms of action, especially for their anabolic effect of accelerating bone formation. We hypothesize that CB2 inverse agonists including our in-house compounds may bind with other OP-related targets for synergic regulation of bone metabolism. Hence, we select six CB2 selective inverse agonists (Their chemical structures are shown in **Figure 6**) from literature (AM630,[82] JTE-907,[83] Sch.036,[84] and SR144528[85]) or synthesized by ourselves (Xie95-1042[43] and Xie95-1171[44] are highlighted in blue in **Figure 6**), and three CB1 selective inverse agonists (Rimonabant,[86] CHEMBL425047,[87] and Ibipinabant[88]) as comparison for the polypharmacology study. The binding affinities (K_i) and selectivity against CB2/CB1 for these 9 compounds are shown in **Table 3**. HTDocking is used to screen out top-ranked OP-related proteins as potential targets with the docking score equal to or bigger than 6.0, which has been proved to be a reliable threshold value for identifying potential targets in the polypharmacology study for marketed OP drugs (Section 3.2). We display the association between the number of predicted targets and individual drugs in **Figure 7**. It is CB2 selective inverse agonists that shows more likelihood to act on other OP-related targets other than CB1 inverse agonists. The number of predicted off-targets of six CB2 inverse agonists ranges from 6 to 23 with an average number of 16.7 per compound.

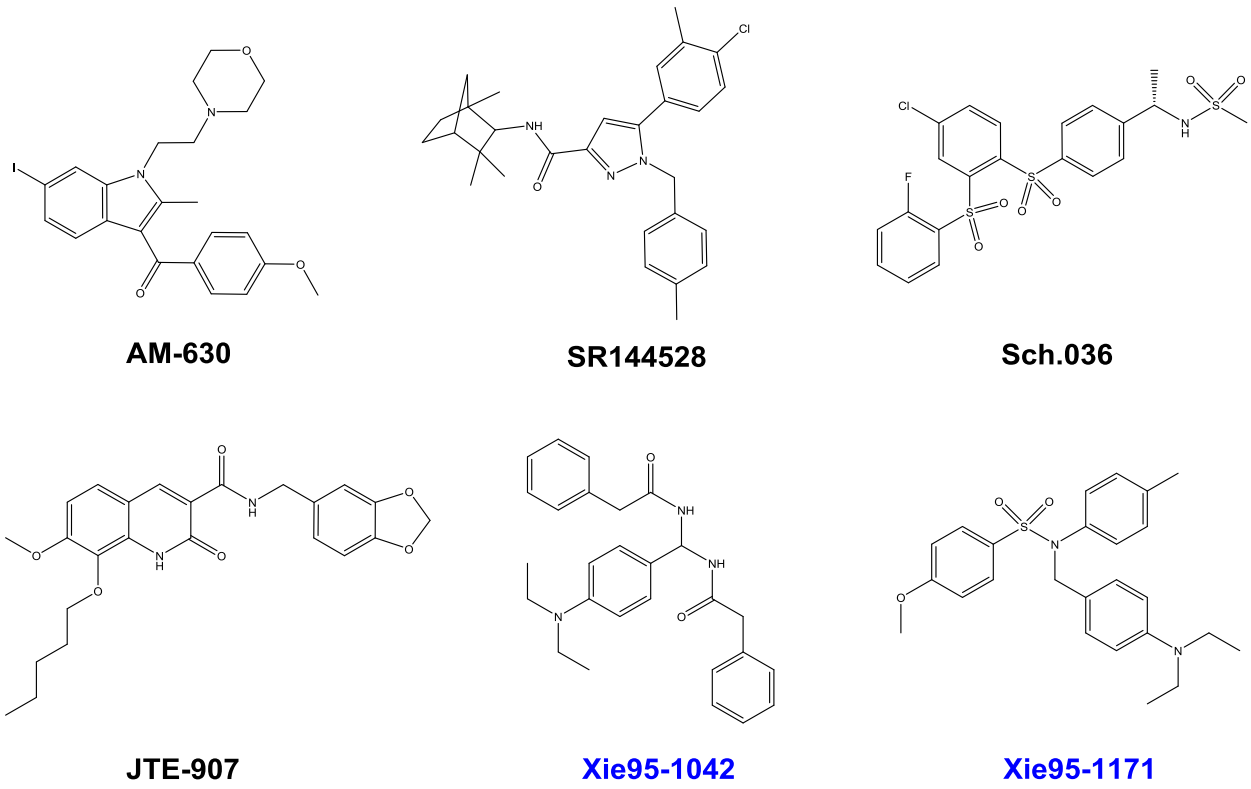


Figure 6. Chemical structures of six representative CB2 inverse agonists. Two in-house compounds (Xie95-1042 and Xie95-1171) are highlighted in blue.

Table 3. Binding affinity (K_i) and selectivity for nine CB2/CB1 inverse agonists

Chemical Names	K_i (n M)		Selectivity	Reference
	CB2	CB1		
JTE-907	**35.9**	**2370**	**66**	[89]
AM-630	**31.2**	**5152**	**165**	[89]
SR144528	**0.6**	**400**	**667**	[89]
Sch.036	**1.3**	**4387**	**3375**	[90]
Xie95-1042	**64**	**20000**	**313**	[43]
Xie95-1171	**0.5**	**1297**	**2594**	[44]
Rimonabant	**1640**	**11.5**	**1/143**	[89]
Ibipinabant	**7943**	**7.8**	**1/1018**	[91]
CHEMBL425047	**10000**	**0.2**	**1/50000**	[87]

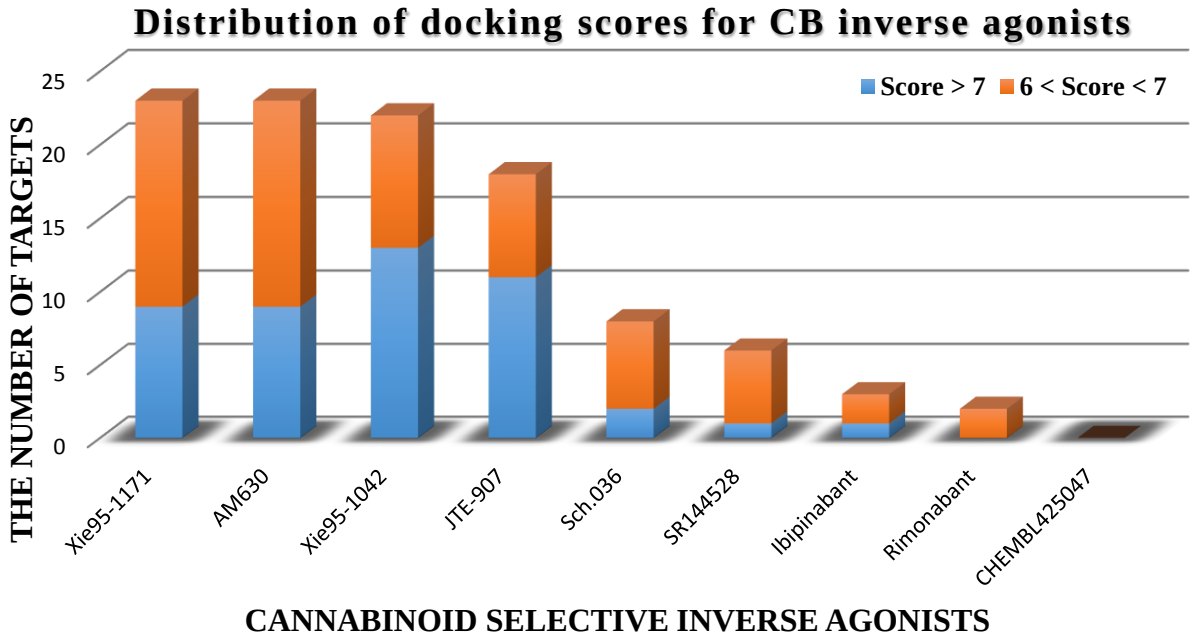


Figure 7. The number of predicted targets associated with CB2/CB1 inverse agonists. The orange color represented the number of targets with docking score ranging between 6 and 7; the blue color stood for the number of targets with docking score larger than 7.

We next build the polypharmacology network for these six compounds and their top candidate targets (docking score ($-pK_d$) ≥ 6) (**Figure 8)**. In the network, circle nodes indicates predicted targets, among which five validated therapeutic targets are labeled in red; rectangle nodes stands for compounds. Both of the target nodes and compound nodes are displayed in a degree sorted circle layout, respectively. The degree of connectivity is also demonstrated by node size and node color. From the network, we find that five known OP targets are identified as possible CB2 inverse agonist targets, and four of them had top degree of connectivity (≥ 4) except androgen receptor (ANDR). This finding is in agreement with the result of OP drugs-off-targets network shown in **Figure 4**, which may suggests that ANDR has a very strict and specific ligand structure's preference. In fact, four available OP drugs targeting ANDR all belongs to the

steroids, including Oxandrolone, Methyltestosterone, Testosterone and Testosterone Propionate. In addition, we list the docking scores of 6 CB2 inverse agonists with top six predicted targets including four potential anti-OP targets (NOS3, DHI1, ALDH2 and TRFL) and two known anti-OP targets (VDR and ESR1) in **Table 4**.

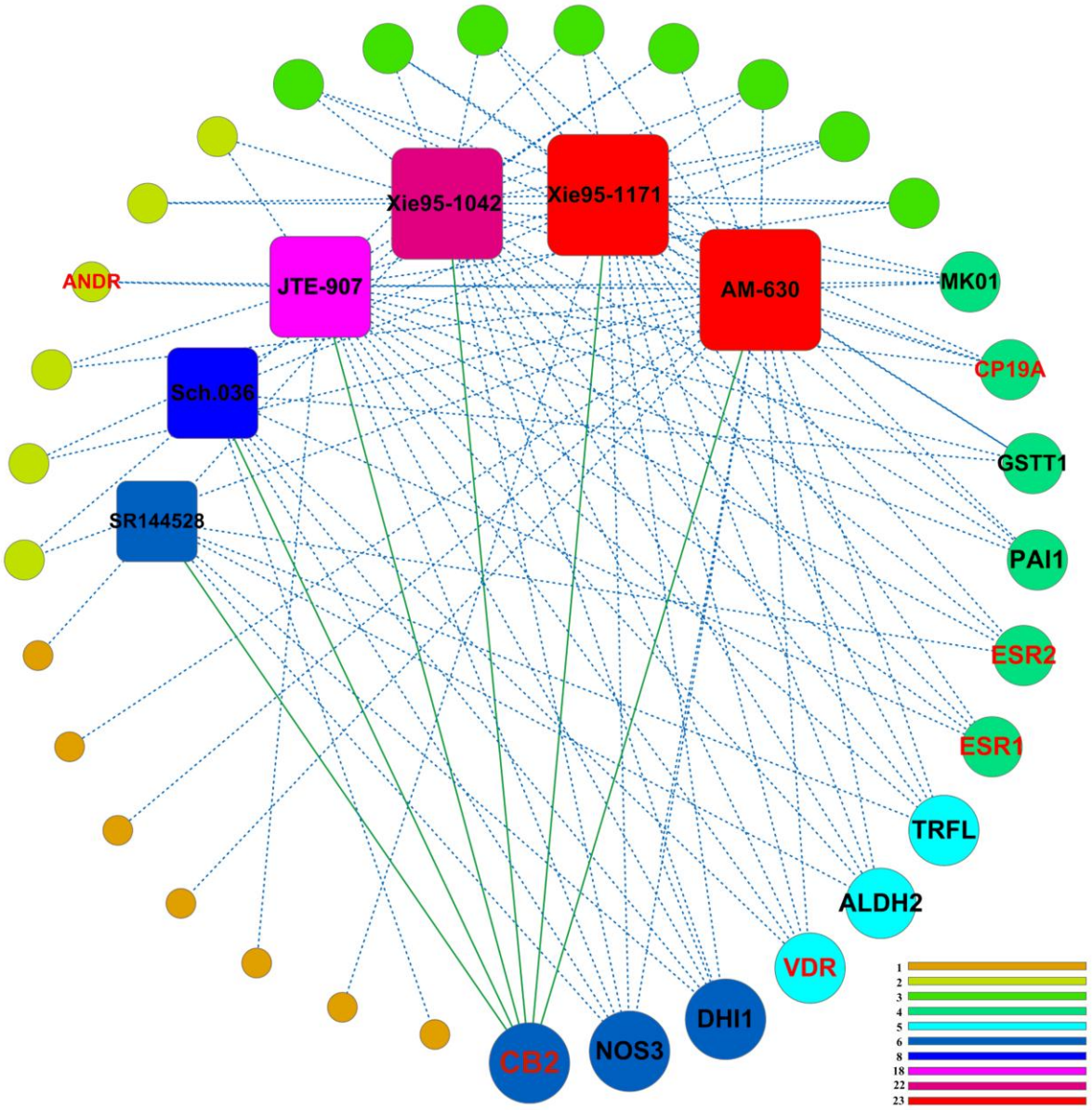


Figure 8. Polypharmacology network for CB2 inverse agonists and predicted OP targets. The circle nodes indicates predicted targets, among which five known targets were labeled in red. The rectangle nodes stood for compounds. Both of the target nodes and compound nodes

were displayed in a degree sorted circle layout, respectively. The degree of connectivity was demonstrated by node size and node color. The association of the degree of connectivity and color is indicated in the right-bottom corner.

Table 4. Docking score of CB2 inverse agonists with anti-OP targets.

Compound	Docking score					
	NOS3	DHI1	ALDH2	TRFL	ESR1	VDR
Xie95-1042	8.54	8.16	7.78	7.48	8.37	7.88
Xie95-1171	7.48	7.68	7.97	7.16	7.01	8.87
AM630	6.22	8.11	7.66	8.16	5.94	6.00
JTE-907	8.94	7.98	7.00	7.71	7.59	6.93
Sch.036	6.01	8.20	5.84	5.65	6.01	6.85
SR144528	7.60	6.36	6.90	6.47	4.67	4.90

Note: Top two docking scores with four potential anti-OP targets for Xie95-1042, Xie95-1171, and AM630 are labeled in red. Docking scores (> 7) with two known anti-OP targets for Xie95-1042 and Xie95-1171 are labeled in blue.

In this thesis, we focus on providing an explanation for the fact that our compound Xie95-1042 can stimulate osteoblast formation and AM630 can increase the number of osteoclasts at high concentration. Meanwhile, we assume that another in-house compound Xie95-1171 may also enhance osteoblast formation, which has not been tested yet. To validate the predicted targets for these three compounds, we docked each compound to its two most likely potential anti-OP targets (top 2 docking scores) (highlighted in red in **Table 4**), as well as to its most likely known anti-OP targets (docking scores >7) (highlighted in blue in **Table 4**). The results and detailed interactions are described in following sections.

3.3.2 Potential anti-OP targets for CB2 inverse agonists

As shown in **Figure 8** and **Table 4**, four OP-related proteins (NOS3, DHI1, ALDH2 and TRFL) are highly shared by six CB2 inverse agonists and thereby considered as potential anti-OP targets. Most of them are the same as those predicted in OP drugs-targets network shown in Figure 4. Furthermore, the docking scores for our compounds and AM630 with these four targets are all higher than 6. Molecular docking is performed on each compound with its two most likely potential targets ((highlighted in red in **Table 4**) for better identification of reliable ligand-protein interactions.

1) Docking result of NOS3 (PDB_ID:1M9K)[92] with Xie95-1042. **(Figure 9)**

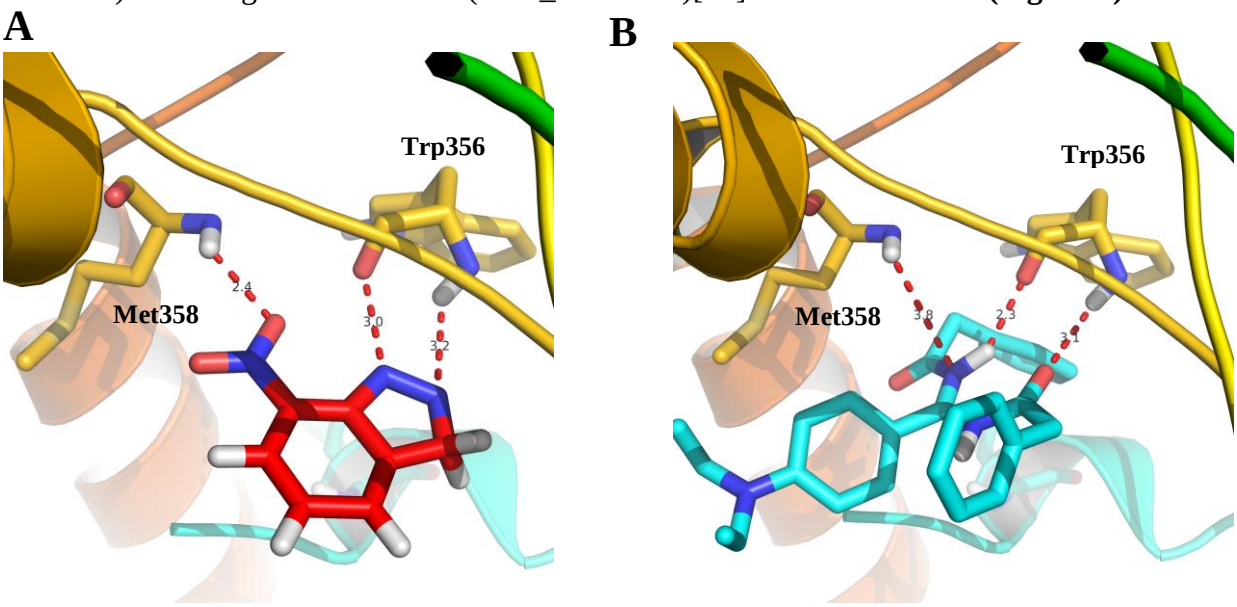
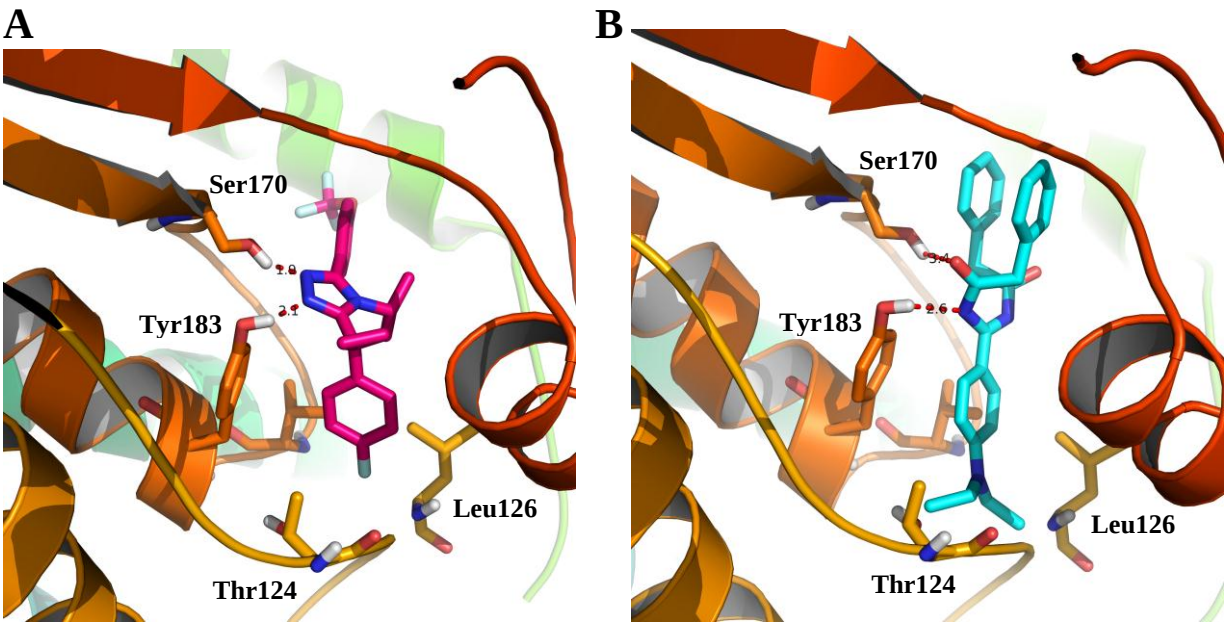


Figure 9. Interaction mode of NOS3 with 7-nitroindazole (A) in co-crystal structure and with Xie95-1042 (B) via molecular docking. Hydrogen bonding is indicated by red dash with measured distance.

From **Figure 9**, we can see Xie95-1042 can form hydrogen bonding with Met358 and Trp356 of NOS3, which ensembles exactly the hydrogen bonding interactions in the co-crystal

structure of NOS3 and 7-nitroindazole (Hydrogen bonding is indicated by red dash with measured distance). Moreover, Xie95-1042 adopts "U-shape" in its binding conformation via intramolecular π-π interactions.

2) Docking result of DHI1 (PDB_ID:3D5Q)[93] with Xie95-1042, Xie95-1171, and AM630. **(Figure 10)**



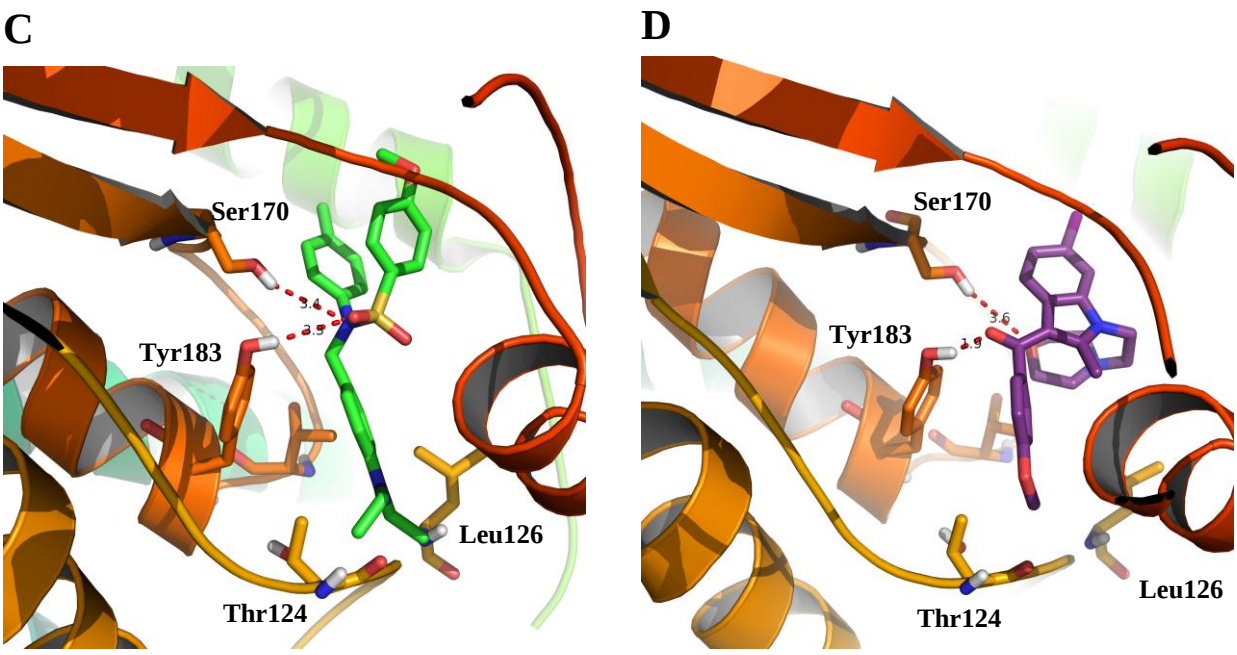


Figure 10. Interaction mode of DHI1 with T30 (A) in co-crystal structure and with Xie95-1042 (B), Xie95-1171 (C), and AM630 (D) via molecular docking. Hydrogen bonding is indicated by red dash with measured distance.

From **Figure 10**, we can see that Xie95-1042, Xie95-1171 and AM630 form two hydrogen bonding with Ser170 and Tyr183 of DHI1 (Hydrogen bonding is indicated by red dash with measured distance). This observation is in agreement with the DHI1-T30 co-crystal structure. In addition, the benzene (at the bottom of molecules) can form π-π interaction with Tyr183, as well as hydrophobic interactions with Thr124 and Leu126. Furthermore, all of Xie95-1042, Xie95-1171 and AM630 adopt U-shape conformation in its binding conformation via intramolecular π-π interactions.

2) Docking result of ALDH2 (PDB_ID:1NZW)[94] with Xie95-1171. **(Figure 11)**

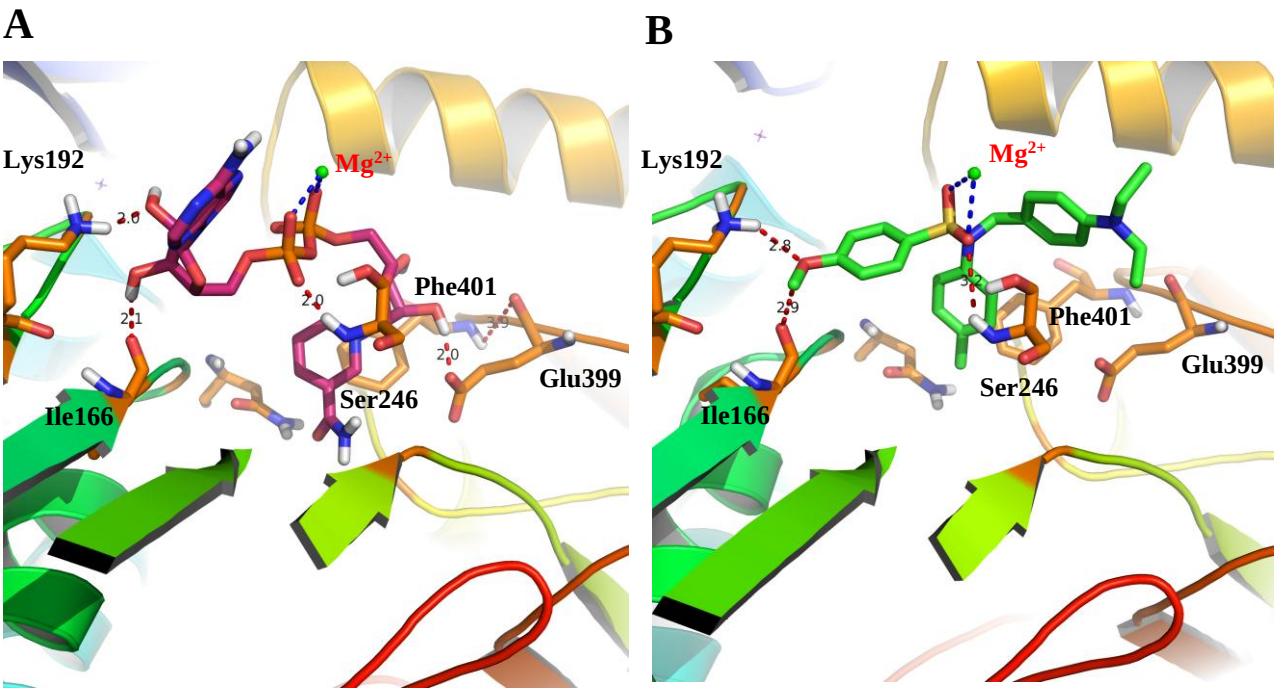


Figure 11. Interaction mode of ALDH2 with NAPH (A) in co-crystal structure and with Xie95-1171 (B) via molecular docking. Hydrogen bonding is indicated by red dash with measured distance. Electrostatic interaction is indicated by blue dash.

From **Figure 11**, we can see that Xie95-1171 can form three hydrogen bonding with Lys192, Ile166 and Ser246 of ALDH2 (Hydrogen bonding is indicated by red dash with measured distance), which is the same as the hydrogen bonding interactions revealed in ALDH2-NAPH co-crystal structure. The π-π interaction is also observed between the benzene ring of NAPH and Xie95-1171 (at the bottom of molecules) with Phe401. Interestingly, the sulfonyl group of Xie95-1171 can chelate with magnesium ion (Mg^{2+}) via π-cation interaction. In fact, the similar interaction appears between bi- carbonyl group in NAPH and Mg^{2+}, which may imply the specificity of binding between ALDH2 with its ligands.

3) Docking result of TRFL (PDB_ID:1CB6)[95] with AM630.

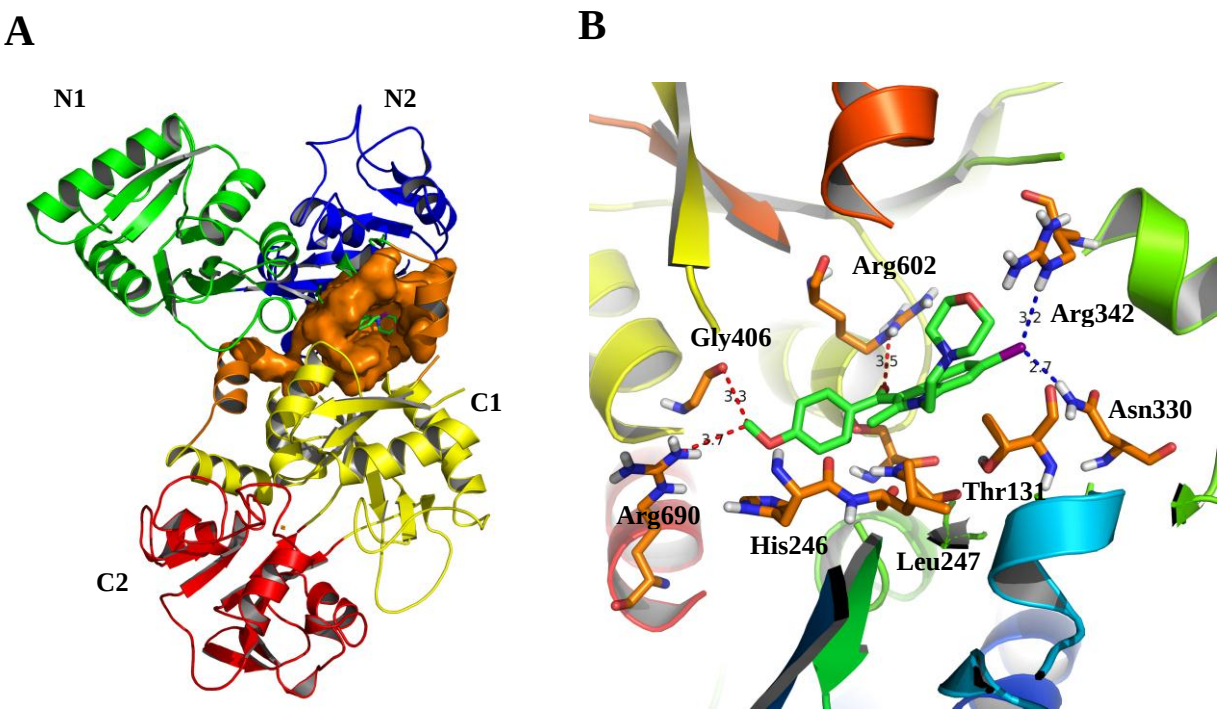


Figure 12. Interaction mode of TRFL with AM630. (A) The composition of TRFL and the predicted binding pocket at the interface of N lope and C lope (shown in orange shape); (B) Detailed interaction of TRFL with AM630. Hydrogen bonding is indicated by red dash with measured distance. Halogen bonding is indicated by blue dash with measured distance.

From **Figure 12A**, we can see that TRFL is composed of two N-terminal domains (N1-lope and N2-lope) and two C-terminal domains (C1-lope and C2 lope). There is no published co-crystal structure for TRFL and its bound ligands yet. We use our published method[66] to explore the potential binding pocket that is predicted to locate at the interface of N-lope and C-lope. This prediction is in agreement with the fact that the inter-domain region of TRFL is the biggest solvent-accessible surface where the ligands prefer to bind with. **Figure 12B** depicts the detailed binding mode. As shown, hydrogen bonding interactions are observed between AM630

and Arg690, Gly406, Arg602, as well as Arg342 (Hydrogen bonding is indicated by red dash with measured distance). Moreover, AM630 can form hydrophobic interactions with His246, Leu247 and Thr131. Specifically, the iodine atom of AM630 can form halogen bonding with Arg342 and Asn330 (Halogen bonding is indicated by blue dash with measured distance).

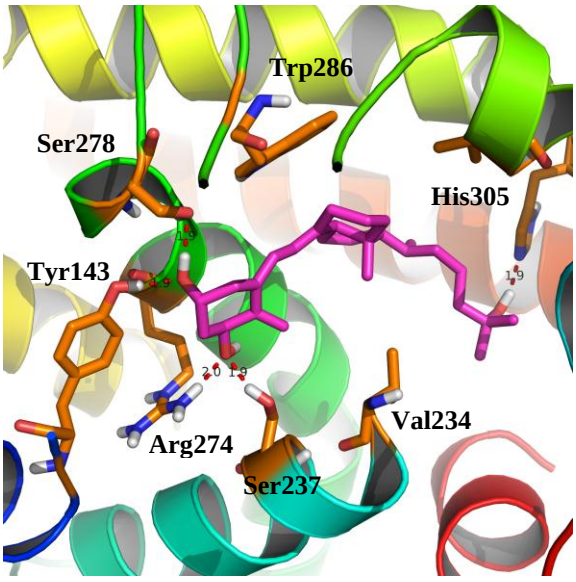
3.3.3 CB2 inverse agonists as VDR modulators

Vitamin D is critically important for bone mineralization via maintaining blood calcium level and restore calcium to bone tissue by activating its receptor (VDR).[96] Upon activation, VDR forms a heterodimer with the retinoid X receptor and binds to hormone response elements on DNA, leading to the expression of certain gene and products that promote calcium absorption in the intestine and reabsorption in the kidneys.[96, 97] Moreover, recent studies reveal the direct effect of vitamin D on osteoblast proliferation, differentiation and mineralization.[98] Also, the transgenic mice overexpressing VDR show increased bone strength.[99] Hence, some vitamin D analogues and other VDR agonists can be used as anabolic agents for preventing fractures and bone loss in osteoporosis therapy.[100, 101]

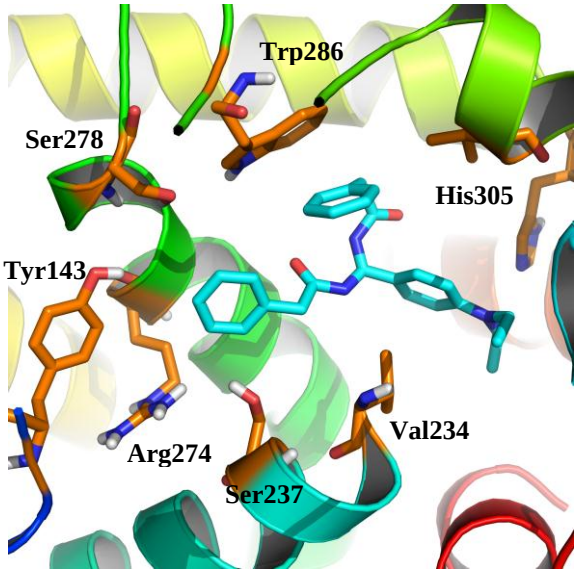
Based on our prediction (**Figure 8** and **Table 4**), our in-house CB2 inverse agonists are prone to bind with VDR. To validate this and explore the possible mechanism of interactions, we dock Xie95-1042 and Xie95-1171 into the VDR protein (PDB_ID: 1DB1).[102] The detailed interaction is shown in **Figure 13** in comparison with its initial co-crystal structure binding with vitamin D. We can see that Xie95-1042 loses all hydrogen bonding shown in VDR-vitamin D co-crystal structure, and Xie95-1171 only keeps two hydrogen bonding with Try143 and Ser278 of VDR. This may be due to the lack of polar groups in the left benzene ring of these two

compounds, which may imply that the binding affinity of VDR and our compounds could be enhanced by chemical modification of the left benzene ring. On the other hand, Xie95-1042 and Xie95-1171 can form similar hydrophobic interactions with nearby residues including Trp286, His305, Arg274, Ser237 and Val234 to those appeared in the co-crystal structure. Among these residues, Trp286 can form π-π interaction with Xie95-1042 and Xie95-1171 other than with initial ligand vitamin D. This new interaction may compensate the missing hydrogen bonding interaction in terms of overall binding affinity. **Figure 13D** shows the nice alignment of our compounds and vitamin D with their binding conformation. We can find that when binding these three compounds adopt very close molecular orientation with very similar molecular shape. That may explain why these three compounds can fit the same binding pocket of VDR very well.

A



B



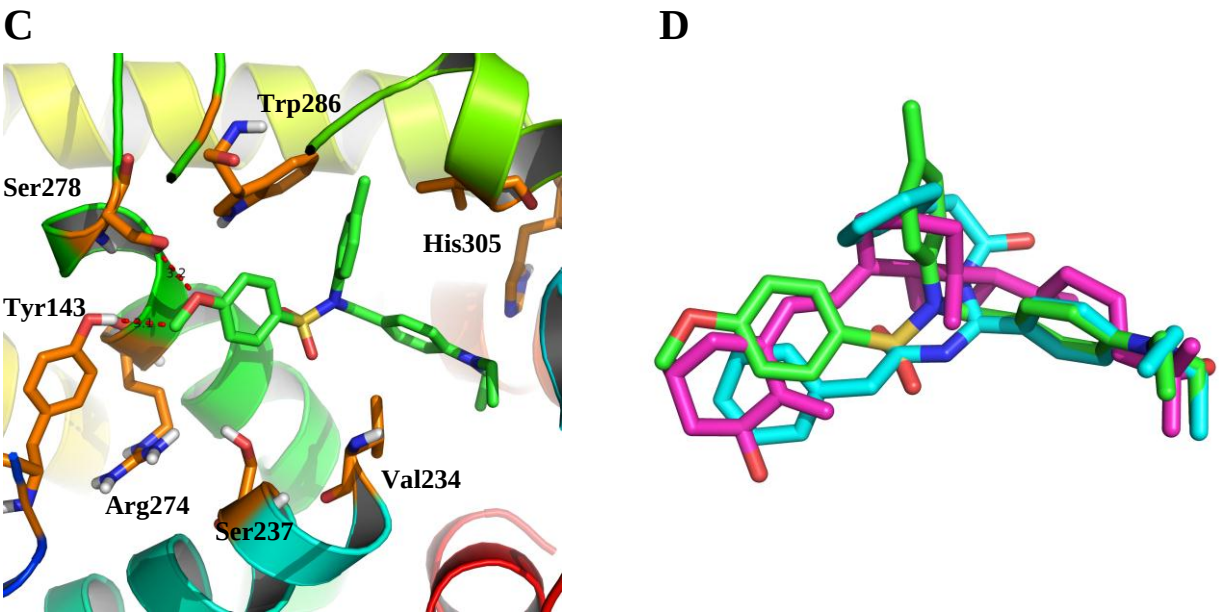


Figure 13. Interaction mode of VDR with vitamin D (A) in co-crystal structure and with Xie95-1024 (B), Xie95-1171 (C) via molecular docking. (D) represents the alignment of these three compounds by adopting their binding conformations. Hydrogen bonding is indicated by red dash with measured distance.

3.3.4 CB2 inverse agonists as ESR1 modulators

It is well-known that selective estrogen receptor modulators (SERMs) possess estrogen receptor antagonistic or agonistic capability depending on target tissue difference.[103] However, in bone tissue, most acted as agonists to exert an estrogen-like effect. For example, Raloxifene, the first approved SERM for osteoporosis therapy, can inhibit osteoclasts formation and bone resorption while also having a stimulatory effect on osteoblasts to facilitate bone formation.[104] On the other hand, ESR1 has been associated strongly with the NOS3 protein using a String protein-protein interaction database search (http://string-db.org/). Relevant literature identifies that ESR1 agonists can enhance the NOS3 level and the release of nitric

oxide (NO), both of which positively contribute for bone formation.[105] Together, ESR1 agonists can promote bone formation by itself or through the activation of NOS3.

Based on our prediction (**Figure 8** and **Table 4**), CB2 inverse agonists are prone to bind with ESR1. There is no direct experimental data to show the binding of CB2 inverse agonists and ESR1, but some strongly related research has been published recently. Prof. Zhao-Hui Song has identified Raloxifene, a marketed estrogen receptor modulator, as a novel CB2 inverse agonist after screening of the 640 FDA-approved drugs.[73] His group confirms two other SERMs Bazedoxifene and Lasofoxifene to be CB2 inverse agonists.[74] Hence, it is reasonable for us to hypothesize that classical CB2 inverse agonists could also be targeting at ESR1. To validate our thought and explore the possible mechanism of interaction, we dock our in-house compounds (Xie95-1042 and Xie96-1171) into the ESR1 protein (PDB_ID: 1ERR).[106] The detailed interactions are shown in **Figure 14** in comparison with its initial co-crystal structure binding with Raloxifene.[106] We observe that both Xie95-1042 and Xie95-1171 have hydrophobic interactions with Met421, Phe404, His524 and Leu525 of ESR1, and form the edge-to-face π-π interaction with Phe404. These observations match quite well this those in Raloxifene-ESR1 co-crystal structure. Moreover, the tertiary amine in Xie95-1042 and Xie95-1171 may be protonated to exert electrostatic interactions with Asp351, which is also in accordance with the observed Raloxifene-ESR1 interaction. However, due to the lack of polar groups in the benzene rings for our compounds, Xie95-1042 and Xie95-1171 lose hydrogen bonding with nearby residues in comparison with co-crystal structure. We assume the missing hydrogen bonding interaction may lead to the decreased binding affinity of our compounds with ESR1. We may design new CB2 inverse agonists with terminal polar benzene rings, which may account for better binding with

ESR1. **Figure 14D** shows the nice alignment of our compounds and Raloxifene with their binding conformation. We can find that when binding these three compounds adopt very close molecular orientation with very similar molecular shape. That may explain why these three compounds can fit the same binding pocket of VDR very well.

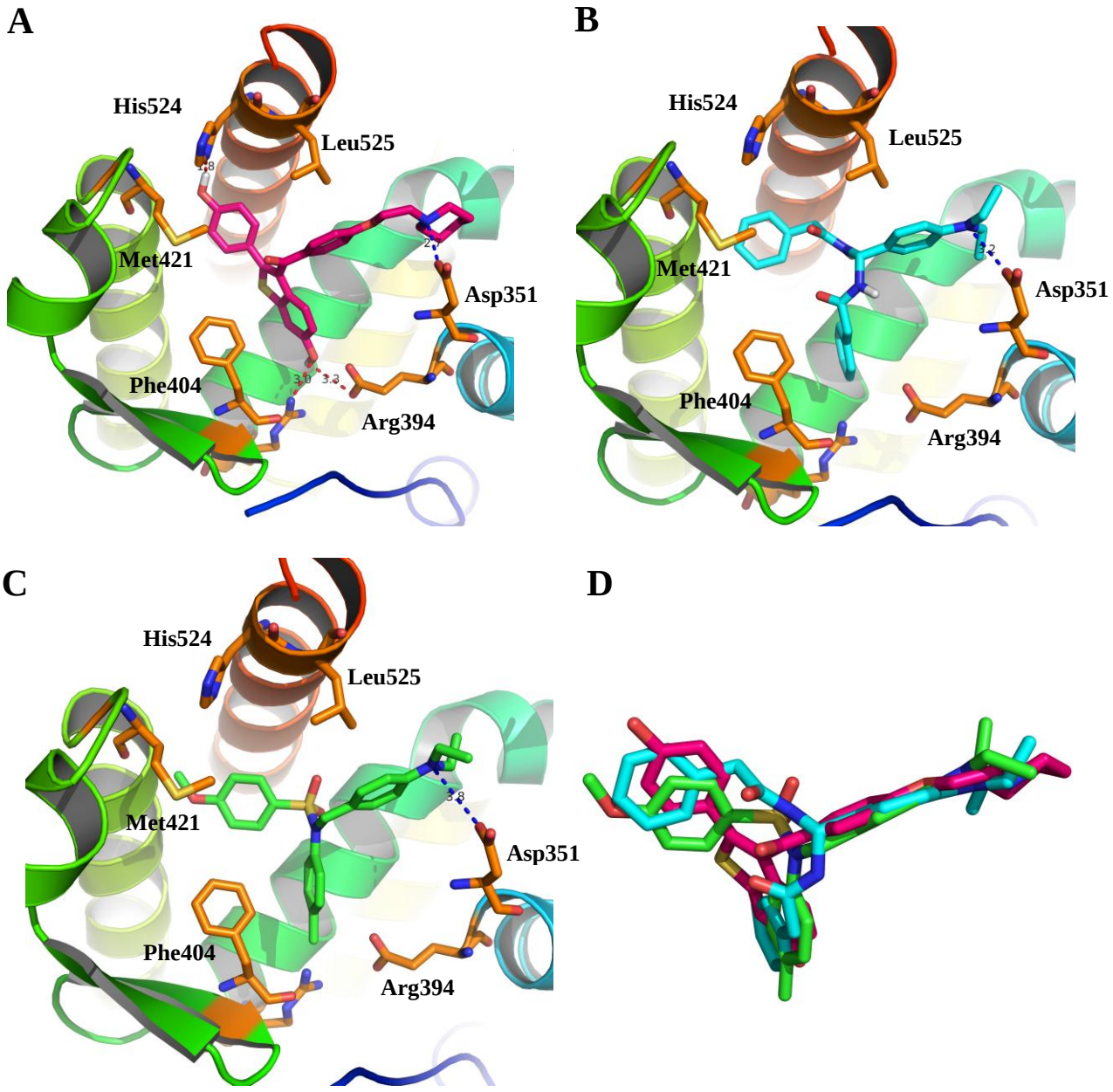


Figure 14. Interaction mode of ESR1 with Raloxifene (A) in co-crystal structure and with Xie95-1042 (B) and Xie95-1171 (C) via molecular docking. Hydrogen bonding is indicated by red dash with measured distance. Electrostatic interaction is indicated by blue dash.

3.3.5 TargetHunter for CB2 inverse agonists off-target prediction

TargetHunter[67] is a complementary tool for off-target prediction, which exerts powerful prediction particularly when protein structures are unrevealed. Herein, we upload two in-house CB2 inverse agonists (Xie95-1042 and Xie95-1171) to our TargetHunter server (http://www.cbligand.org/TargetHunter) where a 2D fingerprint similarity search is performed between queried compounds and the entire CHEMBL database.[63] The identified similar compounds were displayed with the information of their targets, bioactivities and bioassays, and ranked according to similarity scores. First of all, we witness that there is no significant similarity between our compounds and any compound in the CHEMBL database (all pairwise similarity scores ≤ 0.6), suggesting the structural novelty of our compounds. Then we choose the most similar compound(s) for Xie95-1042 (CHEMBL1531234 and CHEMBL1416161 with equal similarity of 0.55) and also for Xie95-1171: that is CHEMBL1329253 with similarity of 0.59. These three CHEMBL compounds shares two targets: aldehyde dehydrogenase 1A1 (ALDH1A1) and glucagon-like peptide 1 receptor (GLP1R), showing biological activity at 10 μM level (**Figure 9**). In fact, two classical CB2 inverse agonists, AM-630 and JTE-907, have shown inhibition against aldehyde dehydrogenase 1A1 with 5.0 μM and 23.8 μM (PubChem assays:1030), respectively. Hence, we propose that our in-house compounds could also bind with ALDH1A1. Recent publications report that ALDH1A1 is treated as a useful marker for prediction of the clinical outcome of breast cancer subsets[107, 108] and shows a potential role

in non-small cell lung carcinogenesis[109] and multiple myeloma.[110] Meanwhile, we know that the CB2 inverse agonist has therapeutic potential in cancer treatment.[18, 45, 111, 112] Thus, our predicted target ALDH1A1 for in-house CB2 inverse agonists may imply novel mechanism for their anti-tumor potency.

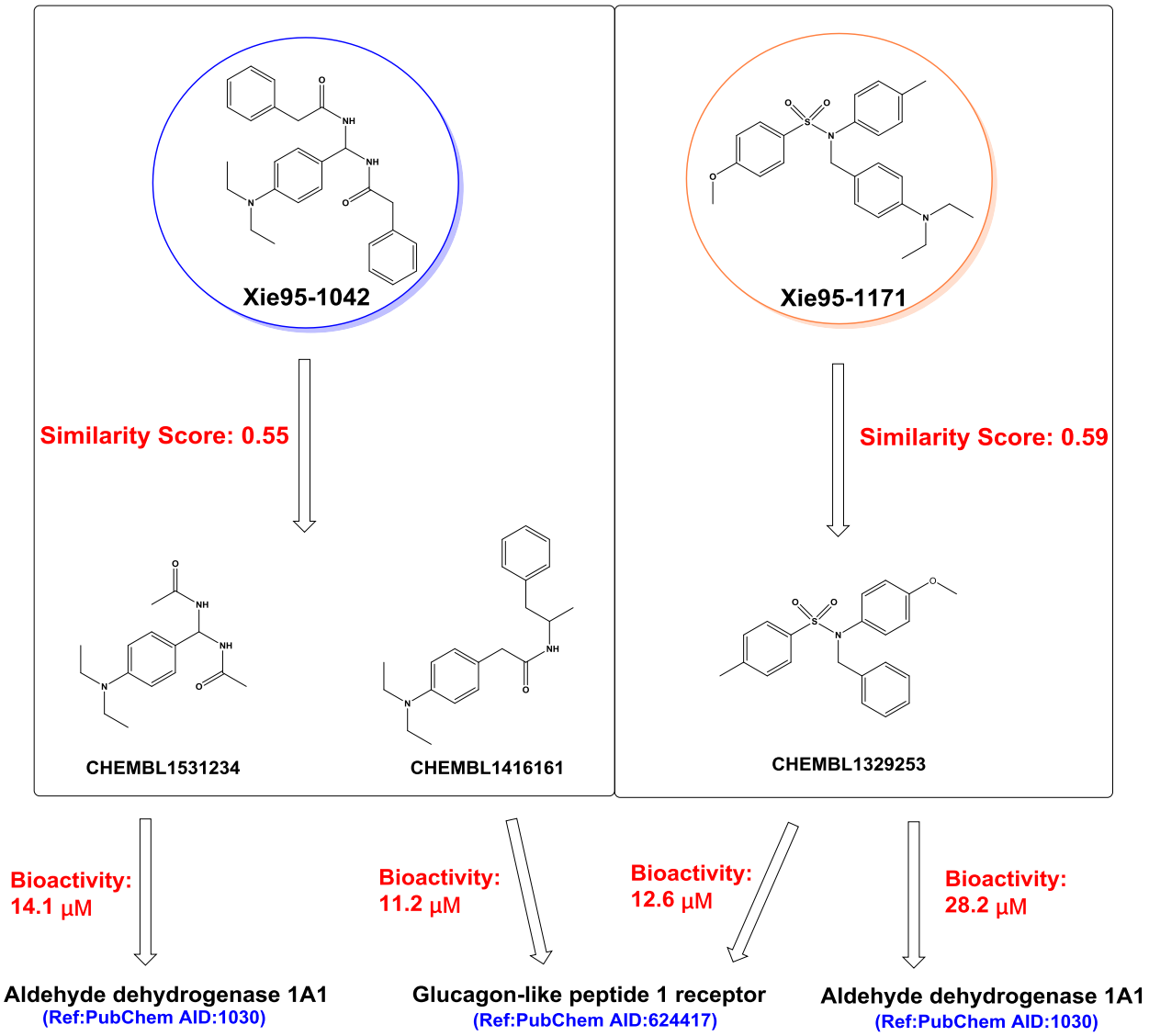


Figure 15. TargetHunter used for off-target prediction for in-house CB2 inverse agonists

4.0 DISCUSSION

In summary, our approach provides a paradigm for polypharmacology study on a specific disease domain, integrating disease domain knowledgebase construction, structure-based method (HTDocking), ligand-based method (TargetHunter) and molecular docking approach (**Figure 11**).

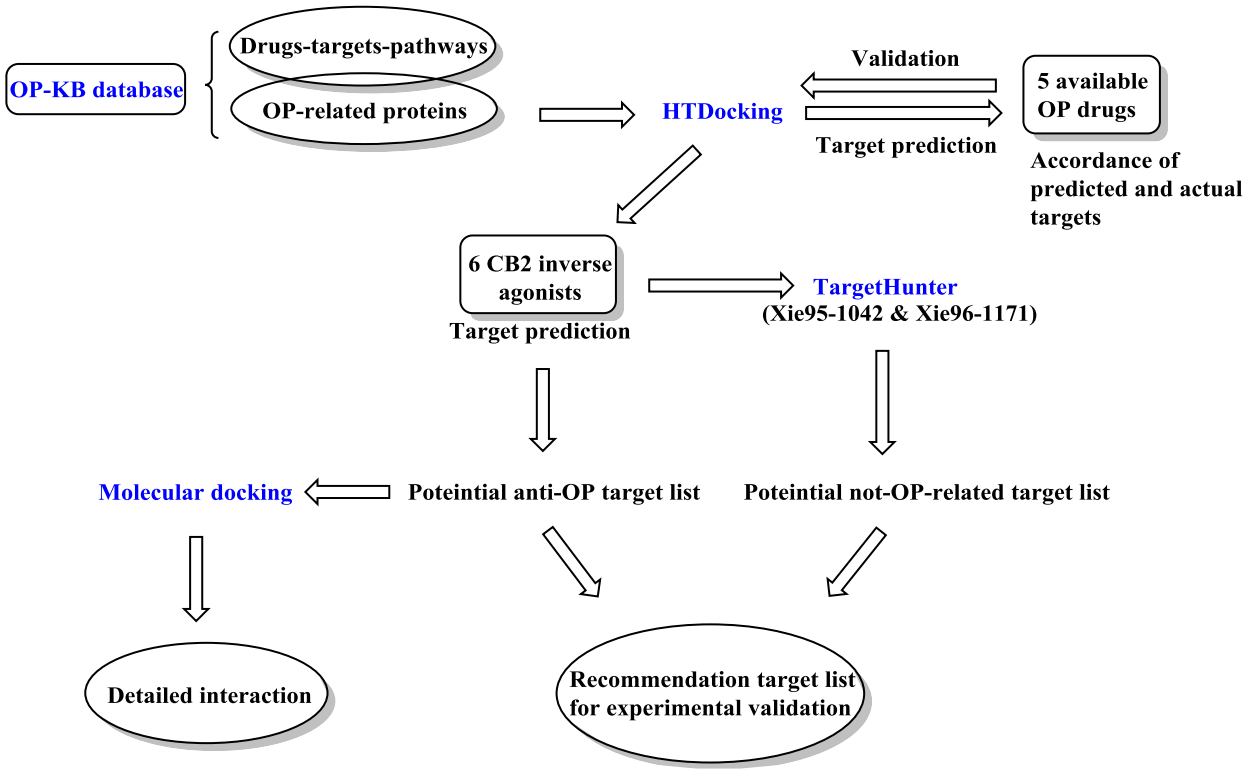


Figure 16. The paradigm for polypharmacology study on OP specific domain

In OP-KB, we compile the data of osteoporosis drugs, targets, related proteins and involved pathways, and do further data analysis. We claim that ESR1 is the most successful anti-OP target because it has the biggest number of marketed drugs (**Table 5** in **Appendix B**). Meanwhile, we observe that osteoporosis and cancer share some signaling pathways. Therefore, some anti-OP drugs could also function as anti-cancer agents. For example, denosumab, which is an antiresorptive agent used for menopausal bone loss, also shows therapeutic potential in mammary tumors.[113] Another case is ESR1 modulator such as Lasofoxifene, which has been reported to reduce breast cancer risk in postmenopausal osteoporotic women.[114]

In the polypharmacology analysis for five anti-OP drugs, we predict their possible anti-OP targets. Apart from the good accordance of known targets with predicted targets, other potential targets and resulting cross-talk interactions are also observed. For instance, Oxandrolone is associated with sex hormone-binding globulin protein (SHBG) as predicted, regardless of its established role as an ANDR agonist. Although there is no direct evidence to show the binding of Oxandrolone with SHBG, previous study convince that the treatment of Oxandrolone can significantly reduce the SHBG level.[115] Again, Letrozole, initially an aromatase inhibitor binding with its cytochrome P450 unit, is predicted to target with ESR1. As reported in a clinical study, Letrozole can induce the regression of estrogen-dependent tumors itself[116] and is commonly used in combination with anti-estrogen drugs for breast cancer therapy.[117] Our prediction may allow drug repurposing for Letrozole as an ESR1 antagonist in the breast cancer therapy.

In the polypharmacology analysis for six CB2 inverse agonists, we predict a list of potential anti-OP targets for these compounds. Most of the predicted targets are identical to those predicted for available anti-OP drugs including the top six candidates (NOS3, DHI1, VDR, ALDH2, TRFL, and ESR1). Detailed interactions are proposed via molecular docking between these targets and three CB2 inverse agonists (Xie95-1024, Xie95-1171, and AM630), which can nicely identify key residues when their binding with initial ligands.

Importantly, the polypharmacology analysis for CB2 inverse agonists also help our understanding of their potential role in enhancing the bone formation. In fact, all of six top predicted targets are expressed in osteoblast and contributable to osteoblast proliferation. Taking NOS3 (the most top possible target) for example, current genetic studies showed some NOS3 gene polymorphisms like Glu298Asp[118] and variable number tandem repeat (VNTR)[119] gene change are implicated in postmenopausal osteoporosis. In coincidence with this, NOS gene-deficient mice exhibited marked abnormalities in bone volume and formation rate and reduced bone mineral density that are mainly due to dysfunctional osteoblasts.[120] Furthermore, DHI1 has been found to be expressed in osteoblasts and modulated by proinflammatory cytokines.[121] It reduces the conversion of cortisone to cortisol that activates glucocorticoid receptor (GR). The monomeric GR can suppress proinflammatory cytokines, leading to decreased osteoblast differentiation.[122] In terms of ALDH2, a recent publication reports that its dominant-negative form, ALDH2*2, can promote osteoporosis due to impaired osteoblastogenesis.[123] Another target, TRFL, has shown positive effects on bone turnover by decreasing bone resorption and increasing bone formation. This finding is indicated by a change

in the levels of distinct bone metabolism markers.[124] As aforementioned, activation of ESR1 can promote bone formation alone or through the stimulation of NOS3.[104, 105]

On the basis of our prediction, we are more interested in the potential role of some CB2 inverse agonists as ESR1 modulators inspired by the fact that some ESR1 modulators have been validated experimentally as CB2 inverse agonists. On the other hand, ESR1 is currently considered as the most successful anti-OP target. We assume that perhaps ESR1 and CB2 share some common signaling pathways that are involved in bone cell proliferation and osteoporosis development. Therefore, based on the literature reports, we draft the putative downstream signaling pathways shared by ESR1 and CB2 (**Figure 15**). As shown, CB2 and ESR1 are both expressed in membrane, which can be activated by the outside signal and accordingly trigger the downstream signaling. These two receptors are believed to share four pathways including PI3K-Akt-anti-apoptosis pathway, PI3K-Akt-NO pathway, PI3K-NF-kB pathway, and Erk1/2-MAPKAPK2-CREB pathway. Ultimately, all of them exert effects on gene transcription and thereby promote cell proliferation. However, we have already known that some compounds such as Lasofoxifene and Xie95-1042 can inhibit CB2 while activate ESR1 in bone tissue. People may wonder the overall effect of these dual function compounds on bone cell proliferation. We consider that the relative binding affinity of compounds with CB2 and ESR1 may count a lot. In addition, the expression level of CB2 and ESR1 in different bone cells and the concentration of compounds are also contributing factors.

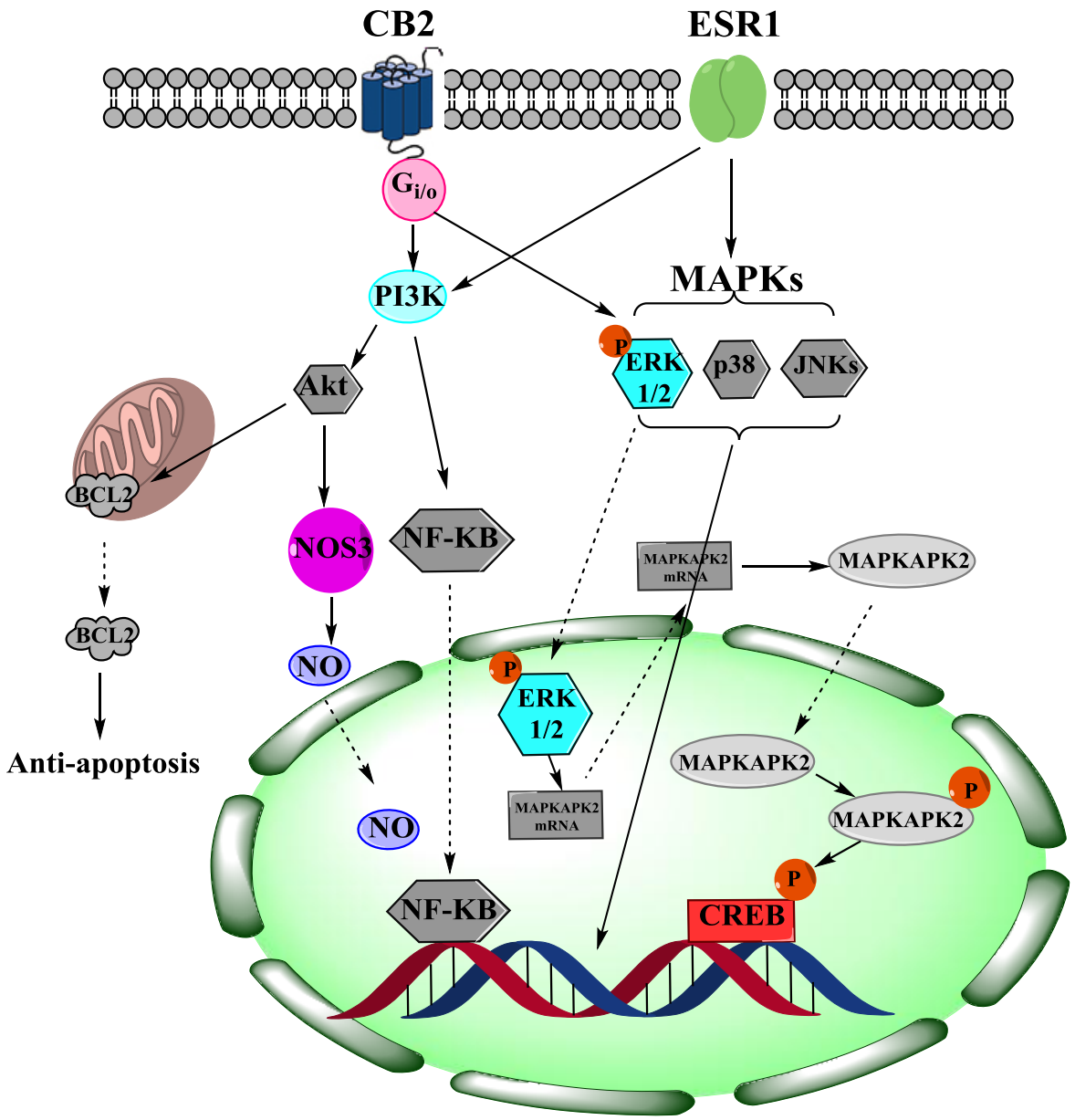


Figure 17. Putative signaling pathways shared by ESR1 and CB2

5.0 FUTURE SPECULATION

In this thesis, we use polypharmacology analysis to predict possible targets for CB2 inverse agonists and some available anti-OP drugs. In the future study, we have some considerations as follows:

1) Validation of our off-target prediction by experiments. We proposed some potential targets for CB2 inverse agonists in osteoporosis therapy or for drug repurposing purpose like NOS3, DHI1, VDR, ALDH2, ESR1, ALDH1A1 and so forth. We are planning to find collaborative labs to test the bioactivities/binding affinities of CB2 inverse agonists for those proteins. Besides, the follow-up biological functional study by targeting those proteins is also necessary to distinguish the inhibition or activation effects. **Table 5** provides a recommendation target list of five anti-OP drugs and three CB2 inverse agonists for future experimental tests.

Table 5. Recommendation target list for experimental validation

Chemicals	Primary Targets	Shared Predicted Targets
Lasofoxifene	ESR1, ESR2	NOS3, DHI1, ALDH2, MK01, PAI1, LOX12, QPCT, PAI1
Estradiol	ESR1, ESR2	ALDH2, SHBG
Calcitriol	VDR	ALDH2,DHI1,SHBG,MK01, TRFL, VTDB
Letrozole	CP19A	NOS3, DHI1, ALDH2,ESR1
Oxandrolone	ANDR	DHI1, SHBG
Xie95-1042	CB2	NOS3, DHI1, VDR, ALDH2, TRFL, ESR1,MK01, ALDH1A1, SHBG, QPCT
Xie95-1171	CB2	NOS3, DHI1, VDR, ALDH2, TRFL, ESR1,ESR2, PAI1, ALDH1A1, LOX12
AM630	CB2	DHI1, ALDH2, TRFL,ALDH1A1, MK01, QPCT

2) Design of multi-target agents for osteoporosis therapy. Our study showed the polypharmacological effects of CB2 inverse agonists. Upon experimental validation, we will focus on how to transfer polypharmacological concept to practical drug design. Based on our published method,[55] we will use fused-pharmacophore modeling approach to design multi-target agents. The procedure is simply shown in **Figure 17**. If we tend to design dual-action anti-osteoporosis agents targeting A and B, we need first build pharmacophore models for both active A and B modulators, respectively. Accordingly, we overlap these two models together by merging common pharmacophore features. The resulting fused-pharmacophore model will keep all distinct pharmacophore features satisfying the dual potency requirements of A and B modulators, and thereby can be used in further virtual screening to find new chemotypes with expected dual activity for A and B.

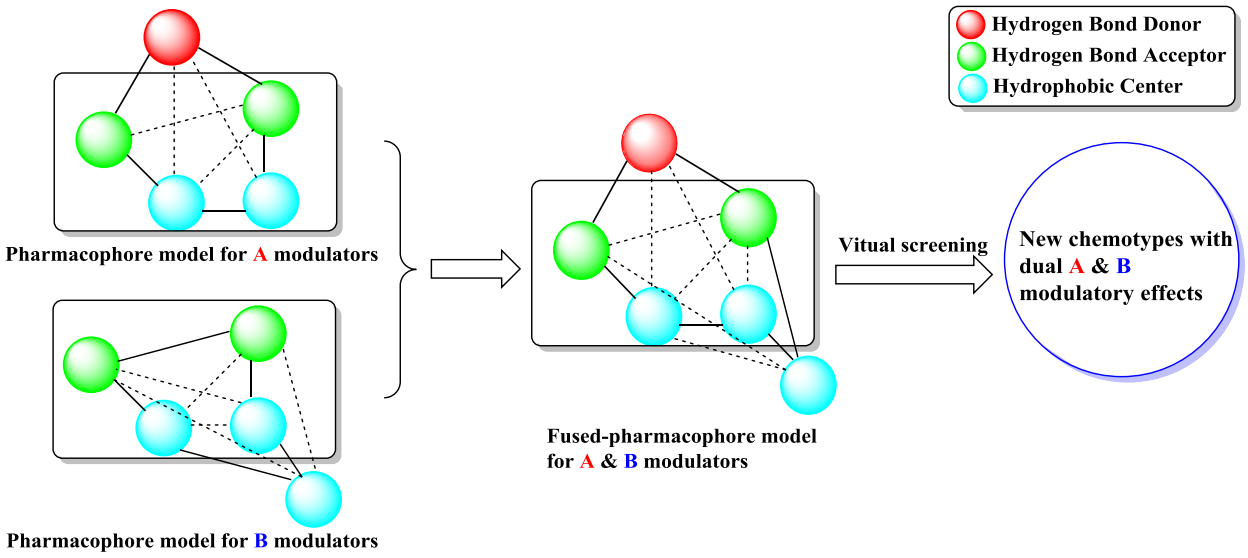


Figure 18. Procedure of fused-pharmacophore modeling in the design and discovery of dual action agents.

APPENDIX A

ABBREVIATIONS

ADT3 = ADP/ATP translocase 3

ALDH1A1 = Aldehyde dehydrogenase 1A1

ALDH2 = Mitochondrial aldehyde dehydrogenase

ANDR = Androgen receptor

ANT3 = Antithrombin-III

AT2C1 = Calcium-transporting ATPase type 2C member 1

CALCR = Calcitonin receptor

CALM = Calmodulin

CATK = Cathepsin K

CB1 = Cannabinoid receptor 1

CB2 = Cannabinoid receptor 2

COMP = Cartilage oligomeric matrix protein

CP19A = Cytochrome P450 19A1

DHB1 = Estradiol 17-beta-dehydrogenase 1

DHI1 = Corticosteroid 11-beta-dehydrogenase isozyme 1

ESR1 = Estrogen receptor

ESR2 = Estrogen receptor beta

FA10 = Coagulation factor X

FAAH = Fatty acid amide hydrolase

FPPS = Farnesyl pyrophosphate synthase

GCR = Glucocorticoid receptor

GLP1R = Glucagon-like peptide 1 receptor

GSTT1 = Glutathione S-transferase theta-1

ICAL = Calpastatin

IGF1R = Insulin-like growth factor 1 receptor

LOX12 = 12S-type arachidonate 12-lipoxygenase

LRP6 = Low-density lipoprotein receptor-related protein 6

MAGL = Monoacylglycerol lipase

MK01 = Mitogen-activated protein kinase 1

NOS3 = Endothelial nitric oxide synthase

PAI1 = Plasminogen activator inhibitor 1

PK3CA = Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform

PRGR = Progesterone receptor

PTH1R = Parathyroid hormone/parathyroid hormone-related peptide receptor

QPCT = Glutaminyl-peptide cyclotransferase

SHBG = Sex hormone-binding globulin protein

ST2A1 = Bile salt sulfotransferase

ST2B1 = Sulfotransferase family cytosolic 2B member 1

TNF11 = Tumor necrosis factor ligand superfamily member 11

TRFL = Lactotransferrin

VATA = V-type proton ATPase catalytic subunit A

VDR = Vitamin D3 receptor

VKOR1 = Vitamin K epoxide reductase complex subunit 1

VTDB = Vitamin D-binding protein

APPENDIX B

ANTI-OSTEOPOROSIS DRUGS ON MARTKET OR IN CLINICAL TRIALS

Table 6. 74 anti-osteoporosis drugs on market or in clinical trials

No.	Status	Target	DRUG	Chemical Structure (SMILES format)
1	Approved	FPPS	Alendronic acid	NCCCC(O)(P(O)(O)=O)P(O)(O)=O
2	Phase I	TNF11	ALX-0141	Humanized single-domain antibody
3	Phase II	NR1H4	Apomine	CC(C)OP(=O)(OC(C)C)C(Cc1cc(c(O)c(c1)C(C)(C)C)C(C)(C)C)P(=O)(OC(C)C)OC(C)C
4	Phase III	ESR1	Arzoxifene	COc1ccc(cc1)-c1sc2cc(O)ccc2c1Oc1ccc(OCCN2CCCCC2)cc1
5	Phase I	CASR	ATF936	O=C1N=C(C2=CC=C(C(C)C)C=C2)C3=CC(OCC#C)=CC=C3N1CC4=CC(OCC)=C(OC)C=C4
6	Clinical trial	Unknown	Avicatonin	CCCC(=O)NC(C)C(=O)NC(CO)C(=O)NC(CC(C)C)C(=O)NC(CO)C(=O)NC(C(C)O)C(=O)NC(CC)C(=O)NC(C(C)C)C(=O)NC(CC(C)C)C(=O)NCC(=O)NC(CCCCN)C(=O)NC(CC(C)C)C(=O)NC(CO)C(=O)NC(CCC(=O)N)C(=O)NC(CCC(=O)O)C(=O)NC(CC(C)C)C(=O)NC(CC1=CN=CN1)C(=O)NC(CCCCN)C(=O)NC(CC(C)C)C(=O)NC(CCC(=O)N)C(=O)NC(C(C)O)C(=O)NC(CC2=CC=C(C=C2)O)C(=O)N3CCCC3C(=O)NC(CCCNC(=N)N)C(=O)NC(C(C)O)C(=O)NC(CC(=O)O)C(=O)NC(C(C)C)C(=O)NCC(=O)NC(C)C(=O)NCC(=O)NC(C(C)O)C(=O)N4CCCC4C(=O)N
7	Phase I	Unknown	AXT914	Unrevealed
8	Phase III	Unknown	BA058	human parathyroid hormone-related peptide
9	Phase II	CATK	Balicatib	CCCN1CCN(CC1)c1ccc(cc1)C(=O)NC1(CCCCC1)C(=O)NCC#N

10	Approved	ESR1	Bazedoxifene	Cc1c(-c2ccc(O)cc2)n(Cc2ccc(OCCN3CCCCCC3)cc2)c2ccc(O)cc12
11	Phase II	SOST	Blosozumab	monoclonal antibody IgG4
12	Phase II	SOST	BPS-804	human IgG2 monoclonal antibody
13	Phase II	Unknown	C3578	Unrevealed
14	Approved	CALCR	Calcitonin	CC(C)CC(NC(=O)C(NC(=O)C1CSSCC(N)C(=O)NC(CO)C(=O)NC(CC(N)=O)C(=O)NC(CC(C)C)C(=O)NC(CO)C(=O)NC(C(C)O)C(=O)N1)C(C)C)C(=O)NCC(=O)NC(CCCCN)C(=O)NC(CC(C)C)C(=O)NC(CO)C(=O)NC(CCC(N)=O)C(=O)NC(CCC(O)=O)C(=O)NC(CC(C)C)C(=O)NC(Cc1cnc[nH]1)C(=O)NC(CCCCN)C(=O)NC(CC(C)C)C(=O)NC(CCC(N)=O)C(=O)NC(C(C)O)C(=O)NC(Cc1ccc(O)cc1)C(=O)N1CCCC1C(=O)NC(CCCNC(N)=N)C(=O)NC(C(C)O)C(=O)NC(CC(N)=O)C(=O)NC(C(C)O)C(=O)NCC(=O)NC(CO)C(=O)NCC(=O)NC(C(C)O)C(=O)N1CCCC1C(N)=O
15	Approved	VDR	Calcitriol	C[C@H](CCCC(C)(O)C)[C@@]1([H])CC[C@@]2([H])\C(CCC[C@]12C)=C\C=C1\C[C@@H](O)C[C@H](O)C1=C |r|
16	Phase I	ESR2	CHF4227	COc1ccc(cc1)C1=C(Cc2ccc(OCCN3CCCCC3)cc2)c2ccc(O)cc2OC1 |c:9|
17	Approved	Unknown	Clodronic acid	OP(O)(=O)C(Cl)(Cl)P(O)(O)=O
18	Approved	VDR	Colecalciferol	CC(C)CCC[C@@H](C)[C@@]1([H])CC[C@@]2([H])\C(CCC[C@]12C)=C\C=C1\C[C@@H](O)CCC1=C |r|
19	Approved	DHB1 ST2A1 ST2B1	Dehydroepiandrosterone	[H][C@@]12CCC(=O)[C@@]1(C)CC[C@@]1([H])[C@@]2([H])CC=C2C[C@@H](O)CC[C@]12C |r,t:17|
20	Approved	TNF11	Denosumab	human IgG2 monoclonal antibody
21	Approved	GCR	Dexamethasone	[H][C@@]12C[C@@H](C)[C@](O)(C(=O)CO)[C@@]1(C)C[C@H](O)[C@@]1(F)[C@@]2([H])CCC2=CC(=O)C=C[C@]12C |r,c:28,t:24|
22	Clinical trial	VDR	Doxercalciferol	CC(C)[C@@](C)([H])\C=C\[C@](C)([H])[C@@]1([H])CC[C@@]2([H])\C(CCC[C@]12C)=C\C=C1\C[C@@H](O)C[C@H](O)C1=C |r|
23	Approved	CALCR	Elcatonin	XSNLSTCVLGKLSQQLHKLQTYPRTNTGSGTX
24	Phase III	VDR	Eldecalcitol	OCCCO[C@@H]1[C@H](O)C\C(=C\C=C2/CCC[C@]3(C)[C@H](CC[C@@]23[H])[C@H](C)CCCC(O)(C)C)C(=C)[C@H]1O |r|
25	Phase II	CASR	Encaleret	[H][C@@](O)(CNC(C)(C)Cc1ccc(Cl)c(F)c1)CO[C@]([H])(C)c1ccccc1-c1ccc(C(O)=O)c(C)c1 |r|

26	Approved	ESR1 ESR2	Estradiol	[H][C@@]12CC[C@H](O)[C@@]1(C)CC[C@]1([H])c3ccc(O)cc3CC[C@@]21[H] |r|
27	Approved	ESR1	Estrone	[H][C@@]12CCC(=O)[C@@]1(C)CC[C@]1([H])c3ccc(O)cc3CC[C@@]21[H] |r|
28	Approved	ESR1	Estropipate	C1CNCCN1.C[C@]12CC[C@H]3[C@@H](CCc4cc(OS(O)(=O)=O)ccc34)[C@@H]1CCC2=O |r|
29	Approved	PK3CA	Etidronic acid	CC(O)(P(O)(O)=O)P(O)(O)=O
30	Discontinued	VDR	Falecalcitriol	C=C1\C(=C\C=C2\CCC[C@@]3(C)[C@@]2([H])CC[C@]3([H])[C@@](C)([H])CCCC(O)(C(F)(F)F)C(F)(F)F)C[C@@]([H])(O)C[C@]1([H])O |r|
31	Approved	ESR1	Fosfestrol	CC\C(=C(\CC)c1ccc(OP(O)(O)=O)cc1)c1ccc(OP(O)(O)=O)cc1
32	Approved	ANT3	Heparin	CC(=O)NC1C(C(C(OC1O)COS(=O)(=O)O)OC2C(C(C(C(O2)C(=O)O)OC3C(C(C(C(O3)CO)OC4C(C(C(C(O4)C(=O)O)O)O)OS(=O)(=O)O)OS(=O)(=O)O)NS(=O)(=O)O)O)OS(=O)(=O)O)O
33	Approved	FPPS	Ibandronic acid	CCCCCN(C)CCC(O)(P(O)(O)=O)P(O)(O)=O
34	Approved	Unknown	Ipriflavone	CC(C)Oc1ccc2c(c1)occ(-c1ccccc1)c2=O
35	Approved	ESR1	Lasofoxifene	Oc1ccc2[C@H]([C@H](CCc2c1)c1ccccc1)c1ccc(OCCN2CCCC2)cc1 |r|
36	Approved	CP19A	Letrozole	C1=CC(=CC=C1C#N)C(C2=CC=C(C=C2)C#N)N3C=NC=N3
37	Phase III	ESR1	Levormeloxifene	COc1ccc2[C@H]([C@H](c3ccccc3)C(C)(C)Oc2c1)c1ccc(OCCN2CCCC2)cc1 |r|
38	Clinical trial	ANDR	LGD2941	FC(F)(F)CN(CC(F)(F)F)c1ccc2[nH]c(=O)cc(c2c1)C(F)(F)F
39	Approved	ESR1 PRGR	Medroxyprogesterone	[H][C@@]12CC[C@](O)(C(C)=O)[C@@]1(C)CC[C@@]1([H])[C@@]2([H])C[C@H](C)C2=CC(=O)CC[C@]12C |r,t:22|
40	Approved	ESR1 PRGR	Medroxyprogesterone acetate	[H][C@@]12CC[C@](OC(C)=O)(C(C)=O)[C@@]1(C)CC[C@@]1([H])[C@@]2([H])C[C@H](C)C2=CC(=O)CC[C@]12C |r,t:25|
41	Approved	ANDR	Methyltestosterone	[H][C@@]12CC[C@](C)(O)[C@@]1(C)CC[C@@]1([H])[C@@]2([H])CCC2=CC(=O)CC[C@]12C |r,t:19|
42	Phase III	FPPS	Minodronic acid	OC(Cc1cnc2ccccn12)(P(O)(O)=O)P(O)(O)=O
43	Phase II	Integrin $\alpha_V\beta_3$	MK0429	COC1=NC=C(C=C1)[C@H](CC(O)=O)N1CCN(CCCC2=NC3=C(CCCN3)C=C2)C1=O

44	Phase II	Unknown	MK5442	CC1=C(C=CC(=C1)C2=CC=CC=C2C(C)OCC(CNC(C)(C)CC3=CC(=C(C=C3)Cl)F)O)C(=O)O
45	Clinical trial	FPPS	Neridronic acid	NCCCCCC(O)(P(O)(O)=O)P(O)(O)=O
46	Phase II	PGH2	Nitroflurbiprofen	CC(C(=O)OCCCCO[N+]([O-])=O)c1ccc(c(F)c1)-c1ccccc1
47	Preclinical	CASR	NPS2143	CC(C)(Cc1ccc2ccccc2c1)NC[C@H](O)COc1cccc(Cl)c1C#N |r|
48	Phase III	CATK	Odanacatib	CC(C)(F)C[C@H](N[C@@H](c1ccc(cc1)-c1ccc(cc1)S(C)(=O)=O)C(F)(F)F)C(=O)NC1(CC1)C#N |r|
49	Clinical trial	FPPS	Olpadronic Acid	CN(C)CCC(O)(P(O)(O)=O)P(O)(O)=O
50	Phase II	CATK	ONO-5334	Unrevealed
51	Approved	ESR1	Ospemifene	OCCOc1ccc(cc1)C(=C(\CCCl)c1ccccc1)\c1ccccc1
52	Discontinued	TNF11	Osteoprotegerin	Protein
53	Approved	ANDR	Oxandrolone	CC12CCC3C(C1CCC2(C)O)CCC4C3(COC(=O)C4)C
54	Approved	GCR	Prednisolone	[H][C@@]12CC[C@](O)(C(=O)CO)[C@@]1(C)C[C@H](O)[C@@]1([H])[C@@]2([H])CCC2=CC(=O)C=C[C@]12C |r,c:27,t:23|
55	Approved	PTH1R	Preotact	Parathyroid hormone human
56	Phase I	Unknown	PTH134	SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNF
57	Clinical trial	Unknown	PTHY	SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNFVALGAPLAPRDAGSQRPRKKEDNVLVESHKSGEADKADVNVLTKAKSQ
58	Approved	ESR1 ESR2	Raloxifene	Oc1ccc(cc1)-c1sc2cc(O)ccc2c1C(=O)c1ccc(OCCN2CCCCC2)cc1
59	Phase I	CATK	Relacatib	CC(C)C[C@H](NC(=O)c1cc2ccccc2o1)C(=O)N[C@H]1CC[C@@H](C)N(CC1=O)S(=O)(=O)c1ccccn1 |r|
60	Approved	FPPS	Risedronic acid	OC(Cc1cccnc1)(P(O)(O)=O)P(O)(O)=O
61	Phase III	SOST	Romosozumab	humanized monoclonal antibody of Immunoglobulin G2

62	Phase II	CASR	Ronacaleret	CC(C)(CC1Cc2ccccc2C1)NC[C@@H](O)COc1cc(CCC(O)=O)cc(F)c1F |r|
63	Discontinued	Integrin $\alpha_V\beta_3$	SB273005	CNc1cccc(CCOc2ccc3C[C@@H](CC(O)=O)C(=O)N(CC(F)(F)F)Cc3c2)n1 |r|
64	Phase I	Unknown	Sotatercept	Homo sapiens ACVR2A, 21-135 precursor fragment (1-115) -threonyl-triglycyl linker (116-119) -gamma1 chain H-CH2-CH3 fragment (120-344)
65	Approved	Unknown	Strontium ranelate	[Sr++].[Sr++].[O-]C(=O)CN(CC([O-])=O)c1sc(C([O-])=O)c(CC([O-])=O)c1C#N
66	Approved	PTH1R	Teriparatide	SVSEIELMHDLKHLDSMERVEWLRKKLEDVHDF
67	Approved	ANDR	Testosterone	[H][C@@]12CC[C@H](O)[C@@]1(C)CC[C@@]1([H])[C@@]2([H])CCC2=CC(=O)CC[C@]12C |r,t:18|
68	Approved	ANDR	Testosterone Propionate	[H][C@@]12CC[C@H](OC(=O)CC)[C@@]1(C)CC[C@@]1([H])[C@@]2([H])CCC2=CC(=O)CC[C@]12C |r,t:22|
69	Approved	VATA	Tiludronic acid	OP(O)(=O)C(Sc1ccc(Cl)cc1)P(O)(O)=O
70	Approved	ANT3 AT2C1 CALM COMP FA10 ICAL	Tinzaparin	Unrevealed
71	Phase I	CATK	VEL0230	CC(C)COC[C@H](CC(C)C)NC(=O)[C@H]1O[C@@H]1C(O)=O |r|
72	Phase III	BGLAP OSTC VKGC	Vitamin K1	CC(C)CCCC(C)CCCC(C)CCC\C(C)=C\CC1=C(C)C(=O)c2ccccc2C1=O |c:20|
73	Approved	VKOR1	Warfarin	CC(=O)CC(c1ccccc1)c1c(O)oc2ccccc2c1=O
74	Approved	FPPS	Zoledronic acid	OC(Cn1ccnc1)(P(O)(O)=O)P(O)(O)=O

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