



## AAUP Journal of STEM and Health Sciences (JSH-AAUP)

# Designing Around Aldehyde Oxidase: Structural Modifications in Brain-Penetrant Heterocycles: Structure–Activity Relationship, Strategies and Mechanistic Insights

Huda Masri <sup>1,\*</sup>

<sup>1</sup>Arab American University, Faculty of Pharmacy

ORCID: <https://orcid.org/0009-0006-1347-8750>

\*Corresponding Author Email: [huda.masri@aaup.edu](mailto:huda.masri@aaup.edu)

Date Received: 09/08/2026

Date Revised: 02/09/2026

Date Accepted: 11/09/2026

Date Published: 30/09/2026

DOI: <https://doi.org/10.35517/JSH-AAUP.v1.02.03>

## Abstract

The review critically examined the metabolic liability of aldehyde oxidase (AO), an important consideration in drug discovery, particularly for CNS drug candidates that must cross the blood-brain barrier (BBB) while remaining metabolically stable. The review compared the structural modification strategies adopted to overcome AO-mediated metabolism in brain-penetrant heterocyclic compounds, with emphasis on the mechanistic rationale and level of evidence, and the translational scope of each approach. Structure–activity relationship (SAR) strategies examined included strategic fluorination, ortho-substitution, and scaffold-hopping methods, notably switching a pyridine ring to a pyrimidine. The analysis of the experimental, computational, and case-study literature indicated that site-specific substitutions (especially at the C2 position of azaheterocycles) consistently reduce AO-mediated oxidation, and that strategic fluorination modulates physicochemical properties relevant to BBB penetration by altering pKa, lipophilicity, and interactions with efflux transporters. An alternative kinetic reduction of metabolic turnover is the incorporation of deuterium at oxidized sites. Crystallography, molecular docking, and quantum chemical modelling have been used to study the mechanistic aspects of the AO reaction and its dynamics of binding to the active site, which led to the elucidation of AO active site architecture and rational drug design. Integrated SAR strategies are demonstrated in case studies with  $\beta$ -secretase (BACE1) inhibitors, GluN2B selective negative allosteric modulators, and c-Met kinase inhibitors, which led to the discovery of potent, brain-penetrant molecules with better PK properties. The problems that still lie ahead are interspecies variability in AO activity, underprediction of AO clearance in vitro, and lack of clinical validation data. Increased attention to multiparametric

optimization processes involving both AO metabolic liability and CNS penetration, using enhanced computational models and standardized in vitro phenotyping tools, would be warranted for future research.

**Keywords:** aldehyde oxidase, brain-penetrant heterocycles, structure-activity relationship, pyridine to pyrimidine, drug metabolism fluorination.

## 1. Introduction

### 1.1 The role of Aldehyde Oxidase in Drug Metabolism

Aldehyde oxidase (AO; EC 1.2.3.1) is a hydroxylase enzyme that contains molybdenum and is expressed primarily in the liver and in the intestine, lungs, and brain. Over the past two decades, AO has been recognized as an important enzyme involved in the metabolism of a large and structurally diverse set of azaheterocycles and aldehydes [1,2]. In contrast to the cytochrome P450 (CYP) enzymes, AO does not require molecular oxygen as a primary electron donor in the classical sense, but it uses water as the source of oxygen for hydroxylation, which produces hydrogen peroxide as a byproduct. With its exceptional substrate promiscuity, this difference in mechanism makes it a very difficult enzyme to account for during drug development [3].

The clinical relevance of AO was highlighted by several major drug failures, such as BIBX1382, an epidermal growth factor receptor inhibitor, and CP-724,714, a HER-2 kinase inhibitor, that both had very high levels of AO-mediated oxidation in human liver compared to preclinical rodent hepatic preparations [4–6]. What these failures revealed, and what remains insufficiently emphasized in subsequent literature, is not merely that AO is a metabolically active enzyme, but that the entire framework of preclinical pharmacokinetic validation is structurally biased against detecting AO liability: rats have lower and differently distributed AO activity than humans, in vitro cytosol preparations suffer from enzyme instability under standard incubation conditions, and no validated mechanistic in vitro-in vivo (IVIVE) model existed at the time of these failures or exists today in a form adequate for prospective clinical prediction [7,8]. This review treated that gap not as a peripheral limitation but as a central organizing problem for the field.

### 1.2 Particular Challenges in CNS Drug Discovery

The problem of AO liability is especially critical in drug discovery for CNS drugs. Brain penetration is a key requirement for any drug to be metabolically stable while meeting certain physicochemical requirements, including low molecular weight, logP, polar surface area, and resistance to efflux pumps like P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) [9]. Nitrogen-containing aromatic heterocycles (including pyridines, pyrimidines, imidazoles, or quinolines) are widely used in drug candidates for CNS drug design, and it is these functional groups that are the targets of preferential AO-catalyzed oxidation [2]. Thus, medicinal chemists developing brain-permeable therapeutics are left with the paradox of achieving inhibition of AO-mediated metabolism, while at the same time maintaining or optimizing BBB penetration, requiring sophisticated and counterintuitive SAR strategies [2,9].

This challenge is compounded by significant interspecies variation in the expression and activity of AO. In rats, which are the most commonly used preclinical species for pharmacokinetic studies, the hepatic AO activity is significantly lower than in humans, and such pharmacokinetic data therefore systematically underpredict AO-mediated clearance when used in human clinical predictions [7,8]. Cynomolgus monkeys have also been

identified as a more suitable surrogate species for human AO metabolism, but due to the cost and ethical considerations, they are not routinely used in early drug discovery [10]. The consequence is that during typical preclinical development, AO clearance is understated and only comes to surface during human pharmacokinetic studies.

A critical gap in the existing literature is that although numerous individual studies address the reduction of AO liability, there is no published, prospective, head-to-head comparison of the strategies (fluorination, C-2 blocking, scaffold hopping, deuterium substitution) applied to the same series of compounds under the same assay conditions. The field consequently rests on a catalogue of precedents, rather than a ranked evidence base from which to inform decisions on strategy selection; this review aims to make that limitation explicit and to partially correct it through critical, comparative analysis.

### 1.3 Scope and Objectives of the Review

This review contributes to the literature on AO metabolism mitigation in CNS drug discovery in three ways. First, it gives a critical assessment of the evidence that supports each major structural modification strategy, distinguishing strategies supported by primarily empirical precedent from those validated by mechanistic data. Second, it characterizes the translation gap between in vitro AO clearance measurement and in vivo human pharmacokinetics by providing sufficient published IVIVE data to show the size and systematic direction of the IVIVE translation error. Third, it pinpoints three unresolved evidence gaps, which are the absence of comparative head-to-head SAR data, the limitations of existing IVIVE frameworks, and the scarcity of integrated multiparametric datasets, as the key objectives for future methodological investments.

To aid the selection of strategies for a specific scaffold and CNS program, a decision-tree framework was proposed here as a synthesis of the reviewed literature. This framework was provided as an organizational tool and not as a definitive rule to be followed. Each strategy was scaffold-dependent, required certain physicochemical conditions for use, and a recommended sequence of experiments to validate — aspects that have been highlighted separately in previous reviews but have not previously been consolidated in a single practical tool.

### 1.4 Literature Search Methodology

This is a narrative review in which the literature was searched in PubMed, Web of Science and Google Scholar using combinations of the following terms: "aldehyde oxidase", "AO metabolism", "AOX1", "azaheterocycle oxidation", "molybdenum hydroxylase", "brain-penetrant heterocycles", "blood-brain barrier permeability", "CNS drug design", "structure-activity relationships AO", "fluorination metabolic stability", "pyridine pyrimidine bioisostere", "deuterium kinetic isotope effect drug metabolism", "scaffold hopping AO", "BACE1 inhibitor metabolism", "in vitro in vivo extrapolation aldehyde oxidase", "AO clearance prediction", "cynomolgus monkey AO surrogate", and "efflux transporter pKa CNS". These terms were used in a variety of combinations and with Boolean operators (AND, OR) as per the requirement of specificity for each topic area. There was no date restriction. Older work from the early 2010s remained relevant and was cited together with more recent literature.

The papers were chosen based on their relevance to the key topics: AO mechanism and substrate scope, strategies to minimize metabolism by AO, optimization of BBB permeability, computational prediction of AO sites, and case studies of drug candidates in which the AO liability was deliberately minimized. Review articles,

primary research papers, and computation-based studies were all included when they contributed substantially to topics not otherwise covered in primary literature. Experimental papers (in vitro metabolite profiling, in vivo pharmacokinetics, structural biology or quantitative SAR) were preferred over speculative or opinion-based commentaries.

## 2. Aldehyde Oxidase: Biology, Substrate Scope, and Mechanism

### 2.1 Enzyme Structure and Catalytic Mechanism

Human AO (hAO1, encoded by AOX1) is a homodimeric enzyme with each monomer (~150 kDa) comprising three functional domains: an N-terminal domain harboring two [2Fe-2S] iron-sulfur clusters, a central flavin adenine dinucleotide (FAD)-binding domain, and a C-terminal domain that accommodates the molybdenum cofactor (Moco) at the active site [11]. The molybdenum center, in its reduced Mo(IV) state, accepts electrons from the substrate and transfers them sequentially through the iron-sulfur clusters to FAD, where molecular oxygen is ultimately reduced to hydrogen peroxide. Structurally, the AO active site is characterized by a large, predominantly hydrophobic substrate-binding pocket adjacent to the Mo–OH catalytic center, with key residues Phe919, Val811, and Leu799 defining the substrate-binding geometry in the human enzyme [11,12].

Computational and crystallographic investigations have revealed that AO preferentially oxidizes electron-deficient carbon atoms situated adjacent to ring nitrogen atoms in aza-heterocyclic substrates, particularly those at positions that allow optimal coordination with the molybdenum center. [12,13]. The identification of these oxidation-prone positions has been central to guiding rational structural modification strategies.

Recent structural biology studies have provided insights into the mechanisms governing AO dimerization and the functional consequences of AOX1 genetic polymorphisms, including the T755I (rs35217482) variant, which reduces the rate of AO dimerization and the rates of substrate metabolism, revealing the potential for interindividual variability in AO activity and pharmacokinetic variability in clinical populations [14]. This polymorphism has not yet been included in PBPK models of AO substrates, which is a methodological gap that may account for some of the unexplained variability in human pharmacokinetic predictions for this enzyme class. Figure 1 illustrates the three-domain architecture of the human AO1 homodimer and the sequential electron transfer pathway from the molybdenum cofactor through the iron-sulfur clusters to FAD, providing the structural context for understanding how substrate binding at the Mo center initiates the catalytic cycle discussed above.

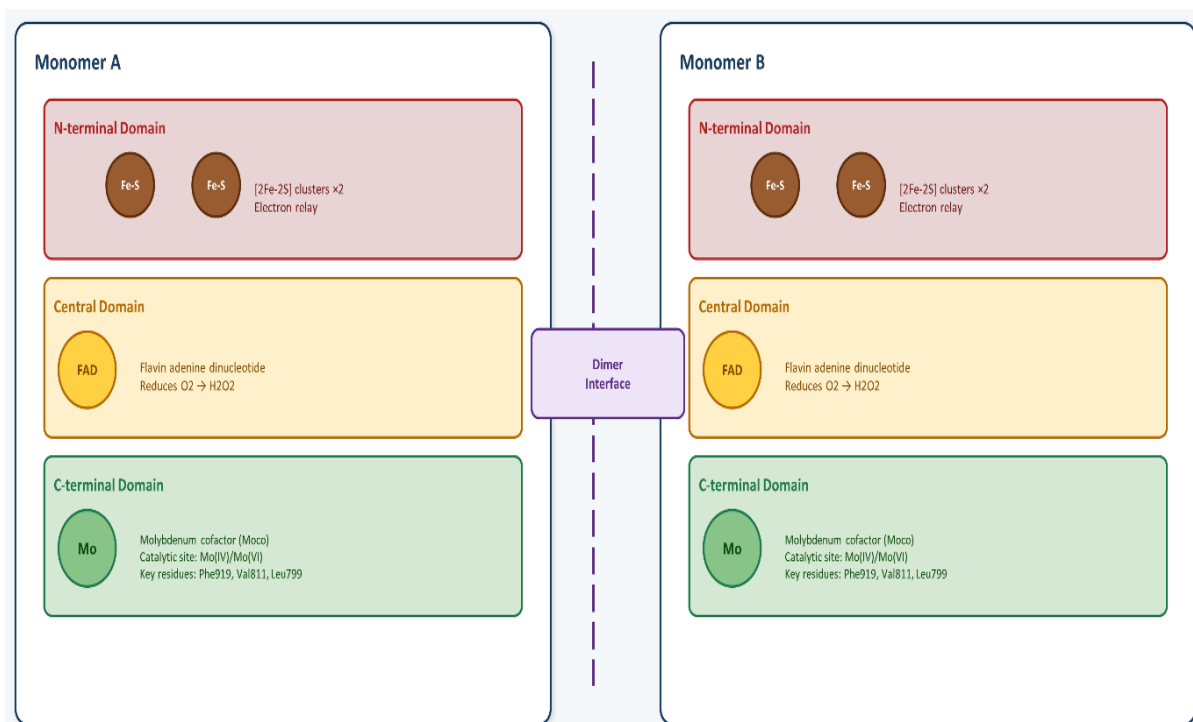


Figure 1. Schematic of the human AO1 homodimer architecture, showing the three functional domains per monomer: [2Fe-2S] iron-sulfur cluster domain (N-terminal), FAD-binding domain (central), and molybdenum cofactor (Moco) domain (C-terminal). The electron transfer pathway from substrate oxidation at the Mo center through FE-S clusters to FAD is indicated by arrows. Structural data adapted from Ferreira et al. [11]. The author prepared this figure using Microsoft PowerPoint (Microsoft Corporation, Redmond, WA, USA) as an original schematic for this review.

## 2.2 Substrate Scope and Species Differences

AO accepts a very broad range of structurally diverse drug candidates as substrates, including quinolines, phthalazines, purines, pyridines, pyrimidines, imidazopyrimidines, and others [2,3]. This promiscuity arises from the large and flexible active site of the enzyme, which can hold structurally different substrates. However, kinetic parameters vary significantly based on the electronic and steric characteristics of the substrate, as seen in systematic studies of zoniporide analogues, where structural changes yielded significant differences in AO oxidation rate [15]. The common scaffolds are summarized in Table 1 below.

The interspecies difference in AO expression and activity constitutes perhaps the most consequential unresolved challenge in the field. Humans have only a single AO isoform (AOX1) expressed at high levels in the liver, whereas rodents have several AO isoforms with different substrate specificities and activities [16]. Standard rodent pharmacokinetic models often underestimate human AO clearance, a discrepancy that has contributed to drug development failures at late stages of development. Empirical analyses have further demonstrated that current in vitro systems—including liver cytosol preparations and recombinant enzyme assays—systematically underpredict AO-mediated clearance in humans, due in part to enzyme instability during cytosol preparation and incubation and variable protein content across preparations [7,17,18]. Critically, none of these sources of error is resolved by the empirical scaling factor approaches currently in use, which improve prediction accuracy for well-characterized substrates but cannot correct for fundamental mechanistic gaps in the extrapolation model [8,17]. Table 1 summarizes different heterocyclic scaffold classes commonly encountered in CNS drug

candidates, showing the primary AO oxidation site, a relative susceptibility rating derived from structure-metabolism relationship studies, the key blocking strategy with established precedents, the anticipated effect of that strategy on BBB penetration, and illustrative literature references.

Table 1. Common heterocyclic scaffolds in CNS drug discovery, their primary AO oxidation sites, relative susceptibility, established blocking strategies, and illustrative references.

| Heterocyclic Scaffold     | Primary AO Site         | AO Susceptibility* | Key Blocking Strategy                          | Effect on BBB                     | Illustrative Reference |
|---------------------------|-------------------------|--------------------|------------------------------------------------|-----------------------------------|------------------------|
| Imidazo[1,2-a]pyrimidine  | C-2/C-8                 | High               | Systematic site blocking, trifluoromethylation | Neutral if small group            | [13,19]                |
| Pyrido[3,4-d]pyrimidinone | C-2                     | High               | C2-substitution (steric + electronic)          | Neutral                           | [20]                   |
| Pyridine (monoaza)        | C-4/C-2                 | Moderate-High      | Pyrimidine ring switch. Fluorination           | Often positive                    | [2,21]                 |
| Quinoline                 | C-2                     | Moderate           | Steric/electronic blocking, deuterium at C-2   | Context-dependent                 | [22,23]                |
| Pyrimidine (diaz)         | C-2 (context-dependent) | Low-Moderate       | Fluorination/ ortho-substitution               | Largely neutral or positive       | [18,24]                |
| Phthalazine               | C-1                     | High               | Scaffold hopping dimer interface modulation    | Requires re-evaluation            | [14,25]                |
| Benzimidazole             | C-2                     | Moderate           | C-2 substitution; fluorination                 | Often positive if logP controlled | [2,26]                 |

\*AO susceptibility rating reflects general trends from structure-metabolism relationship studies; individual compounds within each scaffold class may deviate depending on substitution pattern.

### 3. Structure- Activity Relationship Strategies to Mitigate AO Metabolism

The following critical qualifications apply to all SAR strategies discussed in this section. No prospective, side-by-side comparisons of competing strategies applied to the same scaffold series under the same assay conditions have been published, except for a few case studies. The efficacy data for each approach thus come from different series of compounds and different assay platforms (human liver cytosol vs. recombinant enzyme vs. in vivo rodent clearance), and different measures of the BBB impact. Without a matched control series, causal

attribution of pharmacokinetic improvement to any specific structural modification should be made cautiously. Table 2 presented in section 5.1, provides a comparative summary of the principal AO-blocking strategies alongside the two ortho-substitution variants, rating each on AO reduction magnitude, BBB impact, pKa and efflux effect, evidentiary quality, and main caveat drawn from the reviewed literature.

### 3.1 Blocking Reactive Sites: C2-Substitution and Ortho-Effects

The most direct approach to reducing AO-mediated metabolism is the introduction of steric or electronic blocking groups at the site(s) of preferred oxidation. Systematic studies on imidazo[1,2-a]pyrimidine scaffolds demonstrated that targeted structural modifications at positions susceptible to AO-catalyzed hydroxylation—identified through metabolite profiling, LC- MS/MS analysis, and molecular modelling—effectively reduced AO metabolism while maintaining the pharmacological activity of the parent scaffold [13]. Similarly, investigation of pyrido[3,4-d]pyrimidin-4(3H)-one derivatives established that C2-substitution is particularly effective at blocking AO-mediated oxidation at this preferred site, with both experimental and molecular docking data corroborating the steric blockade mechanism [20,27].

The role of ortho-substituents in modulating AO metabolism has been explored across several compound series. Steric hindrance introduced by substituents at positions flanking the oxidation-prone carbon disrupts substrate positioning within the AO active site, reducing catalytic efficiency. Docking studies of zoniporide analogues provided mechanistic rationalization, correlating the electronic character of substituents with AO active site binding energetics and demonstrating that electron-donating groups at certain positions markedly affect oxidation rates. [15]. In the quinoline-containing c-Met kinase inhibitor series, electron-donating and steric effects at positions adjacent to the AO oxidation site were similarly shown to modulate metabolic half-life in a predictable fashion [22]. Importantly, the effectiveness of site-blocking approaches is scaffold- dependent; modifications that effectively block AO metabolism in one heterocyclic framework may prove ineffective or reduce target potency in another, necessitating empirical evaluation for each new chemotype [2,28].

A limitation that isn't consistently acknowledged in the literature is the scaffold-dependence of blocking effectiveness. A C-2 methyl group provides effective steric protection in one heterocyclic system, while being geometrically mispositioned in another because of differences in ring nitrogen geometry, bond angle, and active site docking geometry. No published study has systematically explored the geometrically constrained factors that contribute to blocking efficacy across more than one scaffold class at a time, forcing medicinal chemists to conduct their own empirical testing for each new scaffold series. This is a huge inefficiency in current practice, which one could potentially overcome by the use of computational structure-based design, making use of the high-resolution hAO1 co-crystal structures [11,12].

### 3.2 Strategic Fluorination

Fluorination has emerged as one of the most versatile and widely employed tactics in medicinal chemistry for modulating both metabolic stability and physicochemical properties [19,28]. With respect to AO metabolism specifically, the introduction of fluorine atoms at or near AO oxidation sites reduces electron density at the susceptible carbon, decreasing substrate reactivity towards the electrophilic AO molybdenum center.

In an effort to attenuate AO-mediated hydroxylation, strategic fluorination has been reported to increase the metabolic stability of several heterocyclic scaffolds, including imidazo[1,2-a]pyrimidines, in which

trifluoromethylation proved to be very effective in enhancing metabolic stability but not compromising biological activity [19].

In addition to its effect on AO-mediated metabolism, fluorination has important secondary effects on properties of particular relevance for BBB penetration. Substitution of fluorine can decrease the basicity (pKa) of adjacent nitrogen containing functional groups, which decreases ionic interactions with efflux transporters and reduces P-gp-mediated efflux, thereby improving CNS exposure [29]. A systematic study of phosphodiesterase 9 (PDE9) inhibitors revealed that strategic fluorination predictably modulated pKa, and further systematic changes in pKa resulted in a predictable change in their bidirectional Caco-2 permeability assays, the efflux ratio [29]. The program described by Hilpert et al. is a good example of the use of the BACE1 inhibitors, which they elegantly showed could be substituted with fluorine to diminish AO liability, decrease efflux ratios, and boost brain exposure in rodent PK studies, and thus make the compounds suitable for further development for clinical trials in Alzheimer's disease [30]. However, the effects of fluorination are not always positive, though. Often, over- or mis-fluorination of a target molecule leads to a rise in its polarity, thereby increasing its susceptibility to efflux or decreasing its potency, which is thus considered as a 'Goldilocks Effect', wherein an optimal balance between steric and electronic perturbation needs to be achieved [31]. This principle was elegantly illustrated in the optimization of phenylethanolamine N-methyltransferase (PNMT) inhibitors, where  $\beta$ -fluorination of a tetrahydroisoquinoline scaffold was found to only result in the desired reduction of AO metabolism, potency, and receptor selectivity when the fluorine was at the exact optimum site [31]. All these observations together highlight the need for fluorination to be used in a mechanism-aware and SAR-driven manner, and not as a one-size-fits-all approach.

This positional criticality is the most important and least appreciated aspect of fluorination strategy. The literature is replete with examples of beneficial fluorination, but the cases where fluorination worsened the pharmacokinetic profile, through polarity increase, P-gp substrate recognition, or potency loss, are underreported due to publication bias. Medicinal chemistry programs that screen multiple fluorine positions and report only the successful variants create an impression of fluorination as a reliably beneficial strategy, when the underlying data frequently show that the majority of fluorine positions tested produced inferior compounds. A systematic, unbiased analysis of fluorine position– outcome relationships across a broad set of CNS scaffolds would be a valuable contribution to the field and does not currently exist. Summary of the effects of fluorination is presented in Figure 2 below.

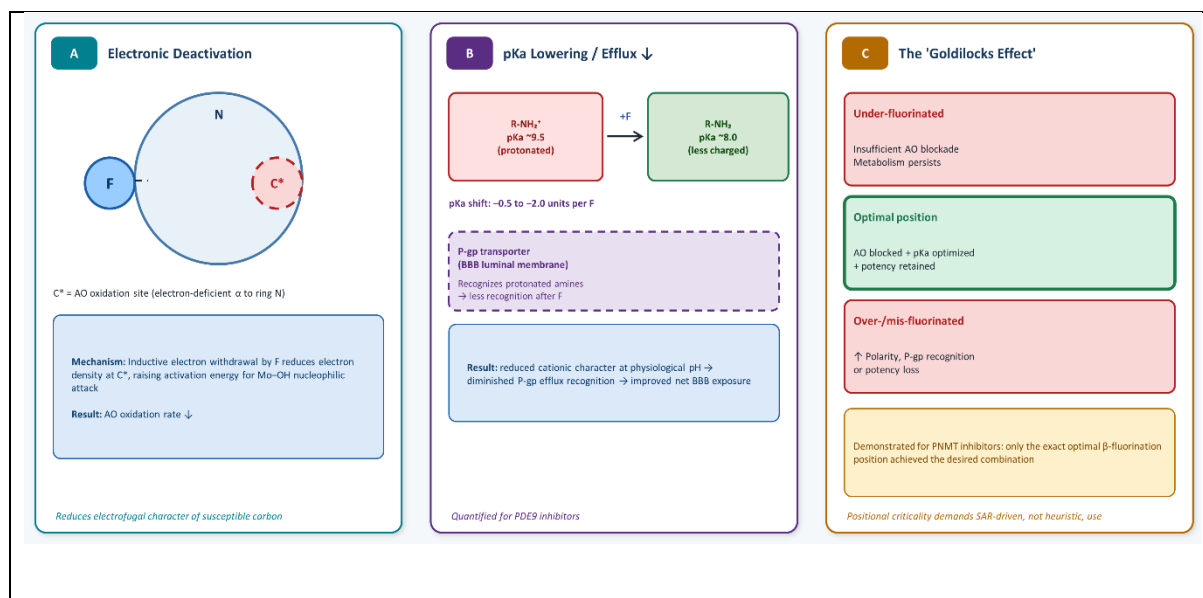


Figure 2. The dual role of strategic fluorination in CNS-targeted AO substrates. (A) Electronic deactivation of the AO oxidation site by fluorine-induced reduction of electron density at the susceptible aromatic carbon. (B) Concurrent lowering of amine pKa by 0.5-2.0 units following fluorine substitution adjacent to the basic nitrogen, reducing cationic charge at physiological pH and diminishing P-gp efflux recognition. (C) The "Goldilocks Effect": Optimal positioning of the fluorine atom for both AO blockade and pKa reduction and target potency — positioning of the fluorine atom is critical, and only the position that achieves the optimal combination of all these three parameters is acceptable; errors in placement may benefit some parameter while harming others. Dotted arrows indicate both changes occurring within the same structural modification event, as documented for BACE1 and PDE9 inhibitor series [30,31]. The author prepared this figure using Microsoft PowerPoint (Microsoft Corporation, Redmond, WA, USA) as an original schematic for this review

### 3.3 Heterocycle Switching: Pyridine to Pyrimidine Scaffold Hopping

Replacement of metabolically labile pyridine rings with pyrimidine or other electron- deficient heterocycles represents a particularly powerful SAR strategy for mitigating AO liability, because it simultaneously alters the electronic environment of the ring and, in many cases, removes or repositions the oxidation-prone carbon relative to the ring nitrogen [2,28].

Pyrimidines are generally less susceptible to AO-mediated oxidation than pyridines, owing to the presence of two ring nitrogen atoms that inductively reduce electron density at susceptible positions. Scaffold hopping from pyridine to pyrimidine has been successfully employed in multiple CNS drug programs, including the development of S3-truncated aminothiazine BACE1 inhibitors by Wu et al., where pyrimidine ring introduction both addressed AO soft spots identified by metabolite profiling and improved oral bioavailability, ultimately enabling demonstration of brain amyloid- $\beta$  reduction in rodent models [21].

Similarly, in the discovery of GluN2B-selective negative allosteric modulators for CNS indications, iterative substitution of a pyrimidine core, guided by metabolite identification and AO liability assessment, yielded a series of potent, brain-penetrant compounds with improved metabolic stability [24]. Metabolic improvement via scaffold switching was directly validated through metabolism studies in the respective species, which showed that AO is the main enzyme oxidizing pyrimidine at the soft spot position, and through in vivo pharmacokinetic studies showing the increased CNS exposure in rodents after scaffold modification [24,32]. Other structures,

such as other aromatic rings replaced by non-AO-substrate bioisosteres, have been investigated as a way to remove AO liability completely, not just encourage its reduction [25]. Lazzara and Moore showed that scaffold hopping away from the electron-rich aromatic systems to more electron-deficient alternatives can be done to achieve both AO and CYP metabolic liability, going beyond the scope of specific pyridine-to-pyrimidine substitution [25]. However, such scaffold modifications should be carefully assessed to make sure the pharmacophoric groups necessary for the interaction towards the target are retained.

A limitation that is not consistently acknowledged in the scaffold-hopping literature is that pyrimidine rings are not universally AO-resistant. Crouch et al. [18] demonstrated that the mGlu5 negative allosteric modulator VU0424238 (Auglurant), which contains a pyrimidine ring, was nonetheless metabolized by AO in a species-specific manner — with the pyrimidine itself serving as the AO substrate [18]. This finding demonstrates that the assumption of pyrimidine AO resistance is a statistical generalization, not a mechanistic guarantee, and that pyrimidine-containing compounds require explicit AO metabolite profiling before AO resistance can be claimed. The failure to make this distinction in the SAR literature perturbs a misleading heuristic that pyrimidine switching equals AO resistance.

### 3.4 Deuterium Substitution as a Kinetic Isotope Approach

The kinetic isotope effect (KIE) of deuterium substitution, exploiting the higher bond dissociation energy of C–D relative to C–H (by approximately 1.2 kcal/mol) to reduce the rate of AO-catalyzed bond cleavage, represents the most mechanistically targeted approach to AO metabolism mitigation [23,33]. The primary KIE for C–H bond cleavage by AO has been measured at 2–7-fold across a range of substrates, depending on the extent to which that bond cleavage contributes to the overall rate-determining step. The c-Met inhibitor MET401 case study showed that deuterium at the quinoline AO oxidation site produced significant improvements in rodent pharmacokinetic parameters, with reduced clearance and prolonged half-life, demonstrating a direct pharmacokinetic consequence of the isotope effect [23].

Two constraints limit the general applicability of this approach that are insufficiently emphasized in the positive case literature. First, the KIE is maximal only when C–H bond cleavage is strictly rate-limiting in the AO catalytic cycle. When other steps — substrate binding, product release, or electron transfer — are partially rate-limiting, the observed KIE will be attenuated (a phenomenon termed the 'commitment to catalysis'), potentially reducing the metabolic improvement below the level required for pharmacokinetic rescue. The magnitude of the commitment to catalysis for AO is substrate-specific and has not been systematically characterized across the scaffold types commonly encountered in CNS discovery. Second, deuterium substitution only affects the metabolic rate at the labeled carbon and does not change any of the underlying physicochemical liabilities that may individually limit BBB penetration, such as P-gp efflux, polar surface area, passive permeability, and thus cannot be used alone as a CNS optimization strategy. In the specific scenario that AO-mediated clearance is the main pharmacokinetic hurdle to CNS penetration with all other parameters for CNS penetration optimized, then its utility is limited.

## 4. Mechanistic Insights into AO Substrate Recognition and Metabolism

### 4.1 Computational Prediction of AO Oxidase Sites

The rational implementation of SAR strategies to block AO metabolism requires accurate prediction of which molecular positions are susceptible to AO-catalyzed oxidation. Computational approaches to this challenge have advanced considerably over the past decade [12,34]. Early structure-based models utilized the available AO crystal structures to identify the geometric and electronic requirements for productive substrate binding within the molybdenum-containing active site [13]. More recent computational workflows have employed quantum chemical transition state analyses to predict the intrinsic reactivity of aromatic carbon atoms towards the AO molybdenum center, providing atom-level predictions of oxidation site regioselectivity for a broad range of azaheterocyclic substrates [12]. Levine et al. conducted a large-scale computational survey of AO substrate reactivity, demonstrating that quantum mechanically derived activation energies for model AO oxidation transitions correlate well with experimentally observed metabolite formation rates [12]. Complementing this atom-level approach, Zhao et al. developed an integrated computational model combining atom-level and molecule-level features—including molecular descriptors encoding electronic properties, steric accessibility, and structural context—to predict both AO substrate classification and site-of-metabolism, validating the model against an extensive curated dataset of in vitro AO metabolism experiments [34]. These predictive tools enable prospective identification of AO liabilities at the hit or lead identification stage, supporting early decision-making regarding structural modification needs.

Lepri et al. [26] assembled a comprehensive SAR dataset by performing structure-metabolism relationship studies on a large structurally diverse set of aza- aromatic and amide compounds, using density functional theory (DFT) calculations together with experimental metabolite identification [26]. The dataset showed that systematic electronic and steric effects govern AO oxidation susceptibility across chemical space, which may be useful for predicting oxidation susceptibility in novel scaffolds found in drug discovery programs [26].

A critical limitation of all current computational AO prediction tools is that they have been validated primarily against in vitro intrinsic clearance data from human liver cytosol or recombinant AO, not against in vivo human clearance data. Given the documented failure of in vitro data to predict in vivo AO clearance accurately (Section 4.3), computational tools trained on and validated against in vitro endpoints inherit the same systematic prediction error. A computational model that accurately predicts in vitro AO clearance may therefore still fail to predict human clinical pharmacokinetics. This limitation is not acknowledged in any of the computational prediction papers reviewed here, and represents a fundamental gap between the performance metrics reported for these tools and the translational objective for which they are intended. Future tool development should prioritize validation against in vivo human pharmacokinetic data where available.

### 4.2 Enzyme-Substrate Interactions from structural and kinetic studies

In addition to computational methods, information on AO–substrate interactions have been obtained through direct experimental studies, which have been very helpful in providing mechanistic information. Dalvie et al. [15] used a molecular docking protocol with kinetic evaluation of a series of zoniporide analogues to establish correlations between the properties of the substrates and the binding affinity of the substrates to the AO and their catalytic efficiency [15]. Such seemingly conservative changes in structure, such as the introduction of methyl or methoxy substituents, were shown to significantly alter the orientation of a substrate in the active site,

and to accompany changes in regioselectivity and metabolic rates [15]. Ferreira et al. [11] reported insights into the conformational dynamics of the AO monomer and homodimer, and how the structural architecture of the enzyme affects the binding and catalytic activity of the substrate [11]. In particular, the homodimeric interface was found to be a region that may regulate substrate access and productive binding, which may be a physiological control mechanism of AO activity [11]. Characterization of AO inhibition mechanisms has also shed mechanistic insight into drug design: Barnes et al. showed that hydralazine inactivates AO by generation of reactive intermediates, a process that is time-dependent and that glutathione can partially protect against, which suggests the possibility of developing selective inhibitors of AO and also allows for the assessment of drug–drug interaction potential [35].

Despite this body of mechanistic data, a fundamental structural gap persists: no high-resolution co-crystal structure of hAO1 in complex with a CNS-relevant drug candidate has been published. The available structural biology data are largely derived from bovine or murine AO homologues or from computational models of the human enzyme, both of which differ in active site geometry from hAO1 in ways that may be pharmacologically significant. Structure-guided design of AO-resistant CNS scaffolds — analogous to the cryo-EM-guided design now possible for amyloid targets — is therefore not yet feasible in a rigorous sense. This represents the most consequential unresolved methodological need in the field.

### 4.3 Persistent Challenges of In-Vitro and In-Vivo Extrapolation: A Quantitative Assessment

Although great progress has been made towards computational prediction and mechanistic understanding of AO metabolism, relevant *in vivo* clearance data in humans remains one of the most formidable challenges in the field [7,8,17]. This challenge is due to several factors. First, the activity of AO enzymes in cytosol preparations from liver is unstable, and significant losses of activity occur during preparation and storage of such samples, which adds to interspecies and inter-laboratory variations [7]. Second, the protein content of AO in human liver cytosol is very donor-dependent, and thus the forward scaling of *in vitro* intrinsic clearance to *in vivo* hepatic clearance is inherently uncertain [17]. Third, AO metabolism in extrahepatic tissues, such as the intestine, may account for *in vivo* clearance in a manner not accounted for by the typical hepatic cytosol assay [8].

Some empirical scaling factor methods have been suggested to resolve these shortcomings to some extent. Izat et al. [8] determined system-specific scaling factors for predicting the clearance of an AO, and showed that the use of calibrated empirical correction factors improves the accuracy of *in vitro*–*in vivo* extrapolation (IVIVE) of the clearance of AO substrates, which was underestimated with the standard scaling factors [8]. Subash et al. [17] also examined the role of activity loss and protein content variability in the underprediction of AO clearance, suggesting a protein-normalized activity factor approach that combines proteomics data with physiologically based pharmacokinetic (PBPK) modelling for more accurate human clearance predictions [17]. These methodological advances are useful, but it should be recognized that empirical scaling factors will be dependent on restricted datasets and may not be generally applicable to the entire chemical space of AO substrates that typically occur in drug discovery [8,17].

The field is therefore in a situation where no validated prospective IVIVE method for AO clearance exists. The magnitude of prediction error is large enough to cause program-level decisions based on incorrect clearance

estimates, and the methodological investment required to close this gap, such as standardized enzyme-stable cytosol preparations, proteomics-normalized scaling, and extrahepatic contribution characterization, has not been made in a systematic, cross-organizational fashion. This is the single highest-priority methodological gap in the field.

## 5. Integrating AO Metabolism Mitigation with BBB Optimization

### 5.1 Physicochemical Determinants of BBB Penetration

In CNS drug design, both BBB penetration and metabolic stability must be simultaneously optimized, a target that is often in conflict and requires a multiparameter optimization [36,37]. Passive BBB permeability depends on the following properties: molecular weight (<450 Da), logP 1–3, polar surface area (<90 Å<sup>2</sup>), and hydrogen bond donor count (<3), which are collectively represented by CNS-specific analogues of the Lipinski rules. In addition to passive permeability, efflux by P-gp and BCRP at the BBB is also a significant obstacle to CNS penetration, and compounds with good passive permeability can have very low brain penetration due to active efflux [38,39].

In practical terms, AO-blocking modifications can be classified by their expected impact on CNS MPO score. Fluorination, C-2 methylation, and pyrimidine switching are 'CNS- neutral or positive' modifications; they typically reduce AO liability without substantially worsening CNS MPO parameters and may improve them through pKa effects (fluorination) or reduced MW (pyrimidine replacing substituted pyridine) [29,30]. By contrast, the introduction of the polar blocking groups (hydroxymethyl, amino, or carboxamide) at oxidation sites improves AO stability but increases topological surface area (TPSA) and HBD count, typically reducing CNS MPO score and increasing P-gp efflux recognition [2,28]. Deuterium substitution has no impact on CNS MPO score by construction, since it does not alter any physicochemical parameter.

Table 2. Comparative effectiveness of AO metabolism mitigation strategies and their effect on CNS drug-like properties. The assessments reflect the authors' synthesis of published data; no head-to-head comparison under identical conditions exists.

| SAR Strategy            | AO Reduction                | BBB Impact               | pKa/Efflux Effect                                             | Evidentiary Quality                                                                                                                                                       | Main Caveat                                |
|-------------------------|-----------------------------|--------------------------|---------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------|
| C-2/ ortho-substitution | Strong; site-specific block | Neutral (if small group) | No direct pKa effect, efflux impact depends on group polarity | Moderate-consistent in vitro precedent across imidazo[1,2-a] pyrimidine and pyrido[3,4-d] pyrimidinone scaffolds; mechanistic validation by docking; limited in vivo data | Scaffold-dependent; may reduce the potency |

|                               |                                   |                                         |                                                                                                       |                                                                                                                                                        |                                                                          |
|-------------------------------|-----------------------------------|-----------------------------------------|-------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------|
| Strategic Fluorination        | Moderate-Strong                   | Often improved (lower pKa, less efflux) | Lowers adjacent amine pKa by 0.5-2 units, reduces P-gp efflux ration in a position-dependent manner   | High mechanistic rationale established; quantitative pKa efflux correlation documented; in vivo brain exposure improvement confirmed in BACE1 series   | Position-critical; risk of over-fluorination                             |
| Pyridine to Pyrimidine Switch | Strong; electronic deactivation   | Largely neutral or positive             | Minimal direct pKa effect unless adjacent to basic amine; can reduce MW, positively affecting CNS MPO | Moderate — multiple CNS programme precedents; mechanistic basis established; pyrimidine AO resistance is not universal                                 | Pharmacophore may shift, pyrimidine itself may be AO substrate           |
| Deuterium Substitution        | Moderate (2-7×KIE)                | Neutral                                 | No pKa efflux effect                                                                                  | Low-Moderate—single well-characterized case study (MET401); KIE magnitude substrate-specific; commitment to catalysis not systematically characterized | Applies only when C-H cleavage is rate-limiting, metabolic shifting risk |
| Scaffold hopping (broad)      | Variable; can eliminate liability | Depends on replacement ring             | Variable; depends on replacement scaffold physiochemistry                                             | Low-Moderate — case-by-case precedent; no systematic cross-scaffold comparison                                                                         | Risk of losing target engagement                                         |
| Ortho-Substitution            | Moderate; steric blockade         | Mixed (depends on group polarity)       | Polar ortho-groups may increase TPSA and reduce                                                       | Low-Moderate — mechanistic rationale from docking studies;                                                                                             | Electronic context matters                                               |

|  |  |  |                                                   |                            |  |
|--|--|--|---------------------------------------------------|----------------------------|--|
|  |  |  | passive permeability;<br>non-polar groups neutral | limited in vivo validation |  |
|--|--|--|---------------------------------------------------|----------------------------|--|

KIE: kinetic isotope effect, TPSA: topological polar surface area, CNS MPO: CNS multiparameter optimization.

## 5.2 Pyrimidines, Morpholines, and Other CNS-Favorable Scaffolds

Several specific scaffold types have emerged as particularly compatible with the dual requirements of AO metabolism resistance and BBB penetration. Pyrimidine-containing scaffolds offer reduced AO susceptibility relative to pyridines and can be designed with the physicochemical properties required for CNS exposure. In the context of multi-target CNS drug design, pyrimidine and pyrrolidine core combinations have been reported to exhibit BBB penetration validated by in vivo efficacy in neuroinflammation models, alongside balanced inhibition of multiple CNS targets [40].

Morpholine-containing heterocycles represent another compound class of particular relevance to CNS drug discovery. Lenci et al. [41] conducted a comprehensive analysis of morpholine-containing CNS drugs, demonstrating that the morpholine ring contributes to favorable BBB permeability through modulation of lipophilicity, pKa, and hydrogen bond acceptor capacity, while also conferring conformational constraints that can improve target selectivity. The physicochemical properties conferred by morpholine make it a privileged scaffold for CNS applications, and its incorporation has been strategically employed in multiple successful CNS drug programs [9,41]. More broadly, Do et al. [42] demonstrated that systematic optimization of lipophilicity and pKa through structural modification of a 2-aminopyridine scaffold yielded potent neuronal nitric oxide synthase (nNOS) inhibitors with low efflux ratios and high brain exposure in rodent pharmacokinetic studies. The study illustrated that multiparametric optimization—simultaneously tracking potency, AO metabolism, and physicochemical/permeability parameters—is both feasible and necessary for the development of efficacious brain-penetrant CNS drugs [42]. Table 3 translates the CNS MPO framework into a practical reference by listing five key physicochemical parameters alongside their established CNS target ranges, the predicted direction of change imposed by common AO-blocking modifications, and the key design consideration for each parameter.

Table 3. CNS drug-like physicochemical targets and predicted impact of common AO-blocking structural modifications

| Parameter                             | CNS Drug Target Range*       | Effect of AO-Blocking Mods              | Key Considerations                       |
|---------------------------------------|------------------------------|-----------------------------------------|------------------------------------------|
| Molecular weight (Da)                 | < 450                        | Minimal change expected                 | Deuterium and F add negligible MW        |
| LogP                                  | 1-3                          | Fluorination can raise logP slightly    | Over-fluorination risks P-gp recognition |
| Polar Surface Area ( $\text{\AA}^2$ ) | < 90                         | Polar blockers increase PSA unfavorably | Prefer small non-polar blocking groups   |
| pKa (basic amine)                     | 7.5-9 (amine); lower for CNS | Fluorination lowers pKa 0.5-2 units     | Lower pKa reduces P-gp efflux            |

|                            |               |                                  |                                             |
|----------------------------|---------------|----------------------------------|---------------------------------------------|
| p-gp efflux ratio (Caco-2) | < 2 preferred | Strategic F reduces efflux ratio | Efflux reduction often parallels AO benefit |
|----------------------------|---------------|----------------------------------|---------------------------------------------|

\*Target ranges reflect CNS MPO (Multiparameter optimization) criteria adapted from [6], pKa ranges reflect practical CNS-penetrant compound experience across multiple therapeutics programs

### 5.3 Efflux Transporter Considerations and pKa Improvement

The relationship between molecular basicity (pKa) and P-gp efflux susceptibility is a recurring theme in CNS drug design. Basic amines, which are common pharmacophoric elements in CNS drugs, are recognized substrates for P-gp when in their protonated form; reduction of amine basicity through structural modification can therefore reduce P-gp efflux and improve CNS penetration [9,29]. Fluorination—as discussed in the context of AO metabolism—additionally reduces pKa of adjacent basic amines, providing a dual benefit of AO metabolism reduction and efflux minimization [29,30]. These effects have been quantitatively documented in a systematic study by Fjelbye et al. [29], which showed a strong correlation between positioning of the fluorine atoms, the pKa shifts, and the in vitro P-gp efflux ratios of the compounds [29].

In addition to fluorination, a modulating physicochemical properties study of tetrahydropyridine-2-amine BACE1 inhibitors was conducted, where, with systematic modification of electron-withdrawing groups, both pKa and efflux susceptibility were predicted and optimized [41]. Such observations highlight the need for simultaneous optimization of all physicochemical properties (metabolic stability, solubility, permeability, and efflux) in the design of brain-penetrant drugs, rather than sequential optimization of each property. Figure 3 represents this multiparametric optimization challenge as a four-axis space, identifying the central green zone that an ideal candidate should occupy and mapping the three most common failure modes as peripheral positions that specific SAR strategies are known to correct.

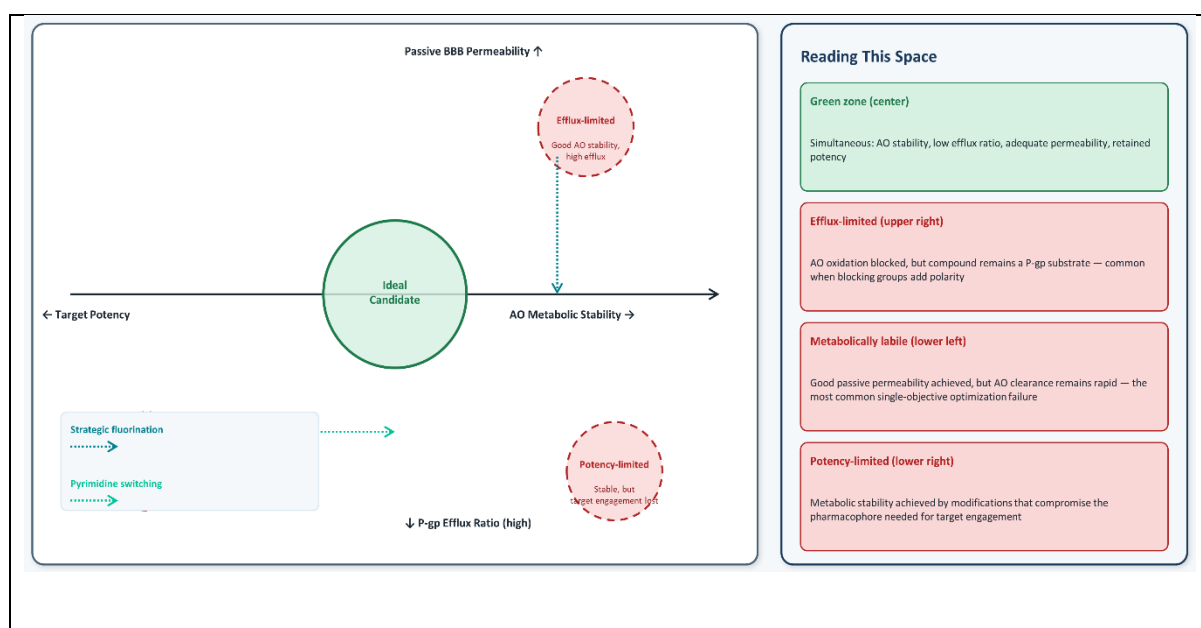


Figure 3. Schematic of the multiparametric optimization space for AO-resistant, brain-penetrant CNS drug candidates. Each axis represents a key parameter (AO metabolic stability, P-gp efflux ratio, passive BBB permeability, target potency). An idealized candidate occupies the center of the optimization window (green zone). Common failure modes are shown: compounds with good AO resistance but high efflux (efflux-limited; upper right), adequate permeability but rapid AO clearance (metabolically labile; lower left), and compounds

that sacrifice potency to achieve metabolic stability (potency-limited; lower right). Strategic fluorination and pyrimidine ring switching tend to move compounds toward the center from multiple directions simultaneously, explaining their prevalence in successful CNS programs. The author prepared this figure using Microsoft PowerPoint (Microsoft Corporation, Redmond, WA, USA) as an original schematic for this review

## 6. Case Studies of Successful Drug Candidates

The case studies presented below represent the best-available evidence for integrated AO mitigation strategies. They are interpreted critically; where evidence supports causal attribution of pharmacokinetic improvement to a specific structural modification, this is stated; where confounding factors limit causal interpretation, these are made explicit

### 6.1 BACE1 Inhibitors for Alzheimer's Disease

Perhaps the most well-documented case studies of successful AO metabolism mitigation in the area of CNS drug discovery are those from the  $\beta$ -secretase (BACE1) inhibitor program. The major AO-mediated oxidation point in BACE1 inhibitors is found in their amino-containing heterocyclic scaffolds, which are inherent hot spots for AO oxidation. Hilpert et al. [30] reported the discovery of a series of highly potent BACE1 inhibitors that were developed by using fluorine-scanning of a CNS-targeted lead series, which resulted in a combination of several properties: blocking AO oxidation, decreasing the efflux by P-gp, lowering the pKa to within an optimal CNS range, and increasing in vivo brain exposure in rodent pharmacokinetic studies. The optimized inhibitors showed high in vivo efficacy and were considered to be suitable for clinical development in Alzheimer's disease, being an example of the potential of fluorination to be a multifunctional SAR tool in CNS drug design [30].

The complementary strategy of scaffold hopping has been used in the BACE1 inhibitor space, for example, by replacing a metabolically labile pyridine ring with a pyrimidine at the primary AO site identified by metabolite profiling (which led to an improvement in oral bioavailability), but also resulted in good brain amyloid- $\beta$  reduction in rodent models. The study is a good example of the ability of pyridine-to-pyrimidine switching, with careful metabolite identification to remove potential AO liability without loss of pharmacological efficacy [21]. The physicochemical aspects of BACE1 inhibitor optimization were further developed in the tetrahydropyridine-2-amine study by systematically varying pKa and efflux with electron-withdrawing substituents, while retaining activity [43].

An important caveat contextualizing both BACE1 case studies is that the BACE1 inhibitors class as a whole failed in Phase III clinical trials for Alzheimer's disease, not only for pharmacokinetic reasons, but also for pharmacodynamic reasons related to on-target toxicity and the inadequacy of amyloid reduction as a therapeutic endpoint [44]. The pharmacokinetic success of these programs is therefore real and instructive, but it did not translate to clinical efficacy, which is a reminder that resolving AO liability is a necessary but not sufficient condition for therapeutic success.

### 6.2 GluN2B Modulators and Neuropsychiatric Drug Candidates

An interesting discovery program in a CNS context where AO requirements are managed, the modulators of GluN2B are an intriguing therapeutic field because the NMDA receptor subunit composition is involved with depression, neuropathic pain, and other neurological diseases [24]. Gelin et al. [28] reported a triazole-

methoxypyrimidine GluN2B modulator series in which iterative SAR-guided AO metabolic profiling and successive structural changes yielded potent, brain-penetrant compounds with improved metabolic stability and in vivo CNS exposure [28]. For the specific neuroinflammation and cholinergic deficit disease area relevant to Alzheimer's, the pyrimidine and pyrrolidine core study showed multi-target inhibition, good BBB penetration, and effective in vivo activity, while maintaining a balance of the properties of the core that must be stable to AO while also meeting the physicochemical properties required for CNS delivery [40]. Such multi-target directed ligand (MTDL) strategies are becoming even more relevant in the field of neurodegeneration, where polypharmacology could provide therapeutic benefits for therapeutic molecules more selective than MTDLs. Within the series, AO-resistant scaffold replacement was achieved by attaching the pharmacophoric element of GluN2B selectivity to the pyrimidine core, a nice convergence of target and metabolic design considerations that bears out the benefit of taking a close look at the metabolic stability of a scaffold early in a CNS program.

### 6.3 c-Met Kinase Inhibitors and Deuterium Strategy

The case study for the c-Met kinase inhibitor MET401 is a good example of an approach to AO metabolism mitigation using deuterium substitution. In vitro studies, which have been carried out, determined that AO is the main enzyme involved in oxidative metabolism of MET401 in rat liver cytosol and that the quinoline ring is the major site of AO-mediated hydroxylation. The incorporation of deuterium in the site of AO oxidation resulted in a deuterated analogue, which demonstrated significantly enhanced pharmacokinetic parameters in rats, such as decreased clearance, prolonged half-life and increased systemic exposure, with no obvious effect on target activity or selectivity. This example is important as it shows that the kinetic isotope effect can lead to substantial improvement in the in vivo PK parameters, and not just the rate reduction, as reported by (Robbins et al.,...). The synthetic methodology contributions of Choi et al.,... (deuterium labeling via dimethylformamide dimethyl acetal-d<sub>3</sub>) and Wright (deuterated enamines with high isotopic fidelity) are important enabling technologies that make the deuterium strategy practically accessible for a broader range of CNS scaffold types. Without reliable synthetic routes to high isotopic purity, the utility of the strategy is limited by isotopic dilution that reduces the effective KIE in vivo. [23,33,45].

A limitation that the MET401 study does not address is the potential for metabolic shifting, which is where blocking an AO oxidation site by deuterium directs metabolism to an alternative site, potentially generating a metabolite with worse toxicological, pharmacokinetic, or pharmacodynamic properties than the AO-mediated product. Metabolic shifting has been documented for deuterium-substituted drugs targeting CYP enzymes (most notably deutetrabenazine/tetrabenazine) and must be considered a contingent risk for the AO deuterium strategy that requires metabolite profiling of the deuterated analogue before its pharmacokinetic improvement can be considered a net benefit [23,46].

### 6.4 Species Surrogacy and Translational Validation

One of the key issues that is sometimes overlooked in the process of drug development for an AO is the identification of a suitable preclinical species for AO metabolism. Hutzler et al. [10] have systemically studied the AO metabolism of BIBX1382, an EGFR inhibitor that is extensively metabolized by AO in humans, but not in rats. The study showed that the hepatic cytosol of cynomolgus monkeys can exactly replicate the human AO metabolite profile and AO clearance rate, making the cynomolgus monkey a valid surrogate species for the assessment of human AO metabolism. This finding has practical implications for the drug development pipeline

and shows that the use of cynomolgus monkeys as a metabolic profiling species, for compounds that are AO-active in human cytosol, can significantly enhance the translatability of preclinical pharmacokinetic information [10].

Due to the practical constraints associated with the use of routine cynomolgus monkey for early drug discovery (cost, ethics, throughput), this is usually used for compounds that passed initial rodent screening and further advanced through candidate selection. The availability of surrogate systems to better mimic the predictive value of the cynomolgus monkey assay, with the ability to incorporate recombinant human AO preparations with normalized enzyme content or fresh hepatocyte systems that retain AO activity, with increased throughput and reduced cost, would be a welcome methodological advance. Currently, there are no validated systems.

## 7. Comparative Analysis of SAR Strategies

### 7.1 Convergent Findings and Robust Strategies

Across the reviewed literature, several consistent themes arose. There is a wide range of compounds that have been site-specifically blocked, either by addition of C2-substituents at the site of the oxidation or by ortho-substituents on scaffolds that do not contain the AO positions, or by scaffold hopping to less AO-prone heterocycles [13,20,28]. The pervasiveness of fluorination as a modification that can simultaneously tackle the dual issues of AO metabolism and BBB permeability is always highlighted, although its most effective application relies on the strategic consideration of positioning [29–31]. Switching of pyridines to pyrimidines is an especially pure and widely applicable strategy, often affording metabolic stability enhancements with little to no impact on drug activity [21,24,25]. Computational and experimental techniques, including metabolite identification, molecular docking studies, quantum chemical reactivity prediction, and in vitro pharmacokinetic characterization, are all deemed essential for successful AO-driven SAR efforts [12,13,26,34]. The SAR conclusions, generally obtained through studies using more than one methodological approach, and utilizing complementary methodologies, were more predictable and more generalizable than studies using one analytical method [12,20,34].

### 7.2 Divergent Findings and Context-Dependence

Although there are some similarities, literature showed significant differences. The ability of a specific structural change to be efficient in blocking AO metabolism is clearly scaffold-dependent, and a change that is efficient in one chemical series can be ineffective or even harmful in another, depending on the difference in active site binding geometry or electronic context [2,28,31]. Likewise, the effects of AO-blocking modification on BBB permeability cannot be universally predicted: modifications that increase metabolic stability by increasing polarity can also decrease passive BBB penetration and/or increase efflux, as seen in some studies where extensive fluorination has caused BBB permeability to drop, even when metabolic stability has been enhanced [29]. It can also be seen that there are inconsistencies in the reliability of in vitro–in vivo extrapolation. Empirical scaling factors provide better prediction of the clearance in well-characterized substrates and enzyme preparations; however, they cannot overcome the basic uncertainty arising from enzymes that are not stable in vivo, interspecies differences in enzyme structure and/or the presence of extrahepatic AO metabolism [7,8,17,18]. Even the most stringent in vitro evaluations have been reported to not be predictive of the degree of AO-mediated clearance found in vivo, suggesting the need for better predictive models [8,17].

The most significant divergence found in the literature, however, is the systematic disagreement provided in the parameters measured from one study to another. Some report only in vitro AO clearance, others report only in vivo rodent pharmacokinetics, and few report BBB permeability, efflux ratio, and target potency data at the same time along with metabolic stability. This form of measurement heterogeneity renders the ability to make cross-study conclusions concerning the effectiveness of the strategies methodologically questionable, and is a systematic weakness in the way the field produces an AO SAR.

### 7.3 Toward Multiparametric Integration

The most important lesson drawn from the comparative analysis of literature from the SAR is that AO metabolism mitigation cannot be effectively addressed without consideration of other pharmacokinetic and pharmacodynamic goals. Optimization of AO metabolic stability while simultaneously neglecting BBB permeability, efflux susceptibility, target potency, or off-target liability risks shifting the bottleneck from one parameter to another without improving overall compound quality [2,9,28,42]. The most successful drug candidates described in the reviewed literature are those for which AO-blocking strategies were implemented within a multiparametric optimization framework, with simultaneous tracking of metabolic stability, physicochemical properties, efflux, and in vivo pharmacokinetics [21,24,30].

Computational tools—including PBPK models that integrate AO clearance predictions with BBB permeability estimates, efflux parameters, and target exposure models—are increasingly enabling prospective multiparametric optimization, though their reliability depends critically on the quality of the underlying experimental data and the accuracy of the component prediction models [12,17,34].

Figure 4 presents a decision-tree framework proposed here as the first synthesis-level tool for strategy selection in AO-CNS drug design, integrating the mechanistic and SAR evidence reviewed in this paper. The framework encodes four key inputs — scaffold class, primary oxidation site, current CNS MPO score, and available assay infrastructure — and maps these to recommended strategy prioritization with explicit rationale and validation requirements.

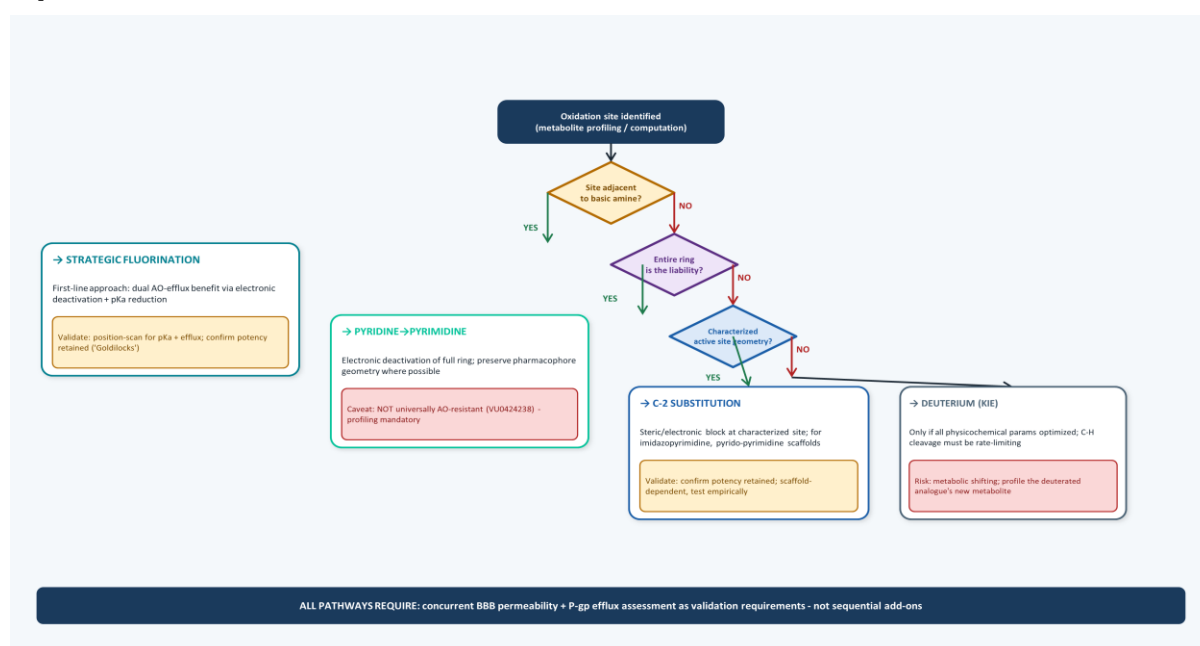


Figure 4. Proposed decision-tree framework for AO mitigation strategy selection in CNS drug discovery programs. Starting from scaffold class and oxidation site identification (from metabolite profiling and/or

computational prediction), the framework guides strategy selection based on: (i) current CNS drug-likeness status (MPO score and key parameters); (ii) nature of the oxidation-prone position (electronic vs. steric blockade feasibility); and (iii) available assay infrastructure. Strategic fluorination is recommended as the first-line approach when the oxidation site is adjacent to a basic amine, due to its dual AO–efflux benefit. Pyrimidine switching is recommended when the entire ring system is the metabolic liability and pharmacophore preservation allows. C-2 substitution is recommended for imidazopyrimidine and pyrido- pyrimidine scaffolds with characterized active site geometry. Deuterium is reserved for scaffolds where all physicochemical parameters are optimized, and only the AO rate at a single C–H position requires reduction. All pathways require concurrent BBB permeability and efflux assessment as validation requirements, not sequential add-ons.

This framework is proposed as a synthesis-level organizational tool by the authors based on the reviewed literature; it does not represent validated prospective guidance and should be tested against novel scaffold series. The author prepared this figure using Microsoft PowerPoint (Microsoft Corporation, Redmond, WA, USA) as an original schematic for this review.

## 8. Limitations of the Current Literature and Future Direction

### 8.1 Limitations

The literature on AO metabolism mitigation in brain-penetrant heterocycles is characterized by five systematic limitations that collectively constrain the development of an evidence-based, generalizable framework for CNS program design.

First and most fundamentally, no prospective head-to-head comparison of the four principal SAR strategies (fluorination, C-2 blocking, scaffold hopping, deuterium substitution) was applied to the same scaffold series under standardized, concurrent assay conditions has been published. This absence means that strategy selection remains precedent-based rather than evidence-based, and that the superiority claims made for individual approaches in case studies cannot be extrapolated to general applicability. Second, the IVIVE problem for AO clearance remained unresolved. Despite methodological advances in empirical scaling and proteomics-normalized approaches, prediction errors of 3–10- fold persisted for structurally diverse AO substrates — errors large enough to cause incorrect go/no- go decisions in drug development programs.

Third, high-resolution co-crystal structures of hAO1 with CNS-relevant drug candidates did not exist in the public literature, limiting the rigor of structure-based drug design for AO-resistant scaffolds.

Fourth, the majority of AO SAR studies conducted on a restricted set of pyrido- pyrimidine and imidazopyrimidine scaffolds, and the SAR conclusions could not be generalized to the broader chemical space of CNS drug candidates.

Fifth, the impact of AO-blocking modifications on BBB permeability is inconsistently assessed alongside metabolic stability, creating an incomplete evidence base for multiparametric optimization decisions.

### 8.2 Future Research Directions

Based on the analysis of the current literature, the papers and experiments identified several priority areas for future research. High priority should be placed on the development and validation of improved in vitro AO assay systems with improved enzyme stability and standardized protein content normalization to reduce the uncertainty in IVIVE and enable more reliable prediction of human AO clearance [8,17,18]. In parallel,

systematic comparative studies of AO metabolism across species using standardized substrates could be conducted to better characterize species differences and validate the cynomolgus monkey surrogate model more broadly [10,32]. High-resolution structural biology studies of human AO1 in complex with diverse substrates and inhibitors would also provide the mechanistic framework needed for structure-based drug design of AO-resistant scaffolds [11,12]. Additionally, integrated multiparametric SAR studies that simultaneously characterize AO metabolism, BBB permeability, efflux susceptibility, and target engagement for the same compound series would provide holistic datasets to support multiparametric optimization [9,30,42].

Further research should also focus on the refinement and validation of computational prediction models for AO substrate reactivity and site of metabolism, incorporating machine learning approaches trained on increasingly comprehensive experimental datasets [12,34]. Systematic evaluation of the long-term safety and metabolic fate of fluorinated and deuterated drug candidates in humans should be applied to assess the potential for unforeseen liabilities introduced by these modifications [28,33,45]. Moreover, characterization of the functional impact of AOX1 polymorphisms and isoform variants on substrate metabolism and interindividual pharmacokinetic variability to inform personalized medicine approaches and clinical pharmacokinetic predictions [14]. Standardization of selective AO inhibitors and establishment of validated phenotyping protocols would also have a significant impact on the field, as these would facilitate more robust AO contribution to a drug's metabolism in complex biological matrices.

In the future, organ-on-a-chip and 3D hepatocyte culture with native AO activity could provide a more physiologically relevant in vitro system for assessing AO metabolism than existing cytosol-based systems, which would help to minimize the gap between prediction and experiment. The application of machine learning models trained on large, curated AO metabolism datasets incorporating structural, electronic, and three-dimensional molecular descriptors represents a promising avenue for improving the speed and accuracy of AO liability prediction at the earliest stages of drug discovery, enabling prospective compound design rather than reactive structural modification.

## 9. Conclusion

Aldehyde oxidase has evolved from a metabolic curiosity to a recognized and consequential enzyme in drug discovery with particular significance for the design of brain- penetrant therapeutics. The body of literature reviewed herein demonstrated that diverse and complementary structural modification strategies—site-specific blocking of AO oxidation through C2-substitution and ortho-substituents, strategic fluorination, pyridine-to-pyrimidine scaffold hopping, and deuterium substitution—can effectively attenuate AO-mediated metabolism in heterocyclic drug candidates. The mechanistic basis for these strategies is increasingly well- understood through the convergence of computational modelling, structural biology, and experimental metabolite profiling, enabling more rational and prospective implementation of AO- resistant designs.

However, the dual requirement for AO metabolic stability and BBB penetration in CNS drug candidates demands an integrated multiparametric approach that transcends the mitigation of any single liability in isolation. None of these liabilities by themselves. Strategic fluorination is a case in point in the context of the foregoing, as it can effectively decrease the metabolism of the AO, affect its pKa to limit efflux, and increase its passive BBB permeability, resulting in higher levels in the CNS. These principles, if applied with rigor, can be demonstrated in case studies of BACE1 inhibitors and GluN2B modulators and in c-Met kinase inhibitors, to

generate brain-penetrant drug candidates with the desired pharmacokinetic properties for CNS drug development. Ongoing difficulties, such as under-prediction of human AO clearance from in vitro studies and the restriction of preclinical rodent pharmacokinetic models, highlight the need for ongoing methodological advances in AO metabolism evaluation. To bridge the gap between in vitro characterization and in vivo pharmaceutical prediction, improved in vitro systems, validated computational prediction tools, and standardized phenotyping protocols will prove key in the future. Finally, a rational design of AO-resistant and brain-penetrant heterocycles will rely on the ongoing development of a multiparametric design optimization, which is driven by mechanistic enzymology and computational chemistry, and will require further systematic development and validation, as detailed in the reviewed literature.

## References

- [1] Garattini E, Terao M. Aldehyde oxidase and its importance in novel drug discovery: present and future challenges. *Expert Opinion on Drug Discovery*. 2013;8(6):641–54. doi:10.1517/17460441.2013.788497
- [2] Mičan J, Lloyd K, Winiwarter PW, Lemoine F, Testa F. Metabolism by aldehyde oxidase: drug design and complementary approaches to challenges in drug discovery. *Journal of Medicinal Chemistry*. 2019;62(24):10955–94. doi:10.1021/acs.jmedchem.9b00875
- [3] Pryde DC, Dalvie D, Hu Q, Jones P, Obach RS, Tran TD. Aldehyde oxidase: an enzyme of emerging importance in drug discovery. *Journal of Medicinal Chemistry*. 2010;53(24):8441–60. doi:10.1021/jm100888d
- [4] Paragas EM, Choughule K, Jones JP, Barr JT. Enzyme kinetics, pharmacokinetics, and inhibition of aldehyde oxidase. In: Nagar S, Argikar UA, Tweedie D, editors. *Enzyme kinetics in drug metabolism: fundamentals and applications*. New York: Humana; 2021. p. 257–84. doi:10.1007/978-1-0716-1554-6\_10
- [5] Inoue K, Kikuchi K, Takahashi K, Hitaoka S, Kusano K, Komori T. In Vitro Metabolism of 2-Methoxy-N-[3-[4-[3-methyl-4-[(6-methyl-3-pyridinyl)oxy]anilino]-6-quinazolinyl]prop-2-enyl]acetamide (CP-724,714) by Aldehyde Oxidase and Predicting Its Percent Contribution Relative to CYP-Mediated Metabolism. *Drug Metabolism and Disposition*. 2023;51(8):962–9. doi:10.1124/dmd.122.000995
- [6] Uehara S, Yoneda N, Higuchi Y, Yamazaki H, Suemizu H. Oxidative metabolism and pharmacokinetics of the EGFR inhibitor BIBX1382 in chimeric NOG-TKm30 mice transplanted with human hepatocytes. *Drug Metabolism and Pharmacokinetics*. 2021;41:100419. doi:10.1016/j.dmpk.2021.100419
- [7] Gajula SNR, Nathani TN, Patil RM, Talari S, Sonti R. Aldehyde oxidase mediated drug metabolism: an underpredicted obstacle in drug discovery and development. *Drug Metabolism Reviews*. 2022;54(4):427–48. doi:10.1080/03602532.2022.2144879
- [8] Izat N, Bolleddula J, Abbasi A, Cheruzel L, Jones RS, Moss D, et al. Challenges and Opportunities for In vitro–In vivo Extrapolation of Aldehyde Oxidase-Mediated Clearance: Toward a Roadmap for Quantitative Translation. *Drug Metabolism and Disposition*. 2023;51(12):1591–606. doi:10.1124/dmd.123.001436

- [9] Xiong B, Wang Y, Chen Y, Xing S, Liao Q, Chen Y, et al. Strategies for structural modification of small molecules to improve blood–brain barrier penetration: a recent perspective. *Journal of Medicinal Chemistry*. 2021;64(18):13152–73. doi:10.1021/acs.jmedchem.1c00910
- [10] Hutzler JM, Cerny MA, Yang YS, Asher C, Wong D, Frederick K, et al. Cynomolgus Monkey as a Surrogate for Human Aldehyde Oxidase Metabolism of the EGFR Inhibitor BIBX1382. *Drug Metabolism and Disposition*. 2014;42(10):1751–60. doi:10.1124/dmd.114.059030
- [11] Ferreira P, Cerqueira NMFS, Coelho C, Fernandes PA, Romão MJ, Ramos MJ. New insights about the monomer and homodimer structures of the human AOX1. *Physical Chemistry Chemical Physics*. 2019;21(25):13545–54. doi:10.1039/C9CP01040H
- [12] Levine DS, Jacobson LD, Bochevarov AD. Large Computational Survey of Intrinsic Reactivity of Aromatic Carbon Atoms with Respect to a Model Aldehyde Oxidase. *Journal of Chemical Theory and Computation*. 2023;19(24):9302–17. doi:10.1021/acs.jctc.3c00913
- [13] Linton A, Kang P, Ornelas M, Kephart S, Hu Q, Pairish M, et al. Systematic Structure Modifications of Imidazo[1,2-a]pyrimidine to Reduce Metabolism Mediated by Aldehyde Oxidase (AO). *Journal of Medicinal Chemistry*. 2011;54(21):7705–12. doi:10.1021/jm2010942
- [14] Ueda H, Narumi K, Furugen A, Saito Y, Kobayashi M. The rs35217482 (T755I) Single-Nucleotide Polymorphism in Aldehyde Oxidase-1 Attenuates Protein Dimer Formation and Reduces the Rates of Phthalazine Metabolism. *Drug Metabolism and Disposition*. 2022;50(9):1126–31. doi:10.1124/dmd.122.000902
- [15] Dalvie D, Sun H, Xiang C, Hu Q, Jiang Y, Kang P. Effect of Structural Variation on Aldehyde Oxidase-Catalyzed Oxidation of Zoniporide. *Drug Metabolism and Disposition*. 2012;40(8):1575–87. doi:10.1124/dmd.112.045823
- [16] Garattini E, Terao M. The role of aldehyde oxidase in drug metabolism. *Expert Opinion on Drug Metabolism and Toxicology*. 2012;8(4):487–503. doi:10.1517/17425255.2012.663352
- [17] Subash S, Singh DK, Ahire DS, Khojasteh SC, Murray BP, Zientek MA, et al. Dissecting Parameters Contributing to the Underprediction of Aldehyde Oxidase-Mediated Metabolic Clearance of Drugs. *Drug Metabolism and Disposition*. 2023;51(10):1362–71. doi:10.1124/dmd.123.001379
- [18] Crouch RD, Blobaum AL, Felts AS, Conn PJ, Lindsley CW. Species-Specific Involvement of Aldehyde Oxidase and Xanthine Oxidase in the Metabolism of the Pyrimidine-Containing mGlu5-Negative Allosteric Modulator VU0424238 (Auglurant). *Drug Metabolism and Disposition*. 2017;45(12):1245–59. doi:10.1124/dmd.117.077552
- [19] Jismy B, Akssira M, Knez D, Guillaumet G, Gobec S, Abarbri M. Efficient synthesis and preliminary biological evaluations of trifluoromethylated imidazo[1,2-a]pyrimidines and benzimidazo[1,2-a]pyrimidines. *New Journal of Chemistry*. 2019;43(25):9961–8. doi:10.1039/C9NJ01982K
- [20] Hayes A, Mok NY, Liu M, Thai C, Henley AT, Atrash B, et al. Pyrido[3,4-d]pyrimidin-4(3H)-one metabolism mediated by aldehyde oxidase is blocked by C2-substitution. *Xenobiotica*. 2017;47(9):771–7. doi:10.1080/00498254.2016.1230245
- [21] Wu YJ, Guernon J, Shi J, Marcin L, Higgins M, Rajamani R, et al. Discovery of S3-Truncated, C-6 Heteroaryl Substituted Amino-thiazine  $\beta$ -Site APP Cleaving Enzyme-1 (BACE1) Inhibitors. *Journal of Medicinal Chemistry*. 2016;59(18):8593–600. doi:10.1021/acs.jmedchem.6b01012

- [22] Zhang JW, Xiao W, Gao ZT, Yu ZT, Zhang JY (Jeff). Metabolism of c-Met Kinase Inhibitors Containing Quinoline by Aldehyde Oxidase, Electron Donating, and Steric Hindrance Effect. *Drug Metabolism and Disposition*. 2018;46(12):1847–55. doi:10.1124/dmd.118.081919
- [23] Zhang JW, Deng HB, Zhang CY, Dai JQ, Li Q, Zheng QG, et al. In Vitro Metabolism by Aldehyde Oxidase Leads to Poor Pharmacokinetic Profile in Rats for c-Met Inhibitor MET401. *European Journal of Drug Metabolism and Pharmacokinetics*. 2019;44(5):669–80. doi:10.1007/s13318-019-00557-9
- [24] Gelin CF, Stenne B, Coate H, Hiscox A, Soyode-Johnson A, Wall JL, et al. Discovery of a Series of Substituted 1H-((1,2,3-Triazol-4-yl)methoxy)pyrimidines as Brain Penetrants and Potent GluN2B-Selective Negative Allosteric Modulators. *Journal of Medicinal Chemistry*. 2023;66(4):2877–92. doi:10.1021/acs.jmedchem.2c01916
- [25] Lazzara PR, Moore TW. Scaffold-hopping as a strategy to address metabolic liabilities of aromatic compounds. *RSC Medicinal Chemistry*. 2020;11(1):18–29. doi:10.1039/C9MD00396G
- [26] Lepri S, Ceccarelli M, Milani N, Tortorella S, Cucco A, Valeri A, et al. Structure–metabolism relationships in human-AOX: Chemical insights from a large database of aza-aromatic and amide compounds. *Proceedings of the National Academy of Sciences of the United States of America*. 2017;114(16):E3178–87. doi:10.1073/pnas.1618881114
- [27] Dyer RMB, Hahn PL, Hilinski MK. Selective Heteroaryl N-Oxidation of Amine-Containing Molecules. *Organic Letters*. 2018;20(7):2011–4. doi:10.1021/acs.orglett.8b00558
- [28] St. Jean DJ Jr, Fotsch C. Mitigating heterocycle metabolism in drug discovery. *Journal of Medicinal Chemistry*. 2012;55(13):6002–20. doi:10.1021/jm300343m
- [29] Fjelbye K, Marigo M, Clausen RP, Jørgensen EB, Christoffersen CT, Juhl K. Elucidation of fluorine's impact on pKa and in vitro P-gp-mediated efflux for a series of PDE9 inhibitors. *MedChemComm*. 2018;9(5):893–6. doi:10.1039/c8md00114f
- [30] Hilpert H, Guba W, Woltering TJ, Wostl W, Pinard E, Mauser H, et al.  $\beta$ -Secretase (BACE1) Inhibitors with High in Vivo Efficacy Suitable for Clinical Evaluation in Alzheimer's Disease. *Journal of Medicinal Chemistry*. 2013;56(10):3980–95. doi:10.1021/jm400225m
- [31] Grunewald GL, Seim MR, Lu J, Makboul M, Criscione KR. Application of the Goldilocks Effect to the Design of Potent and Selective Inhibitors of Phenylethanolamine N-Methyltransferase: Balancing pKa and Steric Effects in the Optimization of 3-Methyl-1,2,3,4-tetrahydroisoquinoline Inhibitors by  $\beta$ -Fluorination. *Journal of Medicinal Chemistry*. 2006;49(10):2939–52. doi:10.1021/jm051262k
- [32] Asano D, Shibayama T, Shiozawa H, Inoue SI, Shinozuka T, Murata S, et al. Evaluation of species differences in the metabolism of the selective Nav1.7 inhibitor DS-1971a, a mixed substrate of cytochrome P450 and aldehyde oxidase. *Xenobiotica*. 2021;51(9):1060–70. doi:10.1080/00498254.2021.1963009
- [33] Choi C, Carlo AA, Cronin CN, Jing K, Kung DW, Liu J, et al. Synthesis of Deuterated Heterocycles with Me<sub>2</sub>NCD(OMe)<sub>2</sub> and Evaluation of the Products for Metabolism by Aldehyde Oxidase. *ACS Medicinal Chemistry Letters*. 2022;13(2):250–6. doi:10.1021/acsmedchemlett.1c00634
- [34] Zhao J, Cui R, Wang L, Chen Y, Fu Z, Ding X, et al. Revisiting Aldehyde Oxidase Mediated Metabolism in Drug-like Molecules: An Improved Computational Model. *Journal of Medicinal Chemistry*. 2020;63(12):6523–37. doi:10.1021/acs.jmedchem.9b01895

- [35] Barnes JP, Yang SM, Thompson TS, Guevarra CM, Pleasant SS, Do TQ, et al. Mechanistic Investigation of the Time-Dependent Aldehyde Oxidase Inhibitor Hydralazine. *Drug Metabolism and Disposition*. 2023;51(6):782–91. doi:10.1124/dmd.123.001257
- [36] Neumaier F, Zlatopolskiy BD, Neumaier B. Drug Penetration into the Central Nervous System: Pharmacokinetic Concepts and In Vitro Model Systems. *Pharmaceutics*. 2021;13(10):1542. doi:10.3390/pharmaceutics13101542
- [37] Hodgetts KJ. Case studies of CNS drug optimization — medicinal chemistry and CNS biology perspectives. In: Di L, Kerns EH, editors. *Blood-brain barrier in drug discovery*. Hoboken: John Wiley & Sons; 2015. p. 425–45. doi:10.1002/9781118788523.ch19
- [38] Pajouhesh H, Lenz GR. Medicinal Chemical Properties of Successful Central Nervous System Drugs. *NeuroRx*. 2005;2(4):541–53. doi:10.1602/neurorx.2.4.541
- [39] Roskoski R. Properties of FDA-approved small molecule protein kinase inhibitors. *Pharmacological Research*. 2019;144:19–50. doi:10.1016/j.phrs.2019.03.006
- [40] Javed MA, Ashraf N, Jan MS, Mahnashi MH, Alqahtani YS, Alyami BA, et al. Structural modification, in vitro, in vivo, ex vivo, and in silico exploration of pyrimidine and pyrrolidine cores for targeting enzymes associated with neuroinflammation and cholinergic deficit in Alzheimer's disease. *ACS Chemical Neuroscience*. 2021;12(21):4123–43. doi:10.1021/acscchemneuro.1c00507
- [41] Lenci E, Calugi L, Trabocchi A. Occurrence of Morpholine in Central Nervous System Drug Discovery. *ACS Chemical Neuroscience*. 2021;12(3):378–90. doi:10.1021/acscchemneuro.0c00729
- [42] Do HT, Li H, Chreifi G, Poulos TL, Silverman RB. Optimization of Blood–Brain Barrier Permeability with Potent and Selective Human Neuronal Nitric Oxide Synthase Inhibitors Having a 2-Aminopyridine Scaffold. *Journal of Medicinal Chemistry*. 2019;62(5):2690–707. doi:10.1021/acs.jmedchem.8b02032
- [43] Rombouts FJR, Hsiao CC, Bache S, De Cleyne M, Heckmann P, Leenaerts J, et al. Modulating physicochemical properties of tetrahydropyridine-2-amine BACE1 inhibitors with electron-withdrawing groups: A systematic study. *European Journal of Medicinal Chemistry*. 2022;228:114028. doi:10.1016/j.ejmech.2021.114028
- [44] Egan MF, Kost J, Voss T, Mukai Y, Aisen PS, Cummings JL, et al. Randomized Trial of Verubecestat for Prodromal Alzheimer's Disease. *New England Journal of Medicine*. 2019;380(15):1408–20. doi:10.1056/NEJMoa1812840
- [45] Wright SW. Synthesis of Deuterated Enaminones with High Isotopic Fidelity. *ACS Medicinal Chemistry Letters*. 2023;14(12):1742–5. doi:10.1021/acsmchemlett.3c00395
- [46] Schneider F, Bradbury M, Baillie TA, Stamler D, Hellriegel E, Cox DS, et al. Pharmacokinetic and Metabolic Profile of Deutetrabenazine (TEV-50717) Compared With Tetrabenazine in Healthy Volunteers. *Clinical and Translational Science*. 2020;13(4):707–17. doi:10.1111/cts.12754