

A Pre-Specified, Structure-Based Cascade Protocol for the Discovery of Exosite-Directed, Zinc-Sparing Inhibitors of Insulin-Degrading Enzyme

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Research Article

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Abstract

Background

Zn²⁺-dependent metallopeptidase that regulates the clearance of insulin and several bioactive peptides. Therapeutic IDE inhibition is complicated by the conservation of catalytic zinc-binding features among metallopeptidases and by the potential consequences of central IDE inhibition.

Objective

To define a reproducible, prospectively specified computational framework for identifying exosite-directed, zinc-sparing IDE inhibitors while explicitly evaluating selectivity against neprilysin (NEP) and angiotensin-converting enzyme (ACE).

Methods

The workflow comprises structural verification and standardized preparation of IDE, NEP, and ACE; self-docking validation of crystallographic reference ligands; curation of non-chelating chemical libraries; parallel three-target virtual screening; quantitative selectivity filtering; pose-based exosite assessment; molecular dynamics and MM-GBSA rescoring; cross-target dynamic controls; and *in silico* ADMET triage, including exclusion of predicted blood–brain barrier penetration.

Results

Protocol verification identified a critical structural assignment error in the original target set: PDB 2G54 contains Zn²⁺-bound IDE complexed with insulin B-chain, whereas the IDE–li1 complex is PDB 3E4A. Accordingly, 2G54 is designated for substrate-engaged structural mapping and 3E4A for li1 redocking validation. The protocol also replaces the previously stated li1 potency value with the approximately 2 nM value reported in the primary study. No virtual screening, molecular dynamics, or ADMET calculations were performed; therefore, no candidate-level computational outcomes are claimed. The corrected protocol is intended to guide subsequent experimental laboratory validation.

Conclusion

This framework establishes explicit, falsifiable decision criteria for prospective IDE inhibitor discovery and is designed to reduce catalytic-site zinc chelation, NEP/ACE cross-reactivity, and central nervous system exposure before experimental testing.

1. INTRODUCTION

Insulin-degrading enzyme (IDE; insulysin; EC 3.4.24.56) is a zinc-dependent metallopeptidase with an atypical inverted HxxEH catalytic motif and a large clamshell architecture formed by N- and C-terminal halves. Structural studies demonstrated that IDE can encapsulate structurally diverse peptide substrates within a gated internal chamber, providing a mechanistic basis for both substrate selectivity and allosteric regulation.^{1,2}

The therapeutic rationale for IDE inhibition derives from its role in insulin catabolism, but pharmacological manipulation of IDE is complicated by its broader substrate spectrum. Rationally designed peptide hydroxamate inhibitors demonstrated potent IDE inhibition and stabilization of the closed enzyme conformation, with the lead compound Li1 reported to have an inhibition constant of approximately 2 nM.³ Subsequent studies with macrocyclic inhibitors showed that IDE inhibition can alter insulin, glucagon, and amylin biology, indicating that target engagement may have effects extending beyond insulin clearance alone.⁴

A structure-based route to selectivity is therefore particularly relevant. Exosite-directed IDE inhibitors can engage substrate-recognition surfaces outside the catalytic zinc center, and dual-exosite inhibitors have demonstrated that potent IDE inhibition can be achieved without direct zinc coordination.⁵ Recent structural analyses likewise emphasize allosteric and exosite mechanisms as promising alternatives to canonical zinc-chelating chemotypes.⁶

Selectivity must nevertheless be tested against physiologically important metallopeptidases with established pharmacology. Human neprilysin (NEP) is a zinc-dependent ectopeptidase whose extracellular-domain structure has been resolved in complex with phosphoramidon, providing a reference architecture for active-site comparison.⁷ ACE is another zinc metallopeptidase of major cardiovascular relevance, with the human lisinopril-bound structure defining its catalytic domain and inhibitor-binding geometry.⁸ Pharmacological inhibition of NEP is clinically active and contributes to the effects of sacubitril/valsartan, whereas ACE inhibition has well-established cardiovascular and renal consequences.

Structure-based virtual screening can reduce experimental search space, but docking scores are imperfect proxies for binding affinity and can be sensitive to receptor preparation, scoring-function behavior, and library composition.⁹ Methodological bias can also arise when comparator proteins are prepared or scored differently, making apparent selectivity difficult to interpret. Molecular dynamics and end-point free-energy methods can provide orthogonal evidence for pose persistence and relative energetic plausibility, although MM-GBSA remains approximate and should not be interpreted as an experimental affinity measurement.¹⁰

The present study therefore develops a prospectively specified, three-target computational framework in which IDE, NEP, and ACE are processed under harmonized structural and computational rules. The central design principle is to prioritize non-chelating interaction with an IDE exosite, impose a quantitative selectivity gate before computationally expensive refinement, and treat predicted exclusion

from central nervous system exposure as a positive design criterion. The manuscript is a protocol-development study: no virtual screening, molecular dynamics, free-energy rescoring, or ADMET calculations were performed for the present report.

2. METHODS

2.1 Study design and computational scope

This work was designed as a hypothesis-generating computational protocol rather than as a completed virtual-screening experiment. All decision rules, structural inputs, exclusion criteria, and downstream analyses were specified before candidate-level computation. Every software tool and database specified in this protocol is freely available, and none requires a paid license, institutional subscription, or proprietary agreement to execute the workflow as described. Because no candidate-level computation was executed, the manuscript reports protocol verification and methodological outputs only.

2.2 Target selection and structural verification

Three human metallopeptidases were selected: IDE, NEP, and ACE. IDE was represented by PDB 2G54 for substrate-engaged structural mapping and by PDB 3E4A for validation of the Ii1 binding mode. PDB 2G54 is the Zn^{2+} -bound IDE structure containing insulin B-chain, whereas PDB 3E4A contains IDE bound to Ii1.^{1,3} NEP was represented by PDB 1DMT.⁷ ACE was represented by PDB 1O86.⁸ Structural identity, ligand identity, chain composition, crystallographic resolution, catalytic-metal status, and residue numbering were verified against the record in the RCSB Protein Data Bank,¹¹ a public-domain, no-cost structural repository maintained by an international academic consortium, requiring no account or license for download or use.

2.3 Protein preparation and metal-site handling

The three receptor structures are prepared using a common protocol. Nonessential crystallographic waters and deposited ligands are removed, hydrogens are added at pH 7.4, and steric conflicts are minimized. Protein parameters are based on AMBER ff14SB.¹² It should be noted that ff14SB is a published, openly available set of force-field parameters, not a proprietary program: it can be applied within any compatible free MD engine — in this protocol, GROMACS (Section 2.8) — without requiring a license for the commercial AMBER simulation package itself. The IDE catalytic Zn^{2+} is retained in the + 2 oxidation state and treated with MCPB.py,¹³ a metal-center parameterization tool distributed since AmberTools20 under a free, no-license BSD-style distribution (part of AmberTools, which is open source and cost-free, independent of the paid AMBER/pmemd simulation engine). The HxxEH catalytic environment is inspected after preparation to confirm that His108, His112, and Glu189 remain geometrically compatible with the deposited coordination environment.¹

2.4 Reference-ligand redocking validation

Self-docking is performed for li1 into PDB 3E4A, phosphoramidon into PDB 1DMT, and lisinopril into PDB 1O86, using AutoDock Vina 1.2.x,^{14,15} free and open source under the Apache 2.0 license, with no registration or fee required. The primary validation criterion is heavy-atom RMSD ≤ 2.0 Å between the best-ranked redocked pose and the deposited ligand pose.

2.5 Chemical-library construction

Three complementary libraries are specified. Library A and Library B draw from ZINC15,¹⁶ a public, no-cost, ready-to-dock chemical database maintained by the University of California, San Francisco, requiring only free registration for bulk downloads, with hydroxamate-containing chemotypes excluded *a priori* to reduce enrichment of canonical zinc-chelating scaffolds. Library C contains reference and control ligands (li1, the active metabolite of sacubitril, lisinopril, captopril). All manifests are deduplicated by canonical SMILES using open cheminformatics toolkits.

2.6 Three-target cascade screening

Every candidate passing library curation is docked against IDE, NEP, and ACE using AutoDock Vina under identical settings. Filter 1 requires a reproducible dominant pose cluster. Filter 2 calculates the Selectivity Index ($SI = \Delta G_{NEP} - \Delta G_{IDE}$), retaining an operational gate of >2.5 kcal/mol. Candidates are also required to avoid favorable ACE docking relative to IDE.

2.7 Exosite-specific pose refinement

The top 50 candidates undergo higher-resolution pose refinement, retained only when the predicted IDE pose engages the exosite region (Phe141, Phe820, Tyr831, with monitoring of Arg429/Ser816) without direct Zn^{2+} coordination.

2.8 Molecular dynamics and MM-GBSA rescoring

The five highest-priority complexes are simulated in explicit solvent for 100 ns using GROMACS,¹⁷ fully open source (LGPL/GPL), free for academic and commercial use, with no licensing fee. A 50-ns NEP simulation serves as a cross-target dynamic control. MM-GBSA is calculated using standard, freely available AmberTools scripts (MMPBSA.py).^{10,18}

2.9 ADMET and CNS-exposure triage

Physicochemical, pharmacokinetic, and toxicity properties are assessed using three no-cost web servers: SwissADME (Swiss Institute of Bioinformatics),¹⁹ pkCSM (University of Melbourne),²⁰ and ProTox-II (Charité – Universität Berlin).²¹ None of these three platforms requires payment, subscription, or institutional login; all are accessible to any user with a web browser.

2.10 Synthetic accessibility and decision framework

Candidates satisfying the structural, selectivity, dynamic, and ADMET gates are subjected to a retrosynthetic feasibility check using AiZynthFinder,²² a Monte Carlo tree-search retrosynthesis planner released under the permissive MIT open-source license and designed to run entirely on local infrastructure, with no account, cloud service, or usage terms required. This substitution ensures that every computational step in the protocol, including retrosynthesis, remains reproducible by any laboratory without a paid license. The synthesis prediction is used only to assess prospective accessibility and does not constitute experimental synthesis.

2.11 Statistical and reporting principles

No inferential statistics are applicable to this protocol-development stage. Future execution should report the complete screened-library denominator, filter-retention counts, score distributions, redocking RMSD, trajectory convergence, MM-GBSA distributions, and ADMET exclusions. All raw poses, parameter files, scripts, and candidate identifiers should be deposited in a public repository, for example Zenodo or OSF, both free and open, before publication of any empirical follow-up.

Table 1. Target structures and predefined structural roles

Target	PDB	Reference ligand/substrate	Role in protocol	Key structural feature
IDE	2G54	Insulin B-chain + Zn ²⁺	Substrate-engaged architecture and exosite mapping	Zn ²⁺ ; substrate chamber
IDE	3E4A	li1	Self-docking validation	li1-bound closed conformation
NEP	1DMT	Phosphoramidon	Comparator and self-docking validation	Zn-dependent catalytic domain
ACE	1O86	Lisinopril	Comparator and self-docking validation	Human ACE catalytic domain

Table 2. Pre-specified cascade acceptance criteria

Stage	Operational criterion	Decision	Planned output
Structural verification	Correct PDB identity, ligand identity, chain assignment, metal status, and residue numbering	Pass before preparation	Verified coordinate set
Redocking	Heavy-atom RMSD ≤ 2.0 Å for each reference ligand	All three targets must pass	RMSD and pose overlays
Library curation	Deduplication; standardized stereochemistry/protonation; non-chelating design rules	Pass/fail	Curated library manifest
Filter 1	Dominant pose cluster across independent runs	Retain reproducible poses	IDE/NEP/ACE docking scores and poses
Filter 2	SI_NEP = $\Delta G_{\text{NEP}} - \Delta G_{\text{IDE}} > 2.5$ kcal/mol and SI_ACE = $\Delta G_{\text{ACE}} - \Delta G_{\text{IDE}} > 2.5$ kcal/mol	Retain computationally selective candidates	Selectivity matrix
Filter 3	Zn ²⁺ coordination	Retain mechanistically consistent poses	Interaction maps
Molecular dynamics	IDE complex stable over 100 ns; predefined contact/RMSD criteria satisfied	Retain dynamically consistent candidates	Trajectory metrics
Cross-target MD	No persistent analogous binding mode in 50-ns NEP control	Retain target-preferential behavior	Comparative trajectory analysis
ADMET	Predicted BBB exclusion; no major predefined CYP3A4, hepatotoxicity, or hERG alerts	Retain developability-compatible candidates	ADMET report
Synthesis planning	Three-step retrosynthetic proposal with chemically plausible transformations	Prioritize experimentally accessible candidates	Route proposal

3. RESULTS

The principal result of protocol verification was correction of the structural identity assigned to the IDE reference complex. PDB 2G54 was confirmed as the Zn²⁺-bound IDE structure containing insulin B-chain, whereas PDB 3E4A is the crystallographic IDE–Ii1 complex.^{1,3} This distinction changes the role of the two structures: 2G54 is appropriate for mapping the substrate-engaged architecture and candidate exosite geometry, while 3E4A is required for self-docking validation of Ii1.

A second correction concerns the historical potency value attributed to Ii1. The primary characterization supports an inhibition constant of approximately 2 nM, and this value replaces the 0.3 nM value used in

the earlier protocol draft.³ This correction is important because reference-compound potency can influence how computational thresholds are justified and should not be conflated with a docking-derived estimate.

The revised protocol also resolves a methodological inconsistency in the original force-field description. Rather than allowing either OPLS4 or AMBER ff14SB to be selected after inspection of results, the workflow specifies a single protein force field and a separately validated Zn²⁺ treatment. This change reduces analytical flexibility and therefore limits a potential source of *post hoc* optimization.^{12,13}

The cascade now separates four evidentiary layers: structural validity, static ligand ranking, dynamic pose persistence, and developability triage. The distinction is intentional. Docking is used to prioritize poses and candidates, molecular dynamics is used to test whether the proposed interaction pattern persists under explicit-solvent conditions, MM-GBSA is used for relative energetic refinement, and ADMET models are used to eliminate candidates with predefined liabilities. No layer is treated as sufficient evidence of biological activity on its own (Figure 1).

No candidate-level docking, molecular dynamics, MM-GBSA, synthetic-accessibility, or ADMET result was generated for this manuscript. Consequently, the study does not establish the existence of an IDE-selective inhibitor, does not demonstrate a numerical IDE/NEP or IDE/ACE selectivity margin, and does not provide evidence of pharmacological efficacy. The substantive output is the corrected and operationalized protocol itself.

4. DISCUSSION

This protocol-verification study resolved three errors that would otherwise have propagated through any downstream virtual-screening campaign against IDE: a mistaken structural assignment of the IDE reference complexes, an outdated li1 potency value, and an unresolved ambiguity in the force-field specification. In practical terms, the study shows that the originally planned cascade could not have been executed as written, because its two most basic inputs, which PDB structure represents which state, and how potent the reference inhibitor actually is, were incorrect. Correcting these inputs before any candidate-level computation is, in itself, the principal safeguard this work provides for the discovery effort that is intended to follow it.

The decision to specify every structural, computational, and decision-gate parameter before candidate-level computation departs from common practice in structure-based virtual screening, where methodological choices are frequently adjusted after inspecting preliminary results. Reviews of computational drug discovery have identified this kind of *post hoc* flexibility, together with inconsistent reporting of software versions, parameters, and filtering criteria, as a recurring obstacle to reproducibility in the field.²⁴ The present cascade addresses this concern directly by fixing the selectivity threshold, the exosite contact set, and the ADMET acceptance criteria in advance, and by requiring redocking validation before any library is screened.

The zinc-sparing design principle adopted here is not without precedent, although it has not previously been formalized as a pre-registered cascade for IDE. In the matrix metalloproteinase field, inhibitors that engage exosites outside the catalytic zinc center have been developed as an explicit strategy to overcome the poor target selectivity of conventional hydroxamate-based zinc chelators.²⁵ Selective, non-chelating MMP-13 inhibitors identified through high-throughput screening achieved low-micromolar to nanomolar potency together with high selectivity against a panel of related proteases, supporting the general premise that exosite engagement can substitute for direct metal coordination without loss of potency.²⁶ The present protocol extends this validated paradigm, established in a different zinc metalloprotease family, to IDE, where an analogous non-chelating strategy has so far been demonstrated only for individual inhibitors rather than embedded in a prospectively specified, multi-target screening cascade.

Within the IDE literature specifically, dual-exosite inhibitors have already shown that potent inhibition without direct zinc coordination is achievable for this particular enzyme.⁵ The present work does not contradict that precedent; rather, it adds a methodological layer that the earlier studies did not require, because they described individual characterized compounds rather than a prospective screening framework intended to nominate previously untested chemical matter across three counter-screened targets simultaneously.

The exosite-centered design principle is supported by the structural biology of IDE. The enzyme forms a gated internal chamber in which substrate recognition depends on contacts across both IDE halves, and conformational opening and closure are central to catalysis. li1 exploits this architecture by stabilizing a closed state rather than behaving simply as a conventional active-site zinc chelator.³ The later development of dual-exosite inhibitors provided an additional proof of concept that potent IDE inhibition can be achieved without direct interaction with catalytic zinc.⁵ These observations justify using exosite engagement as a structural filter, although the protocol appropriately treats the selected residue set as operational rather than definitive.

The exclusion of hydroxamate-rich chemotypes follows the same mechanistic logic. Hydroxamate-containing inhibitors can achieve strong metal-dependent binding, but their mechanism can favor interaction with conserved metalloprotease catalytic motifs and thereby complicate selectivity.⁶ By contrast, the present workflow seeks chemical matter whose principal recognition determinants arise from IDE-specific peripheral surfaces. This strategy does not guarantee selectivity because shallow exosites can be difficult to model and may accommodate chemically diverse ligands.

The three-target comparator set reflects the same structural logic applied to counter-screening. NEP has a defined extracellular catalytic architecture and is a validated drug target, while ACE has a well-characterized zinc-dependent catalytic domain, and both share the conserved zinc-coordination geometry that motivates the zinc-sparing design principle in the first place.^{7,8}

5. LIMITATIONS

First, the current protocol uses static crystal structures as the starting point, whereas IDE undergoes large conformational changes associated with substrate access and catalytic cycling.¹ This limitation is mitigated, though not eliminated, by the subsequent molecular-dynamics stage, which is specifically intended to test whether a statically favorable pose persists once conformational flexibility is introduced.

Second, a single docking engine and scoring function may introduce model-specific ranking errors; consensus or rescoring strategies could be explored prospectively, but should not be added selectively after candidate identities are known.^{9,23} The redocking-validation requirement in Section 2.4 partially addresses this concern by confirming that the chosen engine reproduces the known experimental binding mode for each of the three targets before it is trusted for candidate ranking.

Third, the proposed libraries are chemically constrained by their curation rules and may omit novel scaffolds that fall outside the predefined physicochemical space. This trade-off is deliberate: the same constraints that could exclude some viable chemotypes are also what allow the selectivity and exosite filters to be applied consistently across a chemically homogeneous set.

Fourth, predicted ADMET properties are not substitutes for experimental permeability, metabolic stability, ion-channel, or toxicity measurements.^{19,20} These predictions are used here only as an early triage step to deprioritize candidates unlikely to meet the stated CNS-exclusion and safety objectives, not as a final developability determination.

6. CLINICAL AND PRACTICAL IMPLICATIONS

The therapeutic rationale for IDE inhibition is not unopposed in the literature. Genetic and pharmacological IDE loss-of-function models have been associated with impaired glucose tolerance in some settings, even though a separate line of evidence indicates that certain IDE inhibitors can improve glycemic parameters *in vivo*.^{4,27}

This tension is precisely why the present protocol's selectivity and exosite requirements matter in practical terms: a compound that inhibits IDE through a mechanism distinct from broad, non-selective active-site blockade may be less likely to reproduce the adverse metabolic phenotypes reported for some earlier inhibitor classes, although this remains a hypothesis to be tested experimentally rather than an established outcome. A recent review of IDE-targeted therapeutics for diabetes reached a similar conclusion, emphasizing that both the potential benefits and the potential risks of IDE inhibition depend heavily on the mechanism and selectivity of the specific compound under study rather than on IDE inhibition as a uniform pharmacological class.²⁸

For clinical translation, the practical consequence is that any candidate emerging from this cascade would still require dedicated glucose-tolerance and insulin-clearance studies before its therapeutic

potential could be assessed, in addition to the enzymology and selectivity work already built into the protocol.

7. CONCLUSION AND FUTURE DIRECTIONS

This revised protocol establishes a prospectively specified, three-enzyme computational framework for the discovery of exosite-directed, zinc-sparing IDE inhibitors. Its principal contribution is methodological rather than empirical: verification and correction of the structural, potency, and force-field inputs that would otherwise have propagated through any downstream screening campaign, together with a workflow that integrates harmonized receptor preparation, explicit selectivity gates, exosite-contact requirements, molecular-dynamics cross-target controls, conservative MM-GBSA interpretation, and CNS-exposure triage. Because no candidate-level calculations were performed, the study provides a reproducible framework rather than evidence of inhibitor efficacy.

The take-home message is that a structurally and pharmacologically defensible IDE-inhibitor discovery campaign requires its target identities, reference potencies, and force-field choices to be verified and fixed before any candidate-level computation begins. Future execution of the corrected cascade should include purified-enzyme kinetic assays against IDE, NEP, and ACE, direct assessment of zinc coordination, cellular target-engagement measurements, permeability testing, and the glucose-tolerance studies discussed in Section 6, before any therapeutic inference is drawn from computational results alone. The framework's ultimate value will depend on this prospective experimental validation.

Declarations

Conflict of Interest:

The authors declare no conflicts of interest.

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Figures

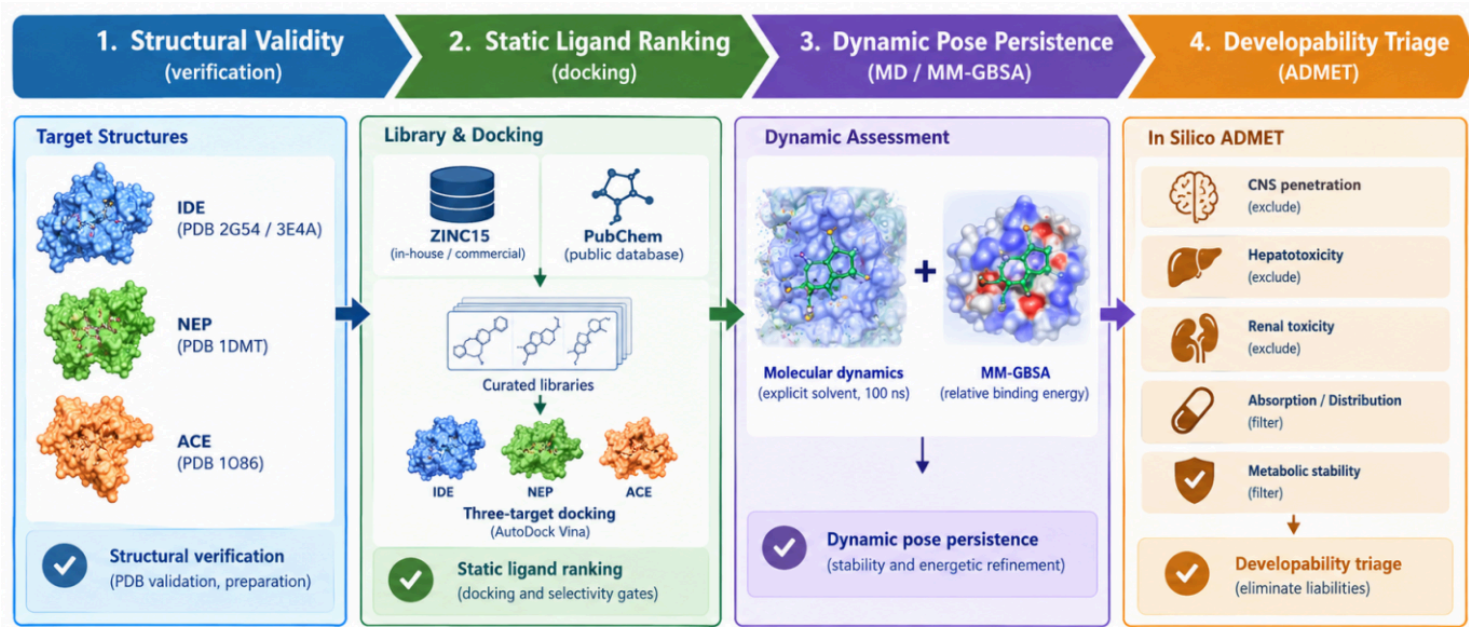


Figure 1

Prospectively specified three-enzyme cascade for IDE inhibitor discovery.

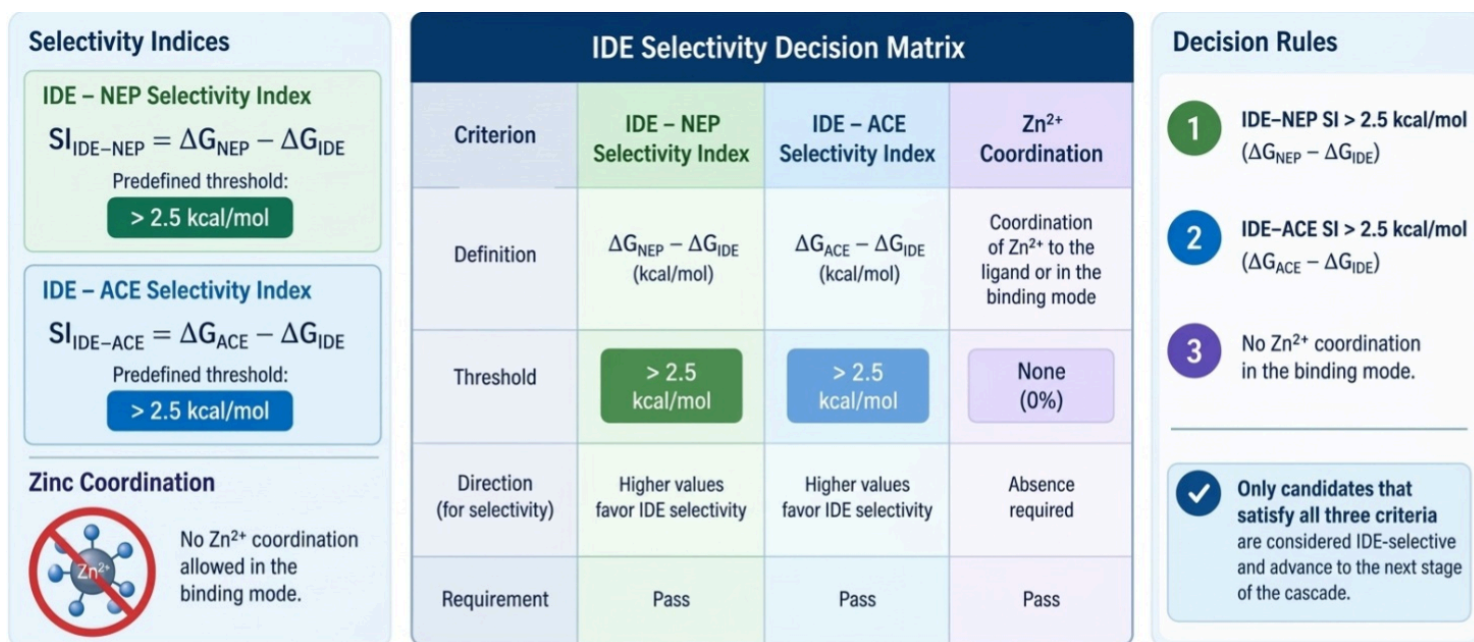


Figure 2

Quantitative selectivity framework.