Reproducible Analysis and Model Identifiability for NNC2215 under Paracetamol-Glucuronide Exposure

A secondary analysis of published data and a conditional occupancy model

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Scope. This work reanalyses published data and presents an assumption-bound calculation. It reports no new biological experiment, validated drug candidate, molecular-dynamics result, or clinical outcome.

Abstract

Glucose-responsive insulin is intended to couple insulin activity to ambient glucose, but non-glucose ligands may perturb the same molecular switch. NNC2215 is a glucose-responsive insulin conjugate, and a subsequent study reported binding of paracetamol glucuronide (PaG) and increased insulin-receptor affinity in the presence of PaG. We reanalysed publisher source data from the NNC2215 glucose-response study, summarized reported glucose and PaG apparent dissociation constants, and evaluated a reduced four-state occupancy model as an explicitly conditional calculation. We also reviewed whether the available source-derived structures and parameterization records support target molecular dynamics. Paired source-data arithmetic reproduced the reported 3.2-fold receptor-affinity increase from 3 to 20 mmol L^{-1} glucose and the 12.5-fold increase from 0 to 20 mmol L^{-1}. Under a single-site competition assumption, a 70 μmol L^{-1} total PaG anchor and 3 mmol L^{-1} glucose corresponded to 13.8% PaG-bound switch states if all PaG were treated as free; the result changed substantially across free-fraction scenarios. The reported receptor-affinity observation is separate and is not predicted by this occupancy model. Structural mapping retains multiple B29 alternatives, and no validated full-conjugate force field or target simulation is available. The analysis therefore establishes neither a new molecule nor a functional or clinical design specification. Extending the published 0-versus-70 μmol L^{-1} PaG receptor-binding relative-affinity comparison with a replicated PaG concentration range and direct occupancy measurements would be needed to identify the relevant binding-response surface.

Keywords: glucose-responsive insulin; NNC2215; paracetamol glucuronide; occupancy model; reproducibility; model identifiability

1 Introduction

Glucose-responsive insulin designs seek reversible changes in insulin bioactivity across clinically relevant glucose concentrations. NNC2215 combines insulin with a glucose-binding macrocycle and a tethered glucoside. In the original study, the conjugate showed glucose-dependent insulin-receptor binding in vitro and glucose-sensitive pharmacology in animal models [1]. These results establish a proof of concept for that compound and study context; they do not validate every molecular-switch mechanism or a successor molecule.

A later study reported that NNC2215 also binds paracetamol glucuronide (PaG), a major paracetamol metabolite, and that PaG increases NNC2215 insulin-receptor affinity, particularly at low

glucose [2]. That study used native mass spectrometry, receptor-binding assays, pig clamp experiments, and pharmacokinetic/pharmacodynamic modelling. Its authors concluded that NNC2215 itself was not a viable therapeutic candidate in light of this interaction and it was not advanced into clinical development; the present analysis does not re-evaluate that conclusion. The study also makes clear that any clinical impact estimate depends on modelling assumptions and requires clinical data for direct quantification.

The present work addresses a narrower question: what can be reproduced from published values, and what does a simple apparent-affinity occupancy calculation identify? We separately document a structural-readiness boundary because a conditional occupancy scenario cannot justify target-binding molecular dynamics. We do not attempt to infer a chemical structure, receptor efficacy, clinical dose, or safety threshold.

2 Methods

2.1 Published glucose-response data

We used the publisher-provided Source Data workbook associated with the 2024 NNC2215 article, worksheet Figure 3d [1, 3]. The workbook supplies the numeric values used here; no values were digitized from a graph. For each of the three independent experiments, we calculated the within-experiment ratio of insulin-receptor affinity at 20 mmol L^{-1} glucose to affinity at 3 mmol L^{-1} and at 0 mmol L^{-1}. We report the arithmetic mean and sample standard deviation of these three ratios. This is a descriptive reproduction, not a new experiment or a refit of the original dose-response curves.

2.2 PaG binding and exposure inputs

The PaG analysis used the glucose and PaG apparent K_d summaries reported by Sturis et al. from native mass spectrometry: 1.91 ± 0.10 mmol L^{-1} for D-glucose and 0.17 ± 0.01 mmol L^{-1} for PaG [2]. The reported fit errors are not biological replicate standard deviations; the native-MS figure specifies single measurements for each D-glucose concentration. We also used the paper's estimated mean maximum human PaG concentration of 70 μmol L^{-1} after a single 1000-mg oral paracetamol dose as an exposure anchor. It is an estimate from the study, not a measured free concentration in the receptor assay or in a person receiving NNC2215.

The 2026 paper reports hIR-A receptor-binding relative-affinity measurements across its tested glucose concentrations with PaG absent or fixed at 70 μmol L^{-1} (N=3). Those measurements used an assay matrix containing 1.5% human serum albumin (HSA). We report the zero-glucose values (0.9% without PaG and 5.4% with 70 μmol L^{-1} PaG) as published observations; we did not digitize or refit the full curves or replicate data, and these values are not outputs of the occupancy calculation [2].

2.3 Reduced apparent-affinity occupancy model

We represented the switch with four statistical states: a closed state with weight 1; an open, externally unbound state with weight L; an open glucose-bound state with weight $L[G]/K_{d,G}^{\text{intrinsic}}$; and an open PaG-bound state with weight $L[P]/K_{d,P}^{\text{intrinsic}}$. Here L is the open-empty to closed-state equilibrium weight, and $[G]$ and $[P]$ denote free glucose and PaG concentrations. The partition function is

$$Z = 1 + L\left(1 + \frac{[G]}{K_{d,G}^{\text{intrinsic}}} + \frac{[P]}{K_{d,P}^{\text{intrinsic}}}\right). \tag{1}$$

The reported apparent dissociation constants do not separately identify L and the intrinsic dissociation constants. After combining the closed and open-empty states and expressing ligand terms

with the apparent constants, the reduced weights are

$$w_G = \frac{[G]}{K_{d,G}^{\mathrm{app}}}, \qquad w_P = \frac{[P]}{K_{d,P}^{\mathrm{app}}}, \qquad D = 1 + w_G + w_P. \tag{2}$$

The model assigns fractions $1/D$, w_G/D, and w_P/D to the combined externally unbound, glucose-bound, and PaG-bound categories, respectively. The PaG share among ligand-bound states is $w_P/(w_G + w_P)$ when at least one ligand is present.

This reduction assumes a single external binding site, mutually exclusive glucose and PaG occupancy of open states, the same conformational linkage for both ligands, and concentrations equal to free equilibrium concentrations. It assigns no receptor-activation efficacy to any state. We show free-PaG fractions of 0.1, 0.5, and 1.0 as stress scenarios applied to the 70 μmol L^{-1} total exposure anchor; these fractions are not estimated probabilities or measurements.

For an illustrative PaG share cap q among ligand-bound states, algebraic rearrangement gives

$$K_{d,P}^{\mathrm{app}} \geq \frac{[P]K_{d,G}^{\mathrm{app}}}{[G]} \frac{1-q}{q}. \tag{3}$$

We report a 5% example only to show the scale implied by a chosen value of q. The value is not an evidence-based therapeutic goal, efficacy requirement, or safety threshold.

2.4 Fit-error sensitivity and structural readiness

As a limited input-error sensitivity analysis, we independently sampled the two reported apparent-K_d fit estimates from Gaussian distributions with the reported means and standard errors (250,000 draws). The resulting central 95% simulation ranges describe only this stated calculation. They omit biological variability, fit covariance, uncertainty in free PaG, uncertainty in the exposure anchor, and uncertainty in the model assumptions; they are not biological confidence intervals.

For the structural-readiness review, a source-derived 507-heavy-atom graph was compared with project-corrected candidate files derived from the publisher-supplied model-built open and closed NNC2215 structures [4]. The graph includes 398 insulin and 109 conjugate heavy atoms. Mapping counts describe matches under the declared graph and coordinate rules; they do not establish a unique atom assignment, complete bond-order or microstate inventory, or validated force field. We recorded target molecular dynamics as not ready unless the full conjugate chemistry and parameters pass independent review.

3 Results

3.1 Published glucose-response arithmetic is reproducible

The paired source-data ratios averaged 3.1576 ± 0.2786 for 20 versus 3 mmol L^{-1} glucose and 12.4720 ± 1.6985 for 20 versus 0 mmol L^{-1} glucose (mean ± sample SD, three independent experiments). Both round to the 3.2-fold and 12.5-fold changes reported in the original article [1]. The source-data reproduction confirms the arithmetic for those published measurements; it does not provide independent experimental validation.

3.2 Conditional PaG occupancy depends on unmeasured free concentration

At the 70 μmol L^{-1} total PaG anchor and 3 mmol L^{-1} glucose, treating all PaG as free gave a conditional PaG-bound fraction of 13.81% and a PaG share of 20.77% among ligand-bound states. At zero glucose, the same assumption gave a PaG-bound fraction of 29.17%. The 5% share example at 3 mmol L^{-1} glucose required an apparent PaG K_d of 0.8468 mmol L^{-1}, about 4.98 times weaker

PaG binding than the reported 0.17 mmol L^{-1}. These are calculations from the assumed model, not measured occupancy or a molecule-level specification.

Table 1 shows how the result changes when only a fraction of the exposure anchor is treated as free PaG. The model's 5% example scales with this assumed free concentration. Because the free fraction is unmeasured, the table is a sensitivity display, not a probability distribution over likely occupancy.

Table 1: Conditional calculations at 3 mmol L^{-1} glucose using the 70 μmol L^{-1} total PaG exposure anchor. The model treats the assumed free concentration as an equilibrium concentration. The 5% share example is arbitrary and is not a therapeutic or safety criterion.

Assumed free fraction	Free PaG (μmol L^{-1})	PaG-bound states (%)	PaG share of bound states (%)	Apparent $K_{d,P}$ for $q = 5\%$ (mmol L^{-1})
0.10	7.0	1.58	2.55	0.0847
0.50	35.0	7.42	11.59	0.4234
1.00	70.0	13.81	20.77	0.8468

When the reported apparent-K_d fit standard errors were sampled independently under the stated Gaussian assumption, the all-free, 70 μmol L^{-1} scenario at 3 mmol L^{-1} glucose gave central 95% simulation ranges of 12.35–15.50% for PaG-bound states and 18.36–23.46% for PaG's share among ligand-bound states. These ranges exclude the larger uncertainties listed in Methods. They should not be interpreted as biological confidence intervals.

The 2026 paper includes a partial matched hIR-A receptor-binding relative-affinity series across its tested glucose levels with PaG absent or fixed at 70 μmol L^{-1} (N=3) [2]. We report its zero-glucose values (0.9% without PaG and 5.4% with PaG) without digitizing or refitting the remaining curves. These binding measurements do not directly measure ligand co-occupancy, and the occupancy equations do not predict them. The published series also does not span a PaG concentration range, so it cannot by itself identify the broader occupancy and receptor-binding response surface considered here.

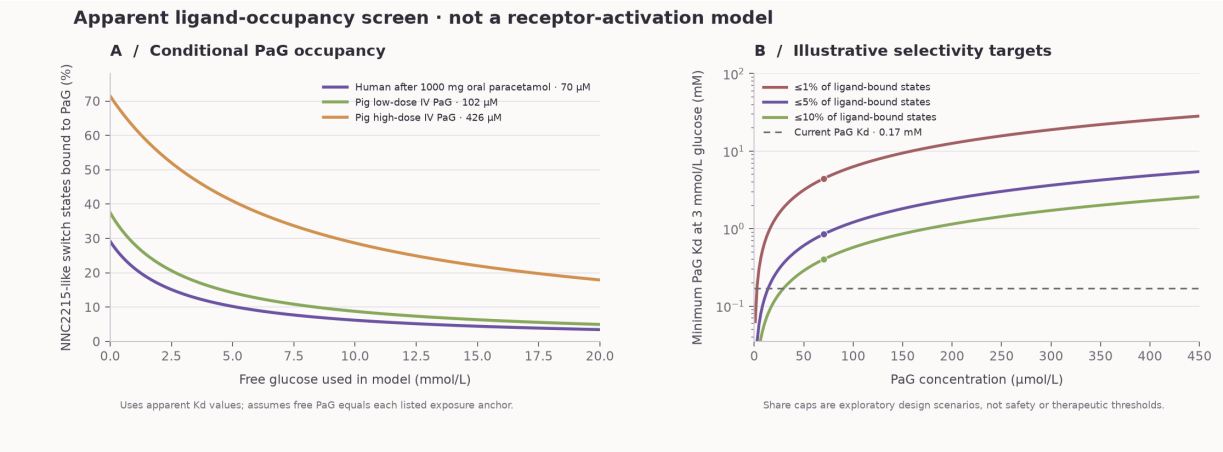


Figure 1: Conditional apparent-affinity occupancy and illustrative selectivity calculations. Panel A varies glucose at fixed listed PaG exposure anchors. Panel B displays the apparent PaG K_d needed to satisfy chosen shares among ligand-bound states at 3 mmol L^{-1} glucose. These curves follow the stated reduced model and use exposure anchors as free concentrations; they do not predict receptor activation, efficacy, safety, or clinical outcomes.

3.3 Coordinate coverage does not establish simulation readiness

The source-derived graph contains 507 heavy atoms and maps to both corrected model-built coordinate candidates under the audit rules. Each state retains one B1 mapping and 16 B29 mapping

alternatives. This is coordinate coverage, not a unique chemical assignment. Two parameterization records describe distinct B29 fragment boundaries: a 92-heavy-atom, net-−3 isolated conjugate fragment with a neutral amino cap, and a 118-heavy-atom, net-−4 capped B27–B29 local-chain context that includes the desB30 terminal carboxylate. The latter is not a full insulin or full-conjugate model. A 256-case matrix (16 coordinate mappings by 16 localized oxygen-label variants) completed parameter-generation tools, but every case remains marked for parameter review; recurring improper-term fallbacks require expert assessment. The unresolved mapping alternatives, hydrogen ownership, full protonation and charge inventory, and cross-boundary parameters remain open. The coordinate files are model-built candidates rather than experimentally resolved NNC2215 structures. The project has not validated a full-conjugate force field, assembled a target OpenMM system, run target molecular dynamics, or estimated target affinity. We therefore report no simulation-based binding or design result.

4 Discussion

This analysis separates three kinds of evidence. First, arithmetic over the publisher's 2024 source data reproduces the rounded glucose-dependent receptor-affinity ratios. Second, the reported 2026 apparent affinities can be placed in a simple equilibrium expression to show how occupancy would behave under explicit competition and free-concentration assumptions. Third, structural coverage and parameterization status determine whether a target simulation is presently defensible. These evidence types answer different questions and should not be collapsed into a claim that a new insulin has been designed.

The occupancy reduction is intentionally transparent but not validated. Its apparent-K_d inputs were measured by native mass spectrometry, while the receptor-binding experiment used a buffer containing 1.5% HSA. The paper's 70 μmol L^{-1} human exposure value is an estimated mean maximum after oral paracetamol, not a measured unbound concentration in either assay. Albumin binding, ligand depletion, non-ideal activities, distinct ligand efficacy, and higher-order allostery are absent from the model. The fit standard errors also do not represent animal- or assay-level biological variability. As a result, the numerical output is best read as a conditional sensitivity calculation.

The model also cannot convert occupancy to receptor affinity or insulin activity. The reported PaG-associated increase in hIR-A relative affinity is evidence from the published receptor-binding assay, not a value derived here. The 2026 study provides a partial receptor-binding series at zero or 70 μmol L^{-1} PaG across glucose levels, while direct mixed-ligand occupancy and a replicated PaG concentration series are not reported. These measurements are needed to test whether glucose and PaG behave as mutually exclusive ligands in the relevant assay context, and whether any occupancy state maps to a measured receptor-binding response.

The structural audit reaches a similarly limited conclusion. Mapping a graph onto model-built coordinates is necessary for molecular simulation, but multiple B29 mappings and unresolved chemical states prevent treating the current files as a validated target topology. The 92- and 118-atom parameterization results are complementary feasibility pilots with different boundaries, not competing versions of a complete insulin model. A separate 16-mapping by 8-neutral-oxygen-label sensitivity audit of the 92-atom fragment found invariant bonded terms and heavy-atom types across the tested set but mapping-dependent partial charges; it did not select an authoritative mapping. The 118-atom calculations do not clear their own parameter-review gate. Independent chemistry and parameter review is the immediate structural gate before attempting even a local OpenMM assembly, and it proceeds separately from the proposed receptor-binding experiment. Short MD cannot repair the missing structural and parameter evidence.

4.1 Next evidence gate

The most informative next experiment for this NNC2215 question is a replicated extension of the existing 0-versus-70 μmol L^{-1} PaG hIR-A receptor-binding series, using a PaG concentration range in one defined assay matrix. It should measure free or otherwise controlled PaG and pair the receptor-binding relative-affinity readout with a direct ligand-occupancy measurement across glucose and PaG concentrations. This would test the occupancy model and estimate a broader binding-response surface. A candidate-specific glucose-response retention criterion and selectivity criterion should be set from assay objectives and biological data before candidate ranking. The current 1%, 5%, and 10% share caps are only algebraic examples and should not be adopted as such criteria.

5 Limitations

This is a secondary analysis of published materials, not a new experimental study. The glucose arithmetic uses three independent published experiments. The PaG binding values are rounded summaries from fit curves; the PaG uncertainty analysis treats reported fit errors as independent and Gaussian. The exposure anchor is estimated and does not specify free PaG. The reduced model assumes competitive occupancy, a shared conformational linkage, and equilibrium. It omits receptor response, pharmacokinetics, albumin binding, ligand depletion, and other allosteric effects. The source structures are model-built; atom mapping, protonation, bond orders, and force-field validation remain incomplete. No new chemical candidate was enumerated or tested, and no target simulation, affinity estimate, dose, clinical efficacy, or safety result is reported.

Data and code availability

The 2024 source workbook is cited below; selected source extracts, derived tables, and audit scripts are available through the project's public research site and GitHub repository. The complete 118-atom 256-case matrix archive remains in the private project repository and is unavailable to readers. A complete, versioned public code-and-data release with a persistent archival DOI is required before journal submission.

Declarations

Author contributions, funding, and competing-interest statements must be completed with all authors before journal submission.

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