

Article

Transition Metal–Metformin Complexes Modulate the Physicochemical Properties of Biguanides and Exhibit Stable Binding at the AMPK ADaM Site

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Abstract

Coordinating metformin to transition metals is a strategy to modulate the pharmacokinetic and pharmacodynamic profile of biguanide antidiabetics while preserving their favorable safety record. Here, we evaluated four previously synthesized and structurally characterized metformin complexes: $[\text{Co}(\text{Met})_2(\text{Met}^-)]\text{Cl}_2 \cdot 2\text{H}_2\text{O}$, $[\text{Ni}(\text{Met})(\text{Met}^-)]\text{Cl} \cdot \text{H}_2\text{O}$, $[\text{Cu}(\text{Met})_2]\text{Cl}_2 \cdot \text{H}_2\text{O}$ and $[\text{Zn}(\text{MetH}^+)\text{Cl}_3]$ (Co-met, Ni-met, Cu-met and Zn-met), as candidate allosteric modulators of the allosteric drug and metabolite site (ADaM) of AMP-activated protein kinase (AMPK; PDB 6C9F), benchmarked against metformin, phenformin and buformin. DFT-derived solvation free energies ($\omega\text{B97X-D/def2-TZVP/def2-SVP}$, SMD), evaluated for the cationic complexes as their neutral chloride ion pairs, placed them above the free biguanides in lipophilicity ($\log P$ 5.17, 3.61 and 2.93 for the cobalt, nickel and copper compounds against 0.71–1.58 for the biguanides), whereas the neutral zinc complex showed no preference between phases. Docking at the ADaM site, validated by redocking the co-crystallized activator R734 to 0.50 Å RMSD ($-11.21 \text{ kcal mol}^{-1}$), ranked phenformin highest among the test compounds ($-7.87 \text{ kcal mol}^{-1}$), followed by Ni-met (-7.10), Cu-met (-7.09) and Co-met (-6.61). Across three independent 200 ns replicas per system (600 ns of aggregated sampling), the Co-, Ni- and Cu-met complexes remained bound to the ADaM pocket in every frame (ligand RMSD 4.2, 5.0 and 6.3 Å) and Zn-met for 88% of the sampling, as did the reference activator R734, which showed the narrowest range of the series (3.3 ± 1.3 Å). These results identified the metformin–transition metal complexes, particularly Co-met, Ni-met and Cu-met, as stable AMPK ADaM-site binders and promising candidates for experimental evaluation as antidiabetic agents.



Academic Editor: Dušan Dimić

Received: 30 July 2026

Revised: 17 September 2026

Accepted: 20 September 2026

Published: 4 October 2026

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Keywords: metallo-drug; biguanides; allosteric modulator; antidiabetic; molecular docking; AMP-activated protein kinase

1. Introduction

Metabolic syndrome (visceral obesity, dyslipidemia, hyperglycemia, and hypertension) is a major public health concern worldwide. Metabolic syndrome, also known as insulin resistance syndrome, is associated with various conditions such as type 2 diabetes and glucose intolerance; both are risk factors for cardiovascular disease [1]. Obesity is one of the most prominent risk factors for type 2 diabetes, along with genetic predisposition, age, and sedentary lifestyle, because a progressive loss of the body's ability to make insulin generates a poor response of the cells to insulin [2]. Biguanide family compounds like metformin (Met), buformin (Buf), and phenformin (Phen), used as first-line drugs for the treatment of type 2 diabetes, have been established as pharmacological agents to manage these metabolic syndromes and their disease derivatives [3]. This biguanide family is known for possessing a wide range of unique biological features, including, among others, antibacterial, anticancer, and cardioprotective effects [3,4]. According to studies, their mode of action enables them to not only lower blood levels of low-density lipoprotein (LDL) cholesterol, but also assist in lowering body weight [3].

The anti-hyperglycemic mechanism of action of biguanides occurs through the reduction in hepatic glucose production. These molecules have been demonstrated to inhibit complex I (NADH dehydrogenase), an enzyme that oxidizes NADH to capture its electrons and move them along the electron transport chain for oxidative phosphorylation, blocking the mitochondrial respiratory chain complex, increasing the AMP/ATP ratio, therefore stimulating the AMP-activated protein kinase (AMPK) pathway in hepatocytes [5], a feature associated with the inhibition of gluconeogenesis and lipogenesis [4,6,7]. Complex I would be a suitable pharmacological target for investigating the modulation of biguanides on metabolic pathways; however, rationally designing compounds with a higher affinity for this complex could lead to catastrophic toxic effects [8]. AMPK is crucial for many metabolic activities, and its activation promotes ATP-generating catabolic pathways, restricting gluconeogenesis by reducing lipid-induced insulin resistance since the lipogenic pathway is downregulated, thereby allowing for insulin to lower hepatic glucose production more effectively [9]. AMPK also inhibits acetyl-CoA carboxylase via phosphorylation, decreasing the synthesis of malonyl-CoA, a natural inhibitor of carnitine palmitoyl transferase I [10,11], which is an enzyme involved in fatty acid catabolism, profiling metformin as an anti-obesity drug. Furthermore, AMPK also phosphorylates 1-phosphatidylinositol 3-phosphate 5-kinase (PIKfyve) [12], a phosphoinositide phosphate kinase, as well as phospholipase D1 [13], which activates ERK, and this further promotes GLUT4 translocation to the plasma membrane, contributing to the reduction of hyperglycemia.

Metformin has been reported to interact directly with the γ subunit of AMPK. This binding may promote AMPK phosphorylation by enhancing its susceptibility to upstream AMPK kinases or protecting it from dephosphorylation by protein phosphatase 2C (PP2C) [14]. Furthermore, the γ subunit specifically appears to play a critical role in both metformin binding and subsequent AMPK activation [15].

Although these molecules show good activity, phenformin and buformin were discontinued in the majority of countries due to an increased risk of lactic acidosis, and only metformin is widely accepted for use as a glucose-lowering agent in the management of type 2 diabetes [3]. In this sense, one strategy to improve biological activity by reducing negative effects is the formation of metallo-drugs [16]. Although some cell metallic micronutrients can become toxic at high concentrations and chronic exposure [17], such as Ni, whose LC_{50} in human cells is around 200 mg L^{-1} [18], in the last 30 years, various vanadium species (IV and V) or their coordination compounds have exhibited insulin-mimetic or antidiabetic effects both in vivo and in vitro [19]. Additionally, several studies have demonstrated the influence of Zn(II) and Cr(III) ions in the potentiation of insulin action

and the levelling of blood glucose concentrations [20–22]. Furthermore, the oral administration of cobalt in diabetic rats has been reported to significantly reduce hyperglycemia levels [23]. On the other hand, cobalt(II), nickel(II), and copper(II) complexes containing metformin and ethylenediamine exhibited antidiabetic activity in vivo [24].

Considering that biguanides are compounds that, in their chemical structure, have functional groups composed of nitrogen which act as hydrogen and electron donor centers (Figure 1), they are excellent candidates to interact with different metallic centers to form new metallo-drugs [4,25]. With this premise, in our research group, we carried out the design, synthesis, and characterization of four metallo-drug complexes from metformin hydrochloride with the metallic centers of Cu(II), Ni(II), Zn(II) and cobalt [26]. The comparison between the solid-state structure and calculated optimized structure results in Co–N distances belonging to low-spin Co(III) species, $[\text{Co}(\text{Met})_2(\text{Met}^-)]\text{Cl}_2 \cdot 2\text{H}_2\text{O}$, in which one biguanide is deprotonated at its bridging nitrogen. These compounds were structurally characterized and their effect on cell viability was assessed by the MTT assay in C2C12 myoblasts and HepG2 hepatocarcinoma cells [26]. The response was concentration dependent, and below $62.5 \mu\text{g mL}^{-1}$ (113–169 μM) the three complexes assayed retained more than 80% viability in both lines; Zn-met was not included. That window lies more than an order of magnitude above the plasma levels reached by metformin in therapy ($\approx 10\text{--}40 \mu\text{M}$), so the complexes are well tolerated over the relevant range, although they are not devoid of cytotoxicity at the highest concentrations tested. Assay conditions and the complete dataset are given in Section S1 and Table S1 of the Supplementary Material.

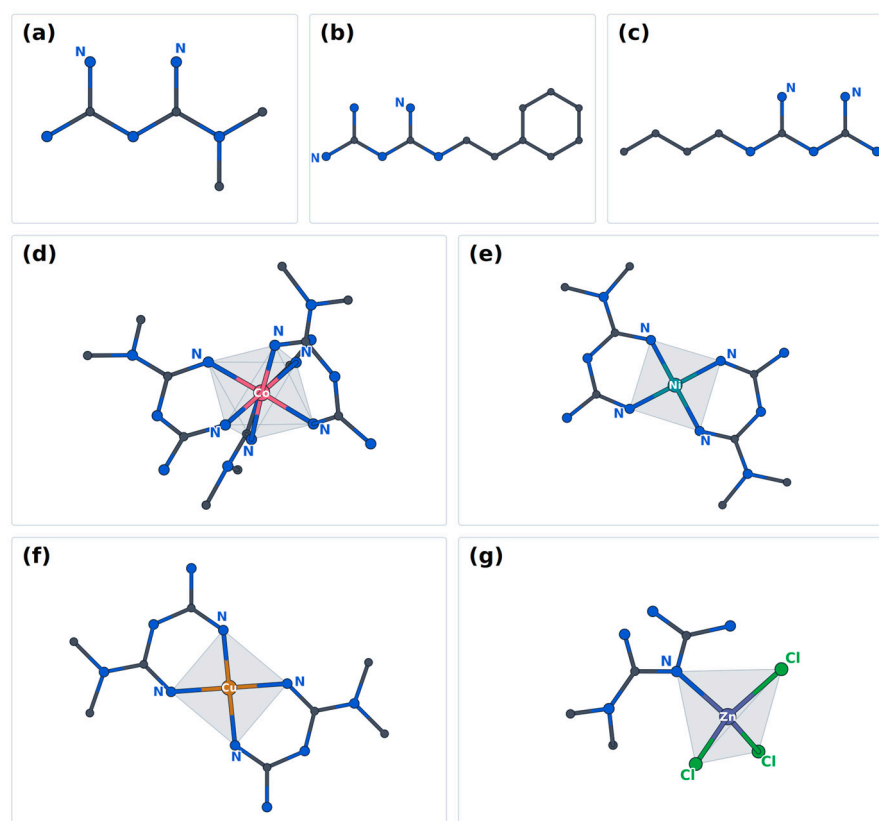


Figure 1. Free biguanides and the coordination spheres of the four complexes. (a) Metformin; (b) phenformin; (c) buformin; (d) $[\text{Co}(\text{Met})_2(\text{Met}^-)]\text{Cl}_2 \cdot 2\text{H}_2\text{O}$, octahedral CoN_6 ; (e) $[\text{Ni}(\text{Met})(\text{Met}^-)]\text{Cl} \cdot \text{H}_2\text{O}$, square-planar NiN_4 ; (f) $[\text{Cu}(\text{Met})_2]\text{Cl}_2 \cdot \text{H}_2\text{O}$, square-planar CuN_4 ; (g) $[\text{Zn}(\text{MetH}^+)\text{Cl}_3]$, tetrahedral ZnNCl_3 . Panels (d–g) are drawn from the crystal structures of ref. [26]. Nitrogen blue, chlorine green, carbon grey; hydrogens, counter ions and lattice water omitted.

In the present work, we extended the characterization of these complexes by performing a predictive physicochemical, pharmacokinetic, and toxicological study. This evaluation aims to assess the potential of these biguanide complexes (Cu-met, Ni-met, Co-met, and Zn-met) as antidiabetic agents relative to conventional therapeutics (Met, Phen, and Buf). Ultimately, we sought to elucidate their interactions with the key metabolic regulator AMPK using computational approaches, specifically molecular docking and molecular dynamics (MD) simulations.

2. Results and Discussion

2.1. Physicochemical and Pharmacokinetics Analysis

The four complexes were first re-examined at the level of theory used throughout, taking the single-crystal metal–ligand distances as an external criterion. Ni-met and Cu-met reproduce them to within 0.05 Å (1.881 against 1.852 Å, and 1.981 against 1.936 Å). For the cobalt complex three electronic states were compared: low- and high-spin Co(II) give mean Co–N distances of 2.065 and 2.137 Å against 1.907 Å in the crystal, whereas low-spin Co(III) gives 1.930 Å. From the perspective of structural optimization, the data are consistent with the diamagnetic Co(III) species $[\text{Co}(\text{Met})_2(\text{Met}^-)]\text{Cl}_2 \cdot 2\text{H}_2\text{O}$, and the nickel singlet is likewise favored over the triplet, by the Ni–N distance (+0.029 against +0.114 Å) and by 1.25 kcal mol^{−1} in free energy. The gas-phase optimization of Zn-met lengthens Zn–N to 2.415 Å; re-optimization in SMD(water) brings the mean deviation over the four Zn–ligand bonds to 0.05 Å, and all reported Zn-met properties refer to that geometry. Every wavefunction is stable and every structure is a minimum, the only exception being a soft torsion of Cu-met of which the computed frequency depends on the integration grid (−1.9, −5.3 and −6.2 cm^{−1} in three calculations whose geometries differ by less than 0.001 Å) and is, therefore, a quadrature artefact.

The partition coefficient (log P) is a critical physicochemical parameter that dictates the pharmacokinetic behavior of drug candidates, directly influencing their intestinal absorption, passive transmembrane permeability, and bioavailability. Because empirical 2D descriptor servers cannot parametrize metal coordination spheres, solvation free energies were computed with the SMD continuum model on $\omega\text{B97X-D}$ geometries, using one common geometry for both solvents so that the transfer free energy is a clean difference (Table 1). Three of the four complexes are cations, and for a charged solute a continuum partition coefficient is dominated by the Born term and by ion pairing that SMD does not describe; their log P was, therefore, evaluated on the neutral chloride ion pair instead.

Table 1. Solvation and transfer free energies (kcal mol^{−1}) and calculated log P. Only neutral species are listed; see text for the cations. ^a Neutral ion pair with the chloride counter ions.

Compound	ΔG_{solv} (Water)	ΔG_{solv} (1-Octanol)	$\Delta G_{\text{tr}}^\circ$	Calculated log P
Metformin	−14.71	−15.68	−0.97	0.71
Buformin	−15.81	−17.22	−1.42	1.04
Phenformin	−17.20	−19.36	−2.16	1.58
$[\text{Zn}(\text{MetH}^+)\text{Cl}_3]$ (Zn-met)	−47.26	−47.25	+0.01	≈0.0
$[\text{Co}(\text{Met})_2(\text{Met}^-)]\text{Cl}_2$ (Co-met) ^a	−44.75	−51.81	−7.06	5.17
$[\text{Ni}(\text{Met})(\text{Met}^-)]\text{Cl}$ (Ni-met) ^a	−33.13	−38.05	−4.93	3.61
$[\text{Cu}(\text{Met})_2]\text{Cl}_2$ (Cu-met) ^a	−49.57	−53.56	−3.99	2.93

The calculated log *P*-values of the free ligands reproduce the expected structure–property relationship, increasing with the hydrocarbon substituent from metformin (0.71)

to buformin (1.04) and phenformin (1.58). That ordering is the one found experimentally: the octanol/water values compiled for the three drugs follow the same sequence, -2.6 for metformin, -1.2 for buformin and -0.83 for phenformin [27]. The calculations therefore reproduce the experimental ranking, but displaced by roughly two to three log units towards higher lipophilicity, a systematic offset inherent to continuum estimates at this level, so the computed values are to be read as relative rather than absolute lipophilicities. Zn-met, the only neutral complex, has a transfer free energy of $+0.01 \text{ kcal mol}^{-1}$ and shows no preference for either phase. The cancellation is not accidental: with a gas-phase dipole of 19.1 D it is the most polar species in the set, so its electrostatic preference for water matches that of the ion pairs, while its molecular surface ($287 \text{ \AA}^2$) is barely half theirs and halves the CDS term that drives partitioning into 1-octanol. Two limitations apply. The value is the difference between two terms of $\pm 7.2 \text{ kcal mol}^{-1}$ and is zero only within the $\sim 1 \text{ kcal mol}^{-1}$ accuracy of the model, so it is better quoted as $\log P = 0.0 \pm 0.7$. More importantly, Zn(II) is kinetically labile, and the three chlorides are inner-sphere: the calculation assumes that $[\text{Zn}(\text{MetH}^+)\text{Cl}_3]$ persists intact in water, an assumption that also underlies the bonded model used in the simulations and that would need experimental verification.

For the three cationic complexes the transfer free energies are mutually inconsistent, and that is itself the relevant observation: Co-met and Cu-met carry the same formal charge and are of comparable size, yet their $\Delta G_{\text{tr}}^\circ$ values of -0.18 and $+4.28 \text{ kcal mol}^{-1}$ would give nominal $\log P$ -values differing by more than three units. Such a spread cannot reflect a real difference in lipophilicity between two closely related biguanide cations. The partition coefficients were therefore obtained from the neutral ion pairs the complexes actually form ($[\text{Co}(\text{Met})_2(\text{Met}^-)]\text{Cl}_2$, $[\text{Ni}(\text{Met})(\text{Met}^-)]\text{Cl}$ and $[\text{Cu}(\text{Met})_2]\text{Cl}_2$), which are the species relevant to an organic phase. In the optimized pairs the chlorides remain outer-sphere, with $\text{N-H}\cdots\text{Cl}$ bridges of $2.11\text{--}2.13 \text{ \AA}$, and the metal–nitrogen distances are unchanged to within $0.005 \text{ \AA}$.

What the calculations establish for the bare cations is that they are strongly hydrated, with $\Delta G_{\text{solv}}(\text{water})$ of -148.1 , -59.7 and $-160.4 \text{ kcal mol}^{-1}$ for Co-met, Ni-met and Cu-met against -14.7 to -17.2 for the neutral biguanides. Their uptake is, therefore, expected to depend on carrier-mediated transport, through the OCT1/2/3 and MATE1/2-K carriers that already govern metformin disposition, rather than on passive diffusion. The neutral ion pairs give internally consistent partition coefficients of 5.17 , 3.61 and 2.93 for the cobalt, nickel and copper compounds, spanning 2.2 log units instead of more than five, and all three above the free biguanides. Two caveats apply to the magnitudes: they carry the same systematic offset as the free ligands, and the CDS term, which scales with molecular surface, contributes 14.6 , 10.6 and $11.6 \text{ kcal mol}^{-1}$ to the three transfer free energies and dominates them. The ordering and the contrast with the free biguanides are more robust than the individual values. Testing the continuum itself, adding five explicit water molecules to Cu-met inside SMD changes $\Delta G_{\text{solv}}(\text{water})$ from -160.4 to $-162.3 \text{ kcal mol}^{-1}$, a difference of 1.2% .

2.2. Molecular Docking Analysis

To evaluate whether the metal complexes can act as direct allosteric activators of AMPK, docking was carried out at the ADaM site of the α/β interface. The search box was first validated by redocking the co-crystallized activator R734: with a $22 \text{ \AA}$ box centered on the centroid of the crystallographic ligand, at $(-18.2, 26.7, -39.9)$, the top-ranked pose reproduces the experimental pose to $0.50 \text{ \AA}$ RMSD and recovers 14 of the 16 residues that lie within $4 \text{ \AA}$ of R734 in the crystal structure (Figure 2a). Only after this control were the seven compounds docked. Each was run three times with independent seeds; the scores differ by no more than $0.02 \text{ kcal mol}^{-1}$ between runs except for R734 (0.22), so the ranking

in Table 2 is not an artefact of the stochastic search. R734 remains the best binder by a wide margin (-11.21 kcal mol $^{-1}$), some 3.3 kcal mol $^{-1}$ ahead of the next compound, which is consistent with its role as a potent direct activator. Metformin, by contrast, is generally held to act on AMPK indirectly, through the cellular AMP:ATP ratio [8], although direct binding to the γ -subunit has also been reported [14]. Among the remaining compounds, phenformin scores highest (-7.87), followed by Ni-met (-7.10), Cu-met (-7.09) and Co-met (-6.61); buformin (-5.82), Zn-met (-4.90) and metformin (-4.82) close the series. The order phenformin > buformin > metformin among the free biguanides follows their lipophilicity (Table 1) and their known relative potency [8], which supports the internal consistency of the calculation.

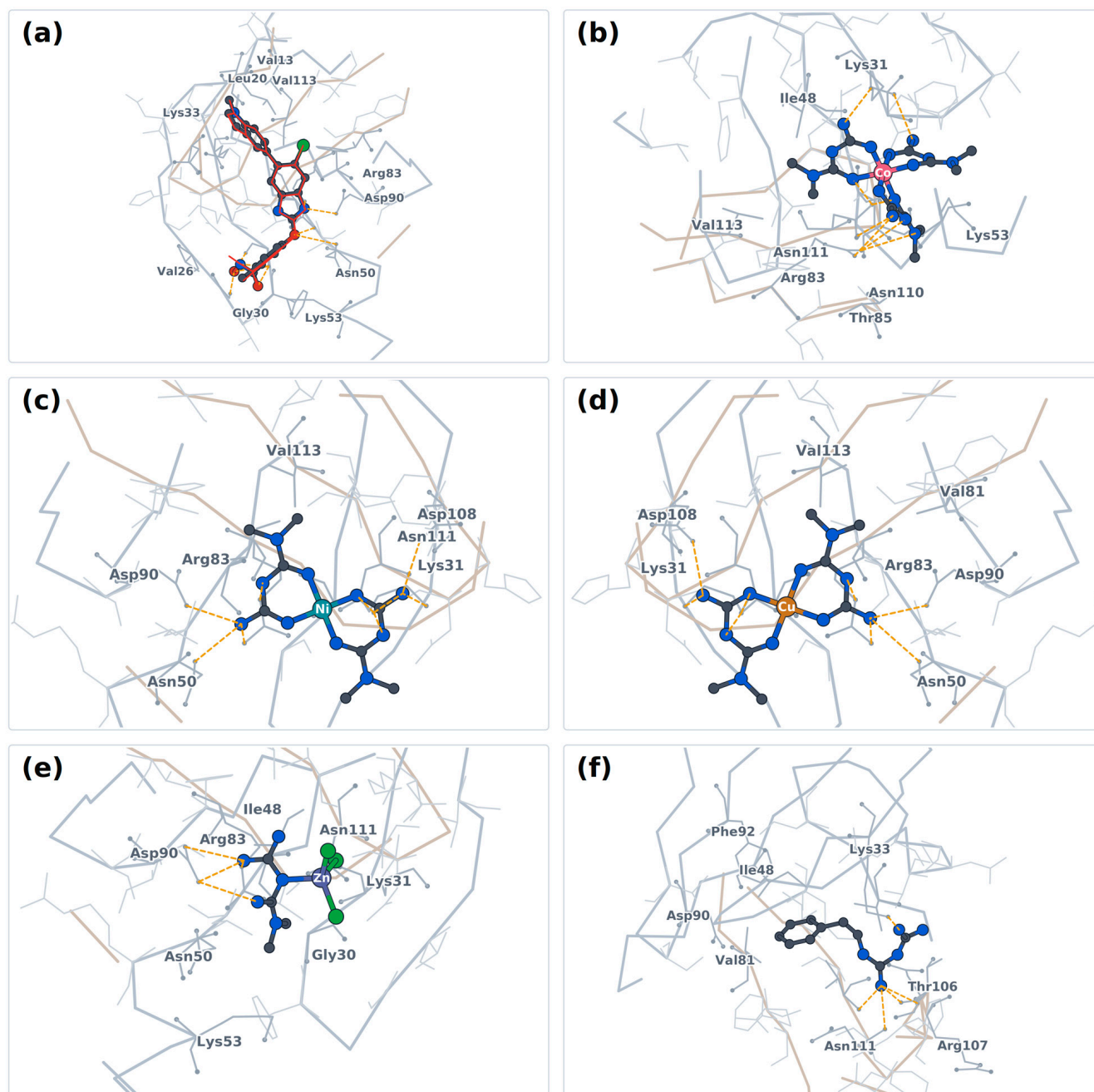


Figure 2. Docked poses at the ADaM site of AMPK (PDB 6C9F). (a) Redocked R734 (ball-and-stick) on its crystallographic pose (red wire-frame), RMSD 0.50 Å; (b) Co-met, (c) Ni-met, (d) Cu-met, (e) Zn-met, and (f) phenformin. α -subunit in cool grey, β -subunit in warm grey; residues within 3.9 Å are labeled and orange dashes mark polar contacts below 3.5 Å. Colors as in Figure 1.

Table 2. Docking scores at the ADaM site of AMPK (PDB 6C9F). Distance is measured between the centroid of the docked pose and that of crystallographic R734; ADaM contacts are those of the 16 reference residues also contacted by the pose.

Compound	Vina Score (kcal mol ^{−1})	SD over 3 Seeds	Distance to the R734 Centroid (Å)	ADaM Contacts Recovered (of 16)
R734 (Reference)	−11.21	0.22	0.17	14
Phenformin	−7.87	0.02	4.94	8
Ni-met	−7.10	0.00	1.78	7
Cu-met	−7.09	0.00	1.84	7
Co-met	−6.61	0.02	3.47	7
Buformin	−5.82	0.01	5.76	7
Zn-met	−4.90	0.00	1.92	8
Metformin	−4.82	0.01	6.26	5

The four complexes occupy the cavity with a common anchoring pattern. Ni-met and Cu-met sit closest to the crystallographic position of R734, with their centroids 1.78 and 1.84 Å away, and both engage Arg83, Asp108 and Val113 of the β -subunit together with Asn50, Asp90 and Lys31 of the α -subunit. Zn-met is equally well centered (1.92 Å) and recovers eight of the reference contacts, the highest count of the series, through its protonated biguanide, while the three chlorides point toward the solvent. Co-met, the bulkiest of the four, is displaced towards the mouth of the cavity (3.47 Å) and contacts Gly30, Ile48, Lys53 and Asn50 of the α -subunit in addition to Arg83. Asn111 of the β -subunit, the residue that recognises the benzimidazole of R734 and of the A-769662 [28] and 991 activators, is contacted by all four complexes.

The free biguanides bind more peripherally. Phenformin inserts its phenethyl group into the hydrophobic part of the cavity, contacting Val13, Leu20, Ile48 and Phe92 of the α -subunit and Val81, Val113 and Phe112 of the β -subunit, which accounts for its score being the best of the three. Buformin reproduces the same pattern with a shorter alkyl chain, and metformin, which has neither an aromatic ring nor the geometric preorganization imposed by a metal center, makes only five of the sixteen reference contacts and sits 6.26 Å from the centroid of R734. Its low score is, therefore, not a failure of the calculation but the expected result for a compound that is not a direct ADaM-site ligand.

Two limitations must be stated. First, the metal complexes were docked as rigid bodies taken from their crystallographic geometries, so their scores are not strictly comparable with those of the flexible free biguanides. Second, and more important, the Vina scoring function contains no explicit electrostatic term, which is precisely the contribution that dominates the interaction of a +1 or +2 species with an acidic protein surface. When the search volume is enlarged to 22 Å, the square-planar Ni-met and Cu-met complexes prefer a second interfacial α/β pocket about 10 Å away, lined by Tyr125, Asp136, Ser138 and Glu139 of the β -subunit, in which the metal center approaches the carboxylate of Glu139 to 2.5 Å; forcing them into the ADaM cavity costs only 1.0–1.2 kcal mol^{−1}. Whether that preference survives an electrostatic treatment must be settled with a scoring function parameterized for metals and with the molecular dynamics described below, and it is the reason why the poses of both sites were carried forward.

Figure 2 shows the docked poses together with the residues that surround them. In all four complexes the metal center remains coordinatively saturated and buried, and the interaction with the protein is made by the peripheral amino and imino groups of the biguanide, which form hydrogen bonds and salt bridges with the polar residues of the

cavity; no direct coordination between the metal and any protein side chain is observed at any point. The highest-ranked pose of each compound was used as the starting structure for the molecular dynamic simulations described in the following section.

2.3. Molecular Dynamics

The dynamic behavior of the eight ligand–AMPK systems was characterized over three independent 200 ns molecular dynamics replicas per system (600 ns of aggregated sampling per ligand) at the ADaM site, monitoring ligand retention (RMSD), protein stability (C α RMSD and radius of gyration, Rg) and per-residue flexibility (RMSF); the binding free energy was estimated by MM-GBSA. Values averaged across the three replicas, with the dispersion between them, are summarized in Table 3, and Figures 3 and 4. The two retention measures in that table are not equivalent: % bound reports occupancy of the cavity, whereas the ligand RMSD is computed without superposition on the reference pose and, therefore, accumulates displacement as well as internal rearrangement.

Table 3. Ligand retention, protein stability and MM-GBSA binding free energy over three independent 200 ns replicas per system (600 ns aggregated). Ligand RMSD is the mean over the three replicas $\pm$ the standard deviation between them, with the maximum reached in any replica; % bound is the fraction of frames with the ligand centroid within 5 Å of the pocket centroid.

System	% Bound	Ligand RMSD (Å)	Max RMSD (Å)	Protein C α RMSD (Å)	Rg (Å)	MM-GBSA ΔG (kcal mol ^{−1})
Co-met	100	4.2 $\pm$ 1.2	5.2	3.7	35.7	−1.9
Ni-met	100	5.0 $\pm$ 1.4	6.3	3.4	35.2	−8.2
Cu-met	100	6.3 $\pm$ 1.8	7.9	6.1	35.8	−10.5
Metformin	99	5.1 $\pm$ 1.6	8.1	4.4	35.8	−19.7
Phenformin	99	3.2 $\pm$ 1.9	6.2	4.8	35.6	−27.2
Zn-met	88	4.3 $\pm$ 1.4	7.2	3.7	35.5	−1.5
Buformin	70	10.3 $\pm$ 6.3	20.6	5.3	36.7	−15.5
R734 (ref.)	100	3.3 $\pm$ 1.3	4.3	5.4	35.1	−14.3

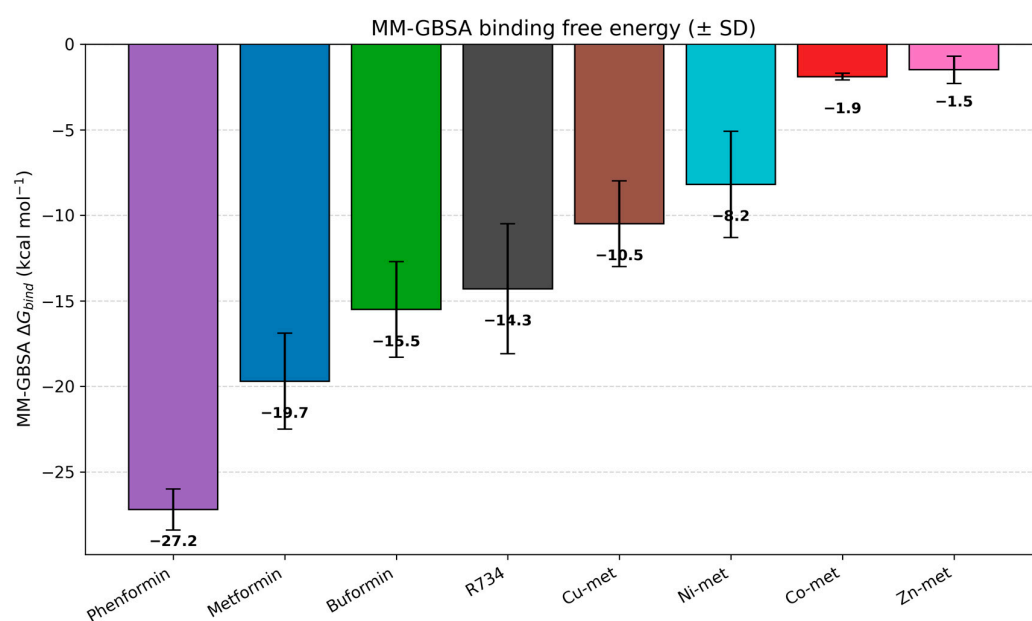


Figure 3. Mean MM-GBSA binding free energy for each system. Error bars are the standard deviation between the three replicas.

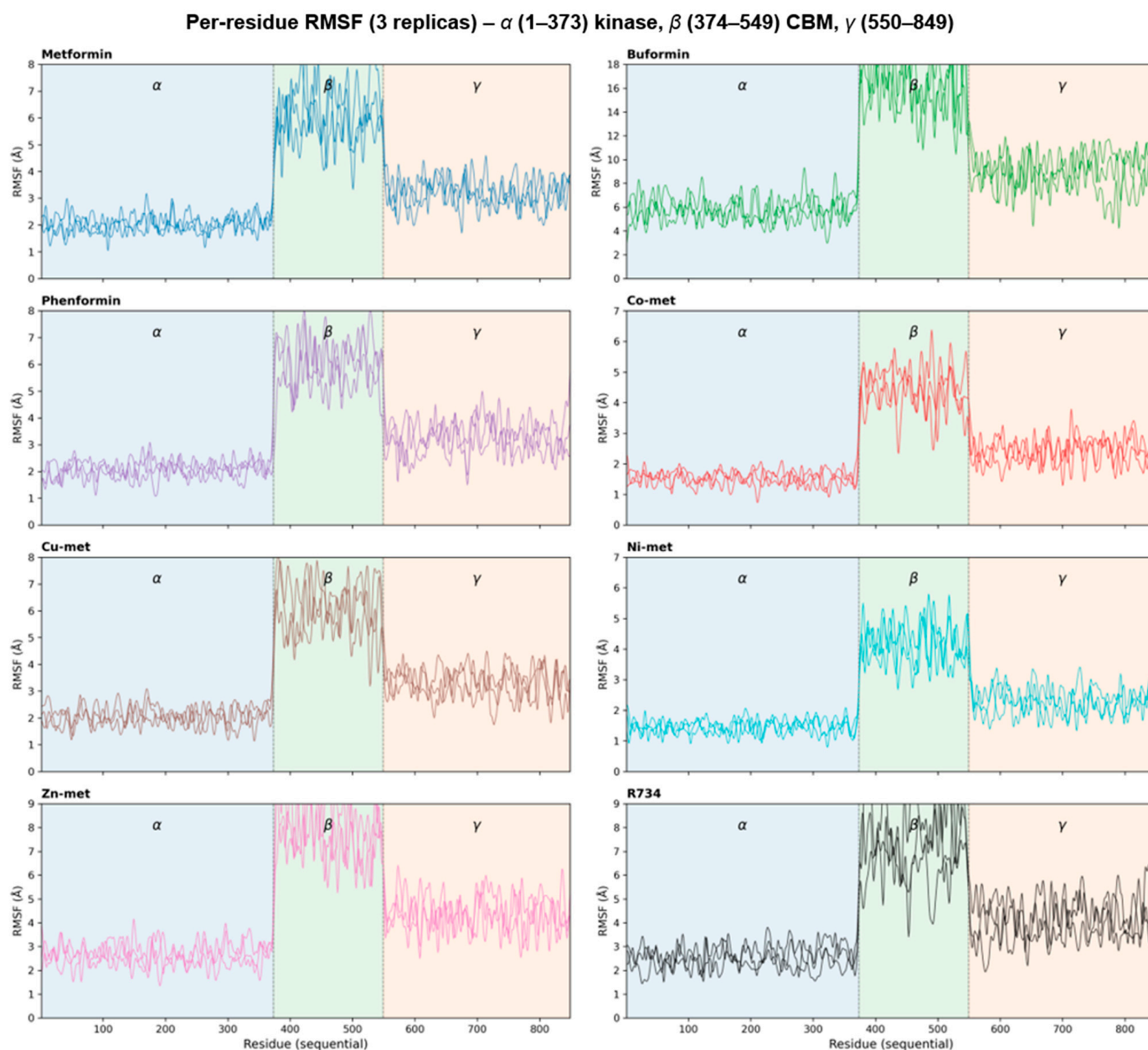


Figure 4. Per-residue RMSF with the α (1–373), β (374–549) and γ (550–849) subunits highlighted.

Because each system contributes three independent replicas, the uncertainty on any mean is dominated by the dispersion between them rather than by the number of frames. With two degrees of freedom the 95% confidence interval on a mean ligand RMSD spans about ± 2.5 standard deviations, so the intervals overlap across the whole series and none of the 28 pairwise comparisons reaches significance (Welch's *t* on the replicate means; the closest, R734 against Cu-met, gives $p = 0.09$). What the replicates establish robustly is, therefore, the retention itself, which is categorical and reproduced in every replica, and not a fine ranking of the systems by mean displacement; the ordering in Table 3 is indicative.

2.4. Ligand Retention

All four metformin–metal complexes remained bound to the ADaM site throughout the three independent simulations: Co-met, Ni-met and Cu-met were retained in every frame (mean ligand RMSD 4.2, 5.0 and 6.3 Å) and Zn-met for 88% of the aggregated trajectory (4.3 Å). The free biguanides metformin and phenformin were equally well retained (99%; 5.1 and 3.2 Å), whereas buformin was only partially retained (70%). The reference activator

R734 remained in the pocket in every replica, with a mean ligand RMSD of 3.3 ± 1.3 Å and a maximum of 4.3 Å, the narrowest range of the series. The coordination complexes, therefore, form stable, long-lived complexes with AMPK over the 600 ns of aggregated trajectory, in agreement with the larger number of interactions observed in the docking analysis.

2.5. Binding Free Energy

The MM-GBSA ranking averaged over the triplicate trajectories placed phenformin (-27.2 kcal mol $^{-1}$) and the free biguanides as the most favorable, followed by R734 and the complexes. For the net-charged complexes (Co $^{2+}$, Cu $^{2+}$, Ni $^{+}$), the electrostatic (EEL) and polar-solvation (EGB) terms are individually large, of opposite sign, and cancel almost completely, which is a well-documented limitation of end-point MM-GB/PBSA methods for charged ligands [29,30], so their absolute ΔG values are only qualitative and likely underestimate the affinity. The strongest evidence for stable binding of the complexes is thus their retention across the three independent replicas rather than the MM-GBSA value; the neutral Zn-met complex, free of this artifact, yields a modest but reliable ΔG (-1.5 kcal mol $^{-1}$).

2.6. Protein Stability and Flexibility

The protein remained folded and compact in every replica ($R_g \approx 35$ Å; C α RMSD plateauing at 3–6 Å), indicating that ligand binding or unbinding did not perturb the global fold. To prevent the artificial inflation of the global structural metrics, an unattached 10-residue capped fragment (residues 478–487) that drifted freely in the simulation box during some replicas was excluded from the RMSD and RMSF calculations. Per-residue RMSF showed that the α catalytic (kinase) domain (residues 1–373) is the most rigid region (RMSF ≈ 2 Å), whereas the β -subunit carbohydrate-binding module (374–549), which forms the ADaM pocket together with the α N-lobe, is the most flexible, consistent with its role in accommodating the ligand. This dynamic flexibility at the α/β interface is essential for locking the catalytic α -domain in an active conformation, thereby facilitating the phosphorylation of downstream substrates such as ACC; the γ -subunit (550–849) shows intermediate flexibility.

3. Computational Details

3.1. Geometry Optimization and Electronic Structure Calculations

The three-dimensional structures of metformin, buformin and phenformin were retrieved in SDF format from PubChem [27]; the initial geometries of the four complexes were taken from their experimental crystal structures. All species were optimized at ω B97X-D/def2-SVP with tight convergence criteria and an ultrafine integration grid, followed by ω B97X-D/def2-TZVP single points for energies and solvation free energies. Optimizations were carried out in the gas phase, except for Zn-met and the neutral ion pairs, for which SMD(water) was applied during the optimization. Harmonic vibrational frequencies were computed for every optimized structure, and the stability of each wavefunction was verified with a stability analysis (stable=opt), including the closed-shell singlets. Three electronic states were optimized for the cobalt complex (high- and low-spin Co(II) and low-spin Co(III)) and two for nickel, and the resulting metal–ligand distances were compared with the single-crystal values (Section 2.1). Partial atomic charges for the docking simulations were derived from Natural Bond Orbital population analysis [31]. All quantum chemical calculations were carried out with Gaussian 16 (Revision C.01) [32].

3.2. Calculation of Pharmacokinetic Parameters and Toxicity Potential

Because empirical cheminformatics servers cannot parametrized transition metal coordination spheres, partition coefficients were obtained from first principles [33]. The

partition coefficient, defined as the ratio of the solute concentration in 1-octanol to that in water, was determined from the solvation free energy (ΔG_{solv}) in the two dielectric environments. Both solvation calculations were performed on the same geometry for each species, so that the transfer free energy is a difference of solvation terms and does not mix in geometric relaxation.

The standard free energy of transfer ($\Delta G_{\text{tr}}^{\circ}$) from the aqueous phase to the organic phase was calculated as the difference in the solvation free energies between 1-octanol and water, according to the following equation:

$$\Delta G_{\text{tr}}^{\circ} = \Delta G_{\text{solv}}(\text{octanol}) - \Delta G_{\text{solv}}(\text{water}) \quad (1)$$

Subsequently, the theoretical octanol/water partition coefficient ($\log P$) was derived using the thermodynamic relationship linking the free energy of transfer to the equilibrium constant at a standard temperature of 298.15 K, as described by

$$\log P = -\frac{\Delta G_{\text{tr}}^{\circ}}{2.303RT} \quad (2)$$

where R is the ideal gas constant ($1.987 \times 10^{-3} \text{ kcal K}^{-1} \text{ mol}^{-1}$) and T is the absolute temperature (298.15 K). Solvation free energies were computed with the SMD model in Gaussian 16 at $\omega\text{B97X-D/def2-TZVP}$ on the def2-SVP geometries [32,34–36], using the external iteration and 1stvac options so that ΔG_{solv} is obtained directly as the gas-to-solution difference, with the non-electrostatic (CDS) term included. For the three cationic complexes the coefficient was evaluated on the neutral ion pair, built by placing the chloride counter-ions at hydrogen bond distance from the most exposed N–H donors on opposite faces of the complex and optimizing the aggregate in $\text{SMD}(\text{water})$. The continuum description was itself tested by recomputing $\Delta G_{\text{solv}}(\text{water})$ for Cu-met with five explicit water molecules included in the optimized aggregate.

3.3. Molecular Docking

Molecular docking was performed on the AMPK heterotrimer (PDB 6C9F, chains A, B and C) for the three free biguanides, the four metal complexes and the co-crystallized activator R734. The receptor was stripped of water molecules and the ligands STU and AMP, the phosphothreonine Thr172 was retained, and hydrogen atoms were added at pH 7.4 with Open Babel 3.1.1 [37]. Flexible ligands were prepared with Meeko and RDKit from the DFT-optimized geometries; the four metal complexes, of which the chelate rings leave essentially no rotatable bonds, were treated as rigid bodies built directly from the molecular fragments extracted from their crystal structures [26]. Docking used AutoDock Vina 1.2.7 [38], of which the scores are not on the same scale as those of AutoDock4 [39]. Specialised metal docking protocols [40] were not required, since the chelate rings leave the complexes without rotatable bonds and they were docked as rigid bodies.

The search space was defined from the crystallographic ligand rather than from a blind-docking prediction. The box was centered on the centroid of R734 in 6C9F, at $(-18.2, 26.7, -39.9) \text{ \AA}$, with an edge of $22 \text{ \AA}$ for the flexible ligands and of $16 \text{ \AA}$ for the metal complexes, so that in the latter case the search was restricted to the cavity itself; the equivalent AutoGrid setting is grid center $-18.2 \text{ } 26.7 \text{ } -39.9$ with $0.375 \text{ \AA}$ spacing and $60 \times 60 \times 60$ or $44 \times 44 \times 44$ points. The definition was validated before any compound was docked, by redocking R734 and comparing the top-ranked pose with the crystallographic one; the protocol was accepted only once that comparison gave an RMSD below $2 \text{ \AA}$. Every compound was then docked three times with independent random seeds (2026, 7 and 101) at an exhaustiveness of 24, keeping the nine best poses of each run, and the dispersion between runs is reported in Table 2 as the standard deviation of the top score. Two descriptors

accompany the score in that table: the distance between the centroid of the docked pose and that of crystallographic R734, and the number of the 16 residues lying within 4 Å of crystallographic R734 that the pose also contacts, taking 4 Å as the contact cut-off. Because the Vina scoring function has no explicit electrostatic term, its absolute values are not comparable with the AutoDock4 scores reported previously and are used here to generate and rank poses within a single series, not to estimate binding affinities. Contacts, distances and figures were computed with in-house Python scripts built on NumPy (version 2.5.0) and SciPy (version 1.18.0).

3.4. Molecular Dynamics Simulation

Molecular dynamics (MD) simulations of the ligand–receptor and complex–receptor systems were performed using the Amber 26 software suite [41]. To ensure high accuracy, a hybrid parametrization strategy was employed. The AMPK protein was modeled using the ff14SB force field [42]. For the free organic ligands (metformin, buformin, and phenformin), parameters were assigned using the General AMBER Force Field version 2 (gaff2), with partial atomic charges derived from the previously described DFT calculations using a restrained electrostatic potential (RESP) fitting scheme [43,44]. To accurately describe the coordination geometry, charge distribution, and electronic environment of the transition metal complexes (Cu-met, Ni-met, Co-met, and Zn-met), custom force field parameters were generated using the Metal Center Parameter Builder (MCPB.py) tool [45]. Bonded and non-bonded parameters for the central metal ions and their primary coordination spheres were explicitly derived from the DFT (ω B97X-D/def2-SVP) optimized structures, whose metal–ligand distances were validated against the single-crystal values of the same compounds (Section 2.1). Because MCPB.py builds a bonded model, whose equilibrium distances and angles are fitted to the DFT Hessian, the coordination sphere fluctuates about that geometry throughout the simulation by construction and cannot rearrange or dissociate; the model also carries fixed charges and therefore does not describe charge transfer or ligand exchange at the metal.

Each docked complex was embedded in a simulation box and solvated using the TIP3P water model, maintaining a minimum buffer distance of 12 Å from the solute to the box edges [46]. The systems were neutralized with counterions and brought to a physiological ionic strength of 0.15 M NaCl. Monovalent ion parameters for the TIP3P water model were taken from the Joung–Cheatham set (frcmod.ionsjc_tip3p) [47]. The simulation protocol began with a rigorous energy minimization process (2000 steps; steepest descent followed by conjugate gradient) to eliminate steric clashes and optimize the solvent geometry. Subsequently, the systems were gradually heated from 0 to 310.15 K over 400 ps under canonical (NVT) conditions using a Langevin thermostat (collision frequency of 2 ps^{-1}), followed by 5 ns of density equilibration in the isothermal–isobaric (NPT) ensemble using a Berendsen barostat to stabilize the system volume and pressure [48].

Production MD was run in the NPT ensemble at 310.15 K and 1 bar as three independent 200 ns replicas per system, differing only in the random seed used to assign the initial velocities (600 ns aggregated per system); coordinates were written every 20 ps, giving 10,000 frames per replica. During all MD steps, the SHAKE algorithm was applied to constrain all bonds involving hydrogen atoms, allowing for a numerical integration time step of 2 fs [49]. Long-range electrostatic interactions were computed using the Particle Mesh Ewald (PME) method with a 10 Å cutoff for short-range (van der Waals and real-space electrostatic) interactions [50]. Trajectory analysis, including the calculation of the Root Mean Square Deviation (RMSD) to determine the structural stability of the complexes over the production replicas, as well as hydrogen bond persistence and interatomic distances, was performed using the cpptraj module of AmberTools (version 26) [51].

Post-simulation trajectory analysis was performed to evaluate the structural dynamics, conformational changes, and binding thermodynamics of the AMPK–complex systems. The Root Mean Square Deviation (RMSD), Root Mean Square Fluctuation (RMSF), and Radius of Gyration (Rg) were calculated using the cpptraj module of AmberTools to assess overall system stability, local residue flexibility, particularly at the active site and the activation loop (Thr-172), and the structural compactness of the complexes over the production replicas. To identify the most representative conformational states of the AMPK–complex systems, a clustering analysis was performed on the MD trajectories using the k-means algorithm implemented in cpptraj. The structural ensemble was grouped based on the C α RMSD, allowing for the extraction of the dominant structural cluster for subsequent interaction analysis. Uncertainties on the trajectory-derived quantities are reported as the standard deviation between the three replicas; 95% confidence intervals were obtained with Student's *t* for two degrees of freedom, and comparisons between systems with Welch's *t* test on the replicate means.

The binding free energy (ΔG_{bind}) of the free ligands and the transition metal complexes within the ADaM allosteric site was calculated using the Molecular Mechanics Generalized Born Surface Area (MM-GBSA) method with the MMPBSA.py script [52]. The generalized Born model of Onufriev, Bashford and Case (igb = 2) was applied with an external (solvent) dielectric constant of 78.3, an internal dielectric of 1.0, a salt concentration of 0.15 M, a nonpolar surface-tension coefficient (surften) of 0.0072 kcal mol^{−1} Å^{−2}, and a maximum Born-radius cutoff (rgbmax) of 25 Å [53]. The calculations were performed over evenly spaced frames drawn from each of the three production replicas (every fourth frame of the 10,000 recorded per replica; 7500 frames per system), after stripping water molecules and counterions (single-trajectory protocol). Configurational entropy contributions were not included. To quantify the specific allosteric interactions driving the binding affinity, a per-residue free energy decomposition analysis was conducted. This allowed for the identification of the primary thermodynamic contributions, including electrostatic, van der Waals, and polar solvation energies, from the interfacial residues to the overall stabilization of the metal–ligand complexes.

4. Conclusions

The four metformin–transition metal complexes were evaluated *in silico* as potential antidiabetic agents using AMP-activated protein kinase (PDB: 6C9F) as the target. DFT-optimized structures were docked at the allosteric ADaM site and refined by three independent 200 ns molecular dynamics replicas per system (600 ns aggregated) together with MM-GBSA. All four complexes recognized the same α/β interfacial pocket as the reference activator R734, anchoring mainly through hydrogen bonds and salt bridges to Arg83, Asp108 and Asn111 of the β -subunit, and remained stably bound throughout the simulations (100% retention for Co-, Ni-, and Cu-met and 88% for Zn-met), with the catalytic α -domain rigid and the β -carbohydrate-binding module accommodating the ligand. Although the absolute MM-GBSA energies of the charged complexes are not quantitatively reliable, their persistent binding and well-defined interaction networks indicate that coordinating metformin to transition metals affords stable AMPK binders. Together with the low cytotoxicity reported for these compounds below 62.5 $\mu\text{g mL}^{-1}$ (Table S1), these results support the metformin complexes, particularly the kinetically stable Co-, Ni-, and Cu-met species, as promising candidates for further experimental evaluation as allosteric AMPK modulators for the treatment of type 2 diabetes and metabolic syndrome; *in vitro* and *in vivo* assays of AMPK activation are recommended to confirm these predictions.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/inorganics14100259/s1>, Section S1. Cytotoxicity of the metformin–transition-metal complexes. Table S1. MTT cell-viability data reported for the metformin–transition-metal complexes; Section S1b. Consistency between the published docking and the revalidated docking. Table S2. Published poses compared with the revalidated ones. Section S2. DFT-optimized Cartesian coordinates for the free biguanides and the metal complexes. Table S3. Cartesian coordinates for the free buformin ligand ($C_6H_{15}N_5$), $\omega B97X-D/def2-SVP$ (gas phase). Charge +0, multiplicity 1; Table S4. Cartesian coordinates for the cobalt complex, $[Co(Met)_2(Met^-)]^{2+}$ ($C_{12}H_{32}N_{15}Co^{2+}$), $\omega B97X-D/def2-SVP$ (gas phase). Charge +2, multiplicity 1; Table S5. Cartesian coordinates for the copper complex, $[Cu(Met)_2]^{2+}$ ($C_8H_{22}N_{10}Cu^{2+}$), $\omega B97X-D/def2-SVP$ (gas phase). Charge +2, multiplicity 2; Table S6. Cartesian coordinates for the free metformin ligand ($C_4H_{11}N_5$), $\omega B97X-D/def2-SVP$ (gas phase). Charge +0, multiplicity 1; Table S7. Cartesian coordinates for the nickel complex, $[Ni(Met)(Met^-)]^+$ (singlet) ($C_8H_{21}N_{10}Ni^+$), $\omega B97X-D/def2-SVP$ (gas phase). Charge +1, multiplicity 1; Table S8. Cartesian coordinates for the free phenformin ligand ($C_{10}H_{15}N_5$), $\omega B97X-D/def2-SVP$ (gas phase). Charge +0, multiplicity 1; Table S9. Cartesian coordinates for the zinc complex, $[Zn(MetH^+)Cl_3]$ ($C_4H_{12}Cl_3N_5Zn$), $\omega B97X-D/def2-SVP$, SMD(water). Charge +0, multiplicity 1. Table S10. Cartesian coordinates for the cobalt ion pair, $[Co(Met)_2(Met^-)]Cl_2$ ($C_{12}H_{32}Cl_2N_{15}Co$), $\omega B97X-D/def2-SVP$, SMD(water). Charge +0, multiplicity 1; Table S11. Cartesian coordinates for the nickel ion pair, $[Ni(Met)(Met^-)]Cl$ ($C_8H_{21}ClN_{10}Ni$), $\omega B97X-D/def2-SVP$, SMD(water). Charge +0, multiplicity 1; Table S12. Cartesian coordinates for the copper ion pair, $[Cu(Met)_2]Cl_2$ ($C_8H_{22}Cl_2N_{10}Cu$), $\omega B97X-D/def2-SVP$, SMD(water). Charge +0, multiplicity 2; Table S13. Cartesian coordinates for the microsolvated copper complex, $[Cu(Met)_2]^{2+}$ with five explicit water molecules ($C_8H_{32}N_{10}O_5Cu^{2+}$), $\omega B97X-D/def2-SVP$, SMD(water). Charge +2, multiplicity 2; Table S14. Cartesian coordinates for the low-spin Co(II) alternative, $[Co(Met)_3]^{2+}$ (doublet, not retained) ($C_{12}H_{33}N_{15}Co^{2+}$), $\omega B97X-D/def2-SVP$ (gas phase). Charge +2, multiplicity 2; Table S15. Cartesian coordinates for the high-spin Co(II) alternative, $[Co(Met)_3]^{2+}$ (quartet, not retained) ($C_{12}H_{33}N_{15}Co^{2+}$), $\omega B97X-D/def2-SVP$ (gas phase). Charge +2, multiplicity 4; Table S16. Cartesian coordinates for the triplet nickel alternative, $[Ni(Met)(Met^-)]^+$ (not retained) ($C_8H_{21}N_{10}Ni^+$), $\omega B97X-D/def2-SVP$ (gas phase). Charge +1, multiplicity 3.

Author Contributions: Conceptualization, J.O.-G. and R.F.D.; methodology, J.O.-G., R.F.D., Y.L.-M., A.P.-D., Y.A.A.-S., J.A.-T. and A.L.O.; software, J.A.-T. and A.L.O.; validation, J.O.-G., R.F.D., J.A.-T. and A.L.O.; formal analysis, J.O.-G., R.F.D., J.A.-T. and A.L.O.; investigation, A.P.-D. and Y.A.A.-S.; resources, J.O.-G. and R.F.D.; data curation, J.O.-G. and R.F.D.; writing—original draft preparation, J.O.-G., R.F.D., J.A.-T. and A.L.O.; writing—review and editing, J.O.-G. and R.F.D.; visualization, J.A.-T. and A.L.O.; supervision, R.F.D.; project administration, R.F.D.; funding acquisition, J.O.-G. and R.F.D. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Universidad del Cauca, grant number 501100005682. This research has been funded by Dirección General de Investigaciones of Universidad Santiago de Cali under call No DGI-01-2026 and project 445–621121–2807.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Acknowledgments: The authors acknowledge the Dirección General de Investigaciones from Universidad Santiago de Cali for the financial support, project 445–621121–2807. A.P.-D. and R.F.D. acknowledge the project SGR BPIN 2022000100068 for young researchers from Chocó, Valle del Cauca, Cauca and Nariño, alliance USC-Univalle-UAO-ICESI-PUJ. R.F.D. acknowledges Universidad del Cauca (grant number: 501100005682) for project 6740.

Conflicts of Interest: The authors declare no conflicts of interest.

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