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Azine or Hydrazone? The Dilemma in Amidinohydrazones

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Azines belong to an important class of compounds which are found to have several applications in medicinal chemistry. Hydrazones are related and more known compounds which carry many biochemical applications. Hydrazones with appropriate substituent can show azine-hydrazone tautomerism. There are many cases in which azines are wrongly considered as hydrazones. In this article, we report the azine and hydrazone tautomeric energy differences and provide structural details of amidinohydrazones which prefer azine structure rather than the hydrazone structure, an important example being the anti-hypertensive drug - guanabenz. The importance of appropriate tautomeric representation of guanabenz has been established in terms of its molecular interactions with a known enzyme.

Introduction

Hydrazones are characterized by the presence of an imine (C=N) linked to an amino (-NHR) moiety (-C=N-NHR).[1] Azines (also known as 2,3-diazabutadienes) carry two 'C-N' double bonds in conjugation with an 'N-N' linker and prefer to exhibit in (*E/E*) configuration (-C=N-N=C-).[2] In recent years, hydrazones and azines have been explored for various chemical and biological applications.[1-19] Hydrazones[3] and azines[4] serve as synthons for the synthesis of many heterocyclic compounds. Formation of stable complexes with most of the transition metal ions has increased the interest in hydrazone[5] and azine[6] complexes, since it was recognized that many of these complexes may serve as models for biologically important species and were reported to act as enzyme inhibitors. Hydrazone derivatives have been suggested for several pharmacological applications like antimicrobial,[7] antimycobacterial,[8] antimalarial,[9] anticonvulsant,[10] antidepressant,[11] analgesic and anti-inflammatory,[12] anticancer[13] and antiplatelet.[14] Similarly azine derivatives are being studied for antibacterial,[15] antifungal,[15] antifilarial,[16] anticancer,[17] opiate antagonist[18] and molluscacidal[19] activities.

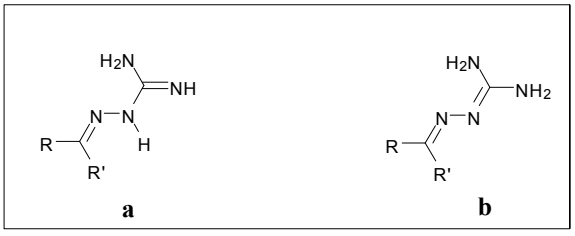

Figure 1. General representations of a) amidinohydrazone and b) 1,1-diamino-2,3-diazabutadiene (azine) tautomer.

Amidinohydrazones (**a**) (also known as 'guanylhydrazones') are a special class of hydrazones containing an amidino group (Figure 1).[20] The amidinohydrazones exhibit several biological activities, for example, Guanabenz (Wy-8678) is an α-2 adrenoreceptor agonist, which is being marketed as an anti-hypertensive agent.[21] Other molecules containing amidinohydrazone moiety such as CNI-1493 (inhibits macrophage activation and subsequent proinflammatory cytokine production),[22] and CGP-48664 (S-adenosylmethionine decarboxylase, SAMdc, inhibitor an anti-proliferative agent),[23] are in clinical trials. Methylglyoxalbisguanylhydrazone (MGBG),[20,24,25] is an agent with a unique mechanism of action of polyamine biosynthesis inhibition (Figure 2). Other pharmacological actions reported by amidinohydrazones are thrombin inhibition,[26] furin inhibition in many bacterial and viral diseases[27] and neurodegenerative disorders.[28] Recently, they have been explored for their use as thermally stable energetic materials.[29]

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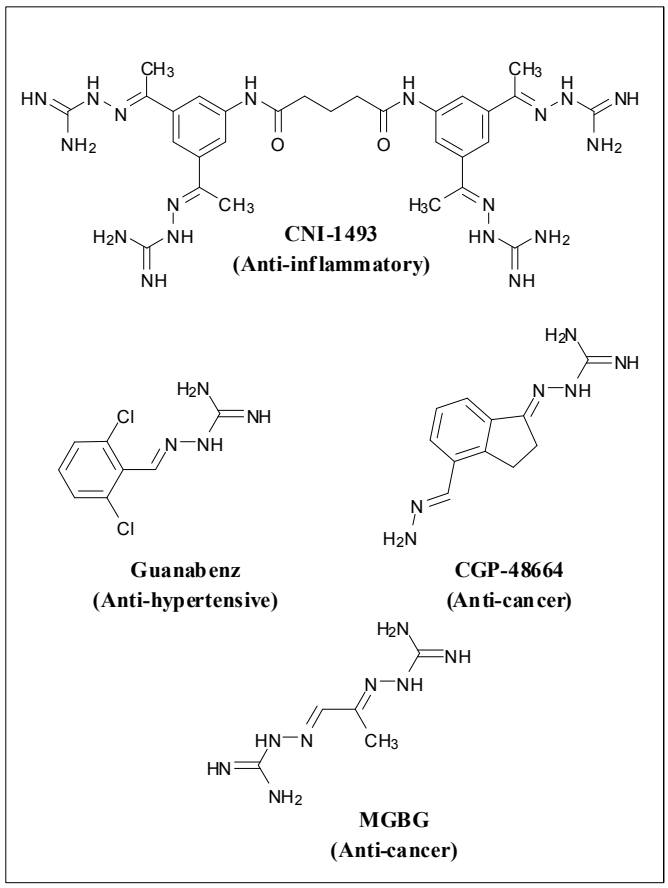

Figure 2. Some important biologically active molecules, containing amidinohydrazone moiety, in their traditional representation.

A notable phenomenon which takes place in drug molecules is tautomerism.[30] Tautomers interconvert with a relatively low activation energy below 20 kcal/mol for the common keto-enol tautomerism.[31] The molecular structure and polarity of solvent define the tautomeric form. pH of the environment is also an important factor.[32] It is important to establish the preferred tautomeric state of drug molecules since drug action can vary as a function of the preferred tautomeric state. Especially when the drug action of any therapeutic agent is being explored using molecular modelling methods, any erroneous representation can lead to undesirable conclusions and expectations. Quantum chemical studies can be used to establish the preferred tautomeric state of any chemical species and to explain the reasons for the same.[31,34] The smaller the ΔE / ΔG between the tautomers, the greater is the possibility of equilibrium between two tautomeric states. The preferred structure of the anti-diabetic agent, metformin and the anti-malarial agent, proguanil, were conclusively established using quantum chemical studies.[33k] Further, design of novel guanyl thiourea derivatives (anti-malarial) was taken up on the basis of the clues obtained, on the tautomeric studies using electronic structure analysis.[33i,35]

Amidinohydrazones are generally represented with the structure **a**. They can also exist in the corresponding tautomeric state **b** as 1,1-diamino-2,3-diazabutadiene (azine) (Figure 1.). In the past, hydrazone-azine tautomerism has been studied and reported (i) in a 14-membered macrocyclic ligand by Bell and co-workers,[36] (ii) in 4-(1-alkylbenzimidazol-2-ylazo)-2-pyrazolin-5-ones by Morkovnik and co-workers[37] and (iii) in aldazines by Silva and co-workers.[38] The crystal structures of glyoxalbisguanylhydrazone (GBG)[39] and its derivatives, EMGBG,[40] MPGBG[41] and PhGBG[42] were indeed established as azines rather than as hydrazones. Similarly, the crystal structure of 2-(1-Phenylethylideneamino)guanidine[43] and guanabenz were established as azines.[21b] Existence of the two tautomeric forms, hydrazone and azine, of amidinohydrazones has been experimentally proven by Zoltan and co-workers.[44] However, in the medicinal chemistry and organic chemistry literature, the title compounds are represented as hydrazones. Especially, molecular docking studies have been performed using the hydrazone structure.[28,45] This leads to a fundamental question on which representation is more suitable (**a** or **b**) for amidinohydrozones? This question is explicitly addressed in this article. Quantum chemical analysis on the azine-hydrazone tautomerism in amidinohydrazones has been carried out and the conditions which influence the tautomeric energy have been explored. Finally, the importance of representing the drug molecule, guanabenz, in the azine form has been evaluated using molecular docking analysis.

Methods of calculation

The quantum chemical calculations were carried out using GAUSSIAN09 package.[46] Full geometry optimizations were carried out using *ab initio* MO methods[47] using MP2[48] and G2MP2[49] methods and DFT calculations[50] using B3LYP,[51] M06[52] and CBS-Q[53] methods. The basis set used was 6-311++G(d,p). Implicit solvent study was performed using B3LYP/6-311++G(d,p) and IEFPCM solvent model.[54] Explicit solvent studies were also carried out in gas-phase using the same method up to four water molecules. Frequencies were computed analytically for all the optimized species to characterize each stationary state as a minimum or a transition state. Gibbs free energy (ΔG) difference between the two tautomers has been considered in all the observations discussed below. The partial atomic charges were estimated using NBO analysis.[55] Electron localization function (ELF)[56] calculations were performed on the most stable tautomers using Multiwfn 3.3.6 software[57] to estimate the total electron density localization at the nitrogen centres. The molecular electrostatic potentials (MESP)[58] were obtained on the B3LYP/6–311++G(d,p) optimized geometries of hydrazone and azine tautomers of amdinohydrazone and superimposed onto a constant electron density (0.004 e/au^3) to provide a measure of the electrostatic potential at roughly the van der Waals surface of the molecules using GAUSSIAN09 software.[46] The colour coded surface provides the location of the positive (deepest blue, most positive) and negative (deepest red, most negative) electrostatic potentials on a molecular surface. The regions of positive potential indicate relative electron deficiency

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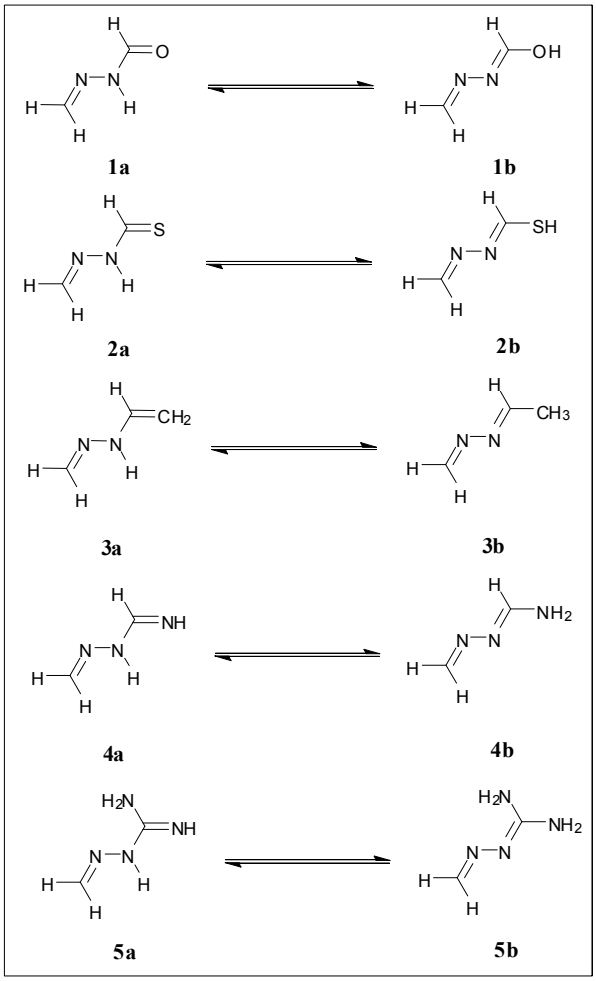
(estimated as a function of the repulsion experienced by a positively charged test probe), and regions of negative potential indicate areas of excess negative charge (estimated as a function of the attractive force experienced by a positively charged test probe). Molecular docking studies were carried out using Glide[59] module of Maestro 9.4 (by Schrodinger Inc.)[60] to demonstrate the importance of tautomeric representation of amidinohydrazone during molecular docking analysis.

Results and Discussions

Quantum chemical calculations were performed on hydrazones (**1a-5a**) and their azine counterparts (**1b-5b**) (Scheme 1). The Gibbs free energy difference between the tautomers was calculated at all levels of theory in the gas-phase and the relative energy of the hydrazone tautomers with respect to the corresponding azine are given in Table 1.

The results show that a prototropic tautomerism in hydrazones, between the sp^3 nitrogen of the hydrazone moeity and the atom (hetero) on the adjacent sp^2 carbon can exist, with azine tautomer being more stable than the hydrazone tautomer in **3a, 4a** (carboxamidrazone) and **5a** (amidinohydrazone). However, in the case of acyl hydrazone (**1a**) and the corresponding thio-analogue (**2a**), the hydrazones are more stable, presumably due to the greater preference of keto groups over the enol groups. In compounds **1**, **2** the tautomerism involves the keto and thioketo functionalities respectively. It is well known that in these species, the keto form is always more preferred.[31-34] On the other hand in acetaldimine, the imine-enamine tautomersism requires ~4 kcal/mol favouring imine tautomer.[33l,33m] Continuing the same in guanidine, the 1,3-hydrogen shift energy difference is zero kcal/mol. In all the substituted guanidine derivatives, the substituent is always found on the iminic nitrogen, instead of the aminic nitrogen.[33k] In compounds **4** and **5** also, the same trend is noticed. In addition to this, the conjugation is playing a role.



Scheme 1. Predicted tautomerism in hydrazones by 1,3-proton shift.

Table 1. The Gibbs Free Energy difference (ΔG) in kcal/mol between hydrazone and azine tautomers with azine structure taken as a base.

Compound	Relative Gibbs Free Energy, ΔG (kcal/mol)				
	MP2	B3LYP	M06	G2MP2	CBS-Q
1a	-7.07	-8.87	-9.31	-6.21	-12.13
2a	-2.01	-5.09	-4.58	-2.48	-5.91
3a	10.88	6.46	6.71	9.69	13.74
4a	5.38	4.30	4.40	4.35	3.96
5a	6.41	4.66	4.19	5.78	6.45

Table 2 shows the ΔG values of a few tautomeric pairs, for example, formamide ⇌ formimidic acid, the tautomeric energy difference is -15.68 kcal/mol (ΔG_1). In **1**, ΔG_2 value is -6.21 kcal/mol. The difference between the two values is 9.47 kcal/mol. If this difference is taken as an indirect measure of stabilization due to conjugation, the gain experienced by **1a** and **1b** is much larger than that of **3** – **5** (see table 2). Even in the presence of such a gain, **1a** and **2a** are the preferred tautomers because of the inherent preference of keto and thioketo groups over their tautomers.[31,33o] In **4a**, the preference for azine structure is of the order 4.35 kcal/mol (G2MP2 based energy calculation) which increases to 5.78 kcal/mol due to the additional NH_2 group in **5a**. All the ΔG values listed in Table 1 are within 10 kcal/mol indicating that all these species can exist in tautomeric equilibrium, in principle. Hydrazone structure is clearly more preferred in case of **1** and **2** whereas azine structure is more preferred in case of **3** to **5**, such a dichotomy is probably responsible for the confusion in the representation of azines as hydrazones. The optimized geometries of **5a** and **5b** are given in Figure 3. Since amidinohydrazones are of medicinal importance, further aspects of tautomerism have been explored on this class of compounds.

Table 2. Free Energy Difference (ΔG, kcal/mol) between tautomer pairs and their comparison so as to estimate the influence of conjugation ($\Delta G_2 - \Delta G_1$), all values are estimated using G2MP2 method.

Tautomer Pair	ΔG_1	Tautomer Pair	ΔG_2	$\Delta G_2 - \Delta G_1$
Formamide ⇌ Formimidic acid	-15.68	**1a ⇌ 1b**	-6.21	9.47
Thioformamide ⇌ Methanimidothioic acid	-9.05	**2a ⇌ 2b**	-2.48	6.57
Acetaldimine ⇌ Vinylamine	+3.90	**3a ⇌ 3b**	+9.69	5.79
Formamidine ⇌ Formamidine	0.00	**4a ⇌ 4b**	+4.35	4.35
Guanidine ⇌ Guanidine	0.00	**5a ⇌ 5b**	+5.78	5.78

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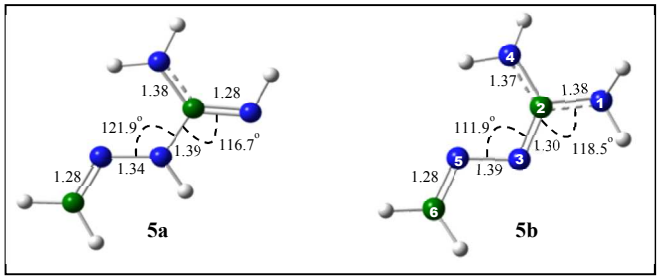


Figure 3. 3-D structures of hydrazone (**5a**) and azine (**5b**) tautomers of amidinohydrazone obtained at B3LYP/6-311++G(d,p) level of quantum chemical optimization (Bond lengths in Å and bond angles in degrees).

Electron distribution in tautomer 5b

5b can show conjugation of electron density unlike **5a** which is the most important reason for the greater stability of **5b** over **5a**. In addition, several second-order delocalization forces stabilize tautomer **5b**, for example, NBO analysis shows that the $n_{N1} \rightarrow \pi^*_{N3\text{-}C2}$ second-order delocalization is very strong (~20.58 kcal/mol) in **5b**. Electron localization function (ELF) analysis showed a bean shaped isosurface at N3 and the population of this basin is 3.19 e signifying electron density accumulation at this center in azine tautomer.

To evaluate the extent of conjugation across the C=N-N=C framework in **5b**, calculations were repeated after freezing the C=N-N=C torsional angle to 90˚. The energy difference between the completely optimized and frozen structure is 8.57 kcal/mol. To further validate, the same calculation was performed using guanabenz and the energy difference obtained is 8.33 kcal/mol. This data clearly establishes that there is a strong conjugation across the C=N-N=C framework of azines, breaking of which requires ~8.5 kcal/mol.

Molecular electrostatic potential (MESP) studies give the details regarding the nuclear and electronic charge distribution on the surface of the molecule which is a result of the force experienced by a positive test probe. The colour contours provide a location of the positive (blue colour) and negative (red colour) electrostatic potentials. MESP calculation for the two tautomers clearly shows the difference in the electron density along the guanidino region wherein electrostatic potential around N1 is partially negative in hydrazone and partially positive in azine and vice-versa at N3 (Figure 4). There is a clear distinction in the surface properties of these two tautomers. Hence, treating the two tautomers individually is important for proper estimation of their interactions with macromolecules.

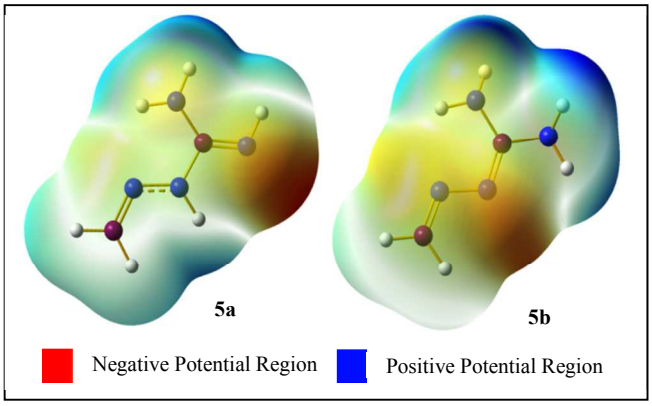


Figure 4. MESP of hydrazone **5a** and azine **5b** tautomers of amidinohydrazone plotted onto a surface of constant electron density (0.004 e/au^3).

Solvent effect

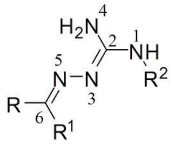
Implicit solvent study shows a gradual decrease in the energy difference between **5a** and **5b** as the dielectric constant increases (Table 3). This decrease is marginal from 4.66 to 3.78 kcal/mol from gas-phase to water medium. This data indicates that under polar solvent conditions, the equilibrium between the two tautomeric states increases. Further, microsolvation using water molecule was studied to examine the extent of difference in energy between the tautomers.

Table 4. Implicit solvent effect on relative Gibbs free energy difference (ΔG) in kcal/mol between **5a** and **5b** with **5b** taken as a base.

Phase	Dielectric Constant (ε)	Relative ΔG (kcal/mol)
Gas	-	4.66
Cyclohexane	2.0	4.21
THF	7.6	3.88
Ethanol	24.5	3.84
Acetonitrile	37.5	3.83
DMSO	46.7	3.87
Water	80.1	3.78

Water molecules were explicitly added to both the systems. In the presence of explicit water molecules, the tautomer energy difference decreased to 3.38 kcal/mol with one water molecule, 2.40 kcal/mol with two water molecules and up to 1.75 kcal/mol with four water molecules (see Supporting Information). This analysis indicates that when the hydrogen atoms and the lone pair electrons of these systems are stabilised by a network of hydrogen bonds in water, the difference

Table 3. Influence of substituents on energy difference (ΔG) in kcal/mol between hydrazone and azine tautomers with azine structure taken as a base.



R	R^1	R^2	Relative Gibbs Free Energy, ΔG (kcal/mol)
H	**H**	**H**	4.66
H	**H**	**$COCH_3$**	2.25
H	**H**	**COPh**	-0.67
H	**H**	**CH_3**	4.12
CH_3	**H**	**H**	5.26
C_2H_5	**H**	**H**	5.11
OH	**H**	**H**	10.76
NH_2	**H**	**H**	8.72
NO_2	**H**	**H**	5.25
CF_3	**H**	**H**	6.37
Cl	**H**	**H**	4.36
F	**H**	**H**	4.68
NH_2	**NH_2**	**H**	9.23
Cl	**Cl**	**H**	6.23
F	**F**	**H**	8.14

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between hydrazone and azine structures become less prominent (in all cases azine tautomer being more stable). This could be one of the reasons why the distinction between the hydrazone and azine tautomers was not explicitly identifiable.

Substituent effect

Influence of various substituents on the energy difference between the two tautomers was estimated in gas-phase using B3LYP/6-311++G(d,p). Various substituents which are of importance in medicinal chemistry are considered in this study. In general, substitution at R group tends to increase the preference for the azine tautomer (Table 4). Substituents which influence the π electron delocalization tend to stabilise the azine tautomer more strongly. The π electron donating groups such as NH_2 and OH, as well as the π electron withdrawing NO_2 group increase the preference for the azine tautomer. On the other hand, substituents at R^2 position tend to decrease the preference for the azine tautomer. The tautomeric energy difference between **5a** and **5b** when substituted with electron-withdrawing groups, COPh and $COCH_3$, at N1 was found to be lower than the unsubstituted form and seems to decrease the preference for the azine form. On the other hand, substituting with an electron-donating group such as CH_3, the energy difference is slightly decreased by a marginal degree compared to the unsubstituted form (Table 4).

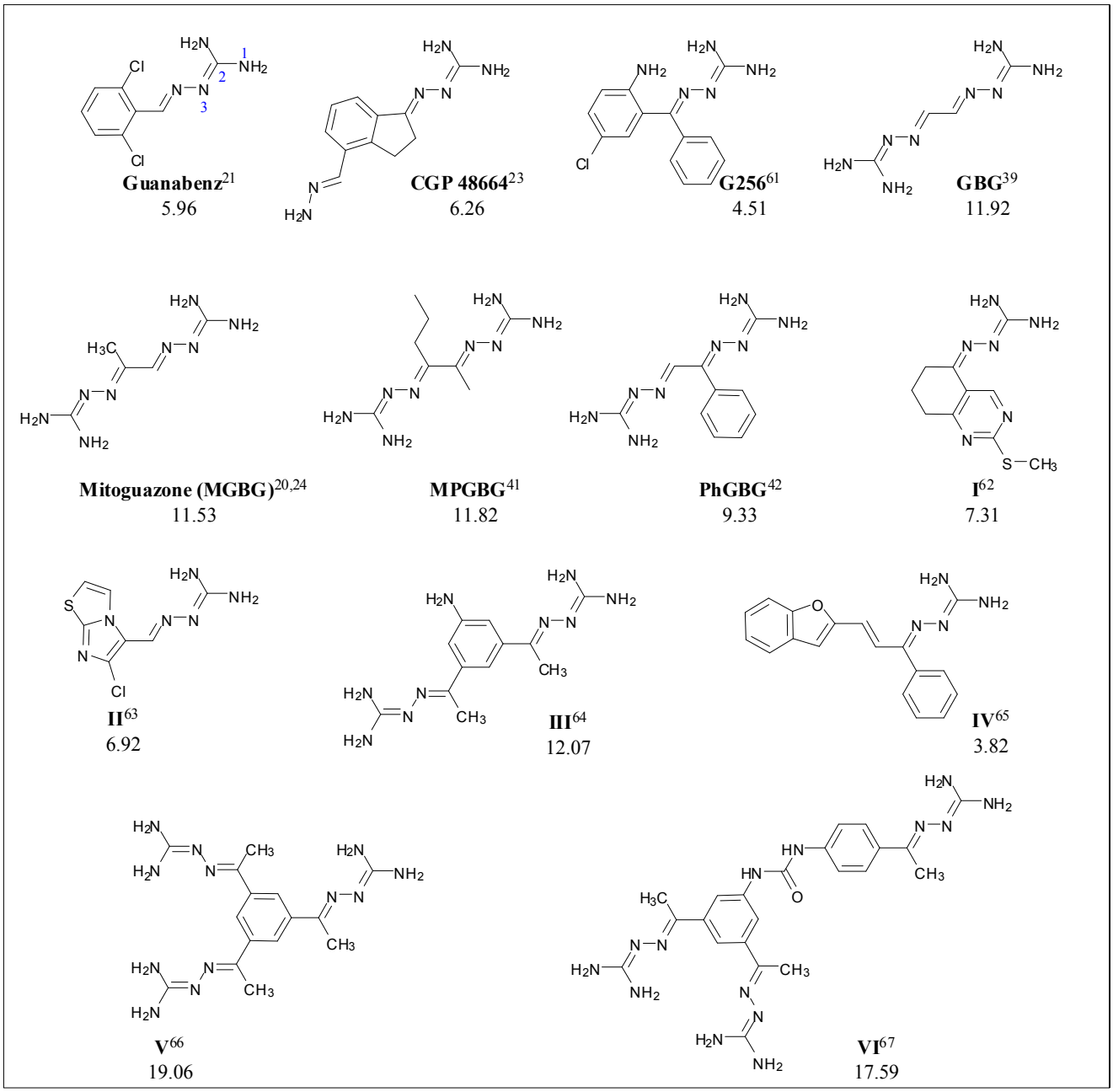

Figure 4. The Gibbs Free Energy difference (ΔG) in kcal/mol (numerical value written under each compound) between hydrazone and azine tautomers of various biologically active molecules, with azine tautomer being more stable than the corresponding hydrazone in each case.

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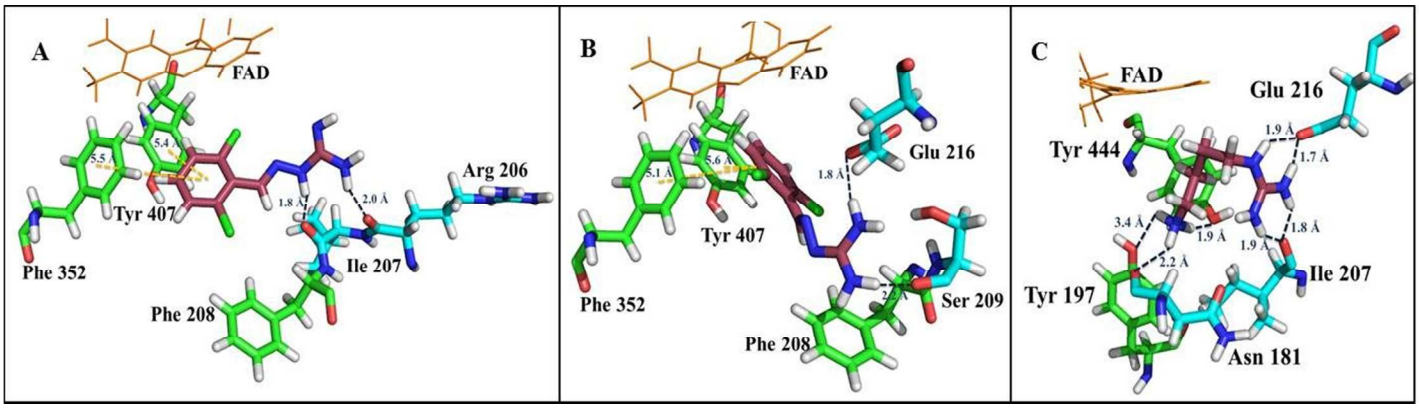

Figure 6. Docking pose of (A) hydrazone tautomer (B) azine tautomer of guanabenz (C) agmatine in the active site (residues marked in green) of MAO-A (2BXS). Hydrogen bond interactions with the nearby amino acids (residues marked in blue) are shown with black dotted lines and hydrophobic interactions shown with yellow dotted lines.

Tautomerism in various biologically active molecules

Several biologically active molecules containing amidinohydrazone group were reported in their 'hydrazone' tautomeric form. Herein, we explored the possible tautomerism in such molecules and calculated the tautomeric energy difference in gas-phase using B3LYP/6-31+G(d) level of quantum chemical calculations. In each case, the azine tautomer is found to be more stable than its corresponding hydrazone form, hence, the structures in Figure 5 are drawn in their azine form. Careful observation of the tautomeric energy differences of these molecules suggests an "additive effect" wherein there is an increase of ~6 kcal/mol for every unit of 1,1-diamino-2,3-diazabutadiene (azine) moiety in the structure of the molecule. For instance, the ΔG of guanabenz (containing a single azine unit) is 5.96 kcal/mol, which increases to 11.53 kcal/mol in MGBG (containing two azine units) and further increases to 17.59 kcal/mol in **VI** (containing three azine units).

Proton Affinity

The drug molecule, guanabenz, is generally supplied as an acetate salt, mainly to improve oral biovailablity. The pK_a of guanabenz is reported to be 8.1,[68] proving it to be basic. To estimate the proton affinity of this class of species, quantum chemical analysis has been carried out. The most preferred site of protonation is N3 of amidinohydrazone moeity in guanabenz with a proton affinity value of 232.70 kcal/mol.

It is important to note that in the protonated state, both hydrazone and azine representations lead to the same structure. This could be an additional reason for ignoring azine versus hydrazone tautomeric preferences in the title compounds. However, considering the large energy difference, especially in the case of **MGBG**, **V** and **VI** (Figure 5), it is advisable to correlate the tautomeric preferences (in neutral state) with the experimental details while exploring the chemistry of these species.

Molecular Docking Analysis

Understanding the tautomerism of drugs is essential in computer aided drug discovery, so as to identify complementary pose of small molecules in the active site of macromolecules. To demonstrate the importance of tautomeric representation of amidinohydrazone, molecular docking analysis was carried out. Docking of guanabenz has been performed in the active site of monoamine oxidase A (MAO-A) which was earlier studied and performed by Ramsay and co-workers.[45d] The active site area is outlined by residues Tyr69, Tyr197, Phe208, Tyr407, Phe352, Tyr444, and the isoalloxazine ring of FAD. Molecular docking experiment has been performed using Glide[59] module implemented in Maestro version 9.4 software package (by Schrodinger)[59] on the crystal structure of MAO-A obtained from Protein Data Bank (PDB ID: 2BXS).[69] The docking protocol followed was as per the one performed by Ramsay and co-workers.[45d] In order to reproduce the reported binding interaction, validation of docking protocol was performed with agmatine on MAO-A active site.[69] The docking pose of agmatine was found to reflect the reported hydrogen bonds with Glu216, Tyr444, Tyr197 and Asn181. The docking pose of both the tautomers of guanabenz are clearly different. In the docked pose, hydrazone tautomer prefers an almost planar arrangement (176°) whereas the azine tautomer undergoes a twist to about 127°. The amino ends of the diaminoazine tautomer formed hydrogen bonds with Ser 209 and Glu 216 unlike the hydrazone tautomer which formed hydrogen bonds between the hydrogen of N5 with Arg 206 and N3 with Ile 207. This provides a clear indication on the importance of tautomerism in drug designing since usage of improper tautomer may conceal certain important interactions with the receptor attributed to its activity. The docking score of the azine tautomer is found to be marginally better (-6.887) in comparison to that of hydrazone tautomer (-6.575). Hence, the pharmacophoric feature varies with each tautomer and in turn affects the binding pattern or interactions with receptor.

In several literature reports of organic and medicinal chemistry, the title compounds were represented as hydrazones,

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presumably due to historic or convenience factor rather than a structural factor. Hence, such a representation potentially misleads the chemistry and surface information since these are different for each tautomer. This information often goes unnoticed by chemists during interpretation of spectroscopic data or in molecular modelling analysis. This needs special attention in all future discussions of this class of compounds.

Conclusions

Quantum chemical calculations have been performed to establish the tautomeric preferences between azine and hydrazone tautomers of titled compounds. The azine tautomers are found to be about 4-6 kcal/mol more stable than the hydrazone tautomers. The electronic structure analysis clearly established the reason for this preference. Substituents and solvent effects influence the azine ⇌ hydrazone tautomerism, mostly preferring the azine tautomeric state. Many of the biologically active molecules have been represented in their hydrazone form which is not appropriate. For example, in guanabenz, the azine form is more stable by 5.96 kcal/mol which showed a better docking score and interactions than its hydrazone form. This study established that azine tautomeric structure is more fundamental and the hydrazone tautomeric structure is the alternative in this class of compounds. The literature gave importance to the hydrazone tautomer and ignored the energetically most preferred azine tautomeric state. The quantum chemical analysis reported in this work suggests that relatively more importance should be given to the azine tautomeric state, especially while carrying out molecular modelling studies.

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Notes and references

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Electronic Supplementary Information (ESI) available: Table S1. Absolute Gibbs free energies (hartrees) in gas-phase of hydrazones and their corresponding azine tautomers mentioned in Scheme 1. Table S2. Absolute Gibbs free energies (hartrees) in implicit solvent phase conditions of hydrazone , **5a** and corresponding azine tautomer, **5b**, of amidinohydrazone. Table S3. Absolute Gibbs free energies (hartrees) in explicit solvent phase conditions of hydrazone , **5a** and corresponding azine tautomer, **5b**, of amidinohydrazone. Table S4. Absolute Gibbs free energies (hartrees) of substituted **5a** and **5b** in gas-phase. Table S5. Absolute Gibbs free energies (hartrees) of various biologically active molecules in gas-phase.
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