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In Silico Toxicological, Anti-Tubercular Effect Evaluation And *In Vitro* Marine Pathogenic Bacteria Inhibition of N-[(3-Chloro-4-Nitro-Phenyl) Methyleneamino]Pyridine-4-Carboxamidine

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ABSTRACT

The hydrazone; N-[(3-chloro-4-nitro-phenyl) methyleneamino] pyridine-4-carboxamidine (**H**) was selected for *in silico* toxicological and *in vitro* bactericidal studies. Toxicological investigation was carried out using software program, such as eMolTox and Gusar, for the toxic substructure determination, and acute rat toxicity prediction respectively. *In vitro* bactericidal effect evaluation was investigated using tow marine pathogenic bacteria; *Vibrio anguillarum* and *Photobacterium damselae*. Computational results determinate toxicophores of (**H**), which are nitro-aromatic part, hydrazine group, and quaternary carbon, were predicted as responsible for Idiosyncratic toxicity metabolic activation, covalent bond with DNA, and hepatotoxicity respectively. In addition, the predicted LD50 of (**H**) are 1086, 244, 1816, and 823.40 mg/kg in intraperitenial, intravenous, oral and subcutaneous administration respectively. For bactericidal results, **H** exhibited an excellent effect with inhibition percentages of 98.65 and 98.83% at the concentrations of 1000 and 500 µg/mL against *Vibrio anguillarum* respectively, the same effect was demonstrated against *Photobacterium damselae* with inhibition percentages of 97.74 and 97.98 % at the same concentrations. For anti-tubercular effect prediction, results revealed that **H** has an excellent effect with probability percentage of 84.6%.

Keyword: Hydrazone, toxicophore, LD50, Anti-tubercular, *Vibrio anguillarum*, *Photobacterium damselae*.

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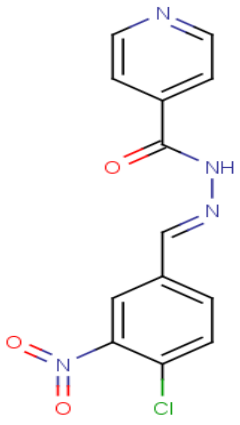
INTRODUCTION

Heterocyclic hydrazones present in many structures, with biological and pharmacological interest. They are a particular class of the Schiff base family, play an important role in medicinal chemistry. This class of compounds has excellent antibacterial, antifungal, antimicrobial and anticonvulsant activity[1]. In this context, heterocyclic hydrazones derived from the anti-tubercular drug; isoniazid (IZD) showed anti-tuberculosis activity against *Mycobacterium tuberculosis*, and some of these compounds are more active and less toxic than isoniazid[2]. For these reason, our objective is to evaluate the toxicity, anti-tubercular activity and the antibacterial effect of new hydrazone derived from isoniside; N-[(3-chloro-4-nitro-phenyl) methyleneamino] pyridine-4-carboxamidine (**H**) (**Scheme 1**).



Scheme 1: Structure of H

MATERIAL AND METHODS

All chemicals and reagents used throughout this study were obtained from commercial sources and used without further purification; Isoniazid (BHD chemicals Ltd Poole England).

Bacterial strains

The antibacterial activity of **H** was tested against:

Photobacterium damselae subsp. piscicida SK 223/04 (CECT 7198), *Vibrio anguillarum* 16 /00. The strains were donned by Dr. MA Moriñigo from the University of Málaga (Spain).

Antibacterial activity evaluation

Two pathogenic marine bacteria *Vibrio anguillarum* and *Photobacterium damselae*, were employed to estimate the bactericidal effect of **H**. The bacteria were grown on tryptic soy agar (TSA) at 25 ° C. After 18h, colonies of 1 to 2 mm were diluted in 5 mL of liquid culture medium (TSB) and cultured for 16 h at 25 ° C. The antimicrobial activity of **H** was determined by evaluating their effects on bacterial growth curves; 10 µL aliquots of each of the bacterial dilutions (1/10) were placed in 96-well flat-bottomed plates (Nunc) and incubated with equal volumes of **H** (concentration of 1000 and 500 µg/mL) for two hours. Then the absorbance of the samples was measured at 620 nm (BMG, Star GalaxyFluoro) at 30 minute intervals for 24 h at 25 ° C. Samples without bacteria were used as blanks (negative control). Samples without **H** were used as positive control[3].

Toxicity predictions

The online software programs used for toxicological studies are summarized in the following **Table 1**

Table 1: **Online software programs for toxicity and bioactivity prediction of H and IZD**

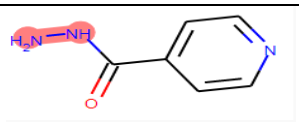
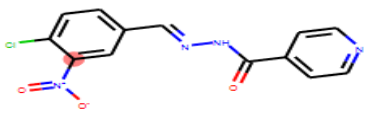
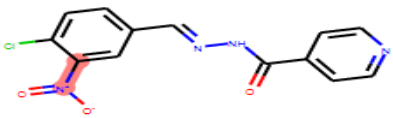
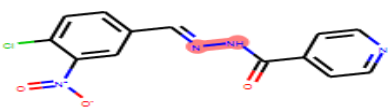
Online software programs	Toxicity prediction
e-MolTOX[4] (http://xundrug.cn/moltox)	Toxic substructure predictions
ProToxII[5] (http://tox.charite.de/protox II)	Mutagenicity
GUSAR eco-toxicity[6] http://www.pharmaexpert.ru/GUSAR/AcuToxPredict/.	Acute rat toxicity
Pass online tool[7] (http://cactus.nci.nih.gov/ncidb2/)	Anti-tubercular activity

RESULTS AND DISCUSSION

Toxic substructure prediction

Drug undesirables and toxic effects were traduced by unfavorable reactions affecting human organs, inducing harmful effects, are the principle problems in drug discovery and development[8]. These toxic actions can be related to chemical structures, or a part of the structures which defined by substructures or toxicophores, which could be an atom or fragments of chemical structure[9]. In order to predict the toxicity of **IZD** and its derivative **H**, **Table 2**, represents toxic substructures (in red color) and their adverse effects. The obtained results defined the toxic fragment in common of all investigated compounds; the hydrazine group (-NH-N-), predicted as responsible in hepatotoxicity effect induction. In addition to the hepatotoxic effect, the hydrazone has other toxic substructures: the nitro-aromatic part of **H**, presents the covalent bond with DNA, in the quaternary carbon atom, the nitro group bond with quaternary carbon, presents toxicity metabolic activation.

Table 2: Toxic substructure prediction of IZD and H

Compounds	Toxic substructure	Alert name
IZD		Covalent bind with DNA Hepatotoxicity
H		Covalent bind with DNA
		Idiosyncratic toxicity metabolic activation
		Hepatotoxicity

Acute rat toxicity prediction

The major advantage of computational approaches for the acute toxicity and the toxicity classification of the investigated hydrazone, is to give important information before they are synthesized. Additionally, due to the ethical consideration, the time consumption and the high cost in animal models, the computational methods were used as a substitute in the *in vivo* toxicological studies.

LD_{50} values and the predicted toxicity class of the **IZD** and its derivative were predicted by GUSAR software in rodent with different administration routes (intraperitoneal, intravenous, oral and subcutaneous). Results in **Table 3,** showed that the intraperitoneal route was the low toxic way of **H** with LD_{50} value of 1086 mg/kg, classified in the class **5**, the LD_{50} of the investigated hydrazone in oral administration route were 1816 mg/kg.

Table 3: Acute rat toxicity prediction of IZD and H.

Compounds	Administration route	LD50 log10(mmol/kg)	LD50 (mg/kg)	Predicted toxicity class
INH	Intraperitoneal (IP)	0.354	309.600	4
	Intravenous (IV)	0.271	256.100	4
	Oral administration	0.817	899.70	4
	Subcutaneous (SC)	0.326	290.20	4
H	Intraperitoneal (IP)	0.552	1086.00	5
	Intravenous (IV)	-0.096	244.00	4
	Oral administration	0.775	1816.00	4
	Subcutaneous (SC)	0.432	823.40	4

Mutagenicity prediction

Mutagenicity is defined as the induction of changes in genes and/or chromosomes that are permanent or transmissible in cells or organisms[10]. Probability of **IZD** and its derivatives to introduce genetic changes showed total mutagenic effect of **INH** with probability of 100%. From **H**, the predicted results depicted in **Table4,** indicate the reduction in the mutagenic effect from 100% to 71% in. In the literature, studies indicated that the hepatotoxic effect of **IZD** can lead to the genetic variations in enzymes in Asian populations (Caucasian population)[11]. These variations could be attributed to the interaction between **IZD**-reactive metabolites with DNA.

Table 4: Mutagenicity prediction of IZD, and H

Compounds	Prediction	Probability %
IZD	Active	100
H	Active	71

The high mutagenic effect of **H** when it was detected in substructure prediction could be explained by the presence of nitro-aromatic fragment in the structure. In the biotransformation, aromatic nitro group is involved in enzymatic reduction, catalyzed by both cytosolic and microsomal enzymes, leading to the formation of aromatic hydroxylamine intermediate. Then, this intermediate is activated to nitronium ion intermediate that covalently bind to DNA and as a result causes mutation[9] (**Fig. 1**).

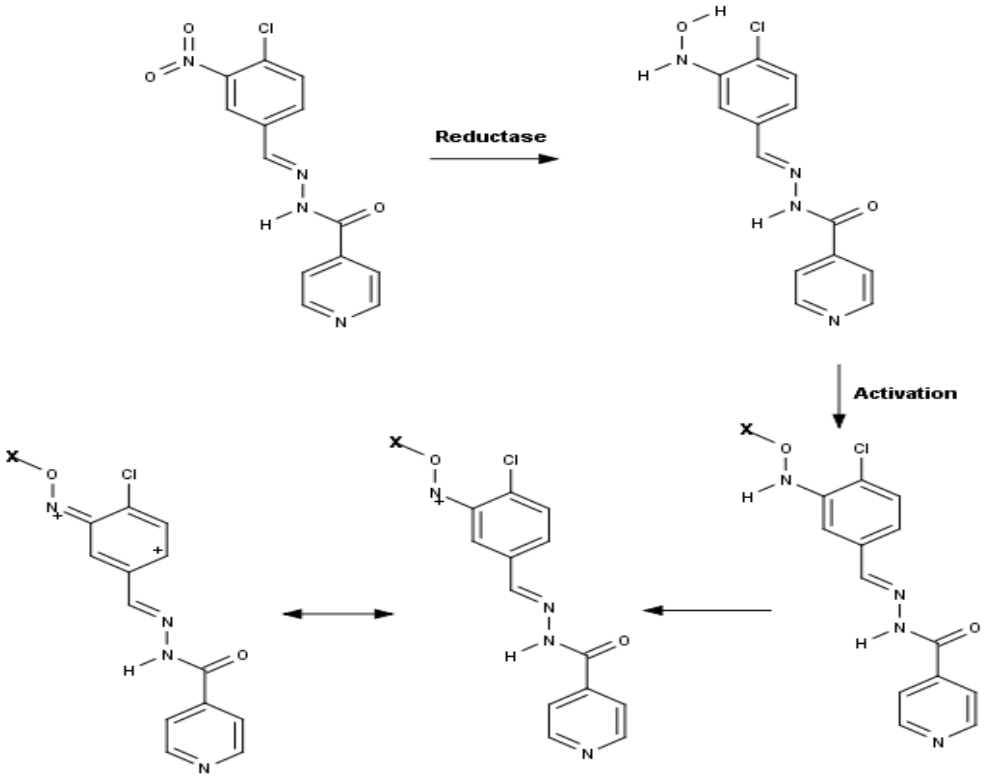


Fig.1: Proposed mechanism of H biotransformations to their reactive metabolite, nitrenium ion intermediate[9].

Antibacterial effect evaluation

Fish infection caused the decrease in marine productivity, and heavy economic costs[12]. This problem led to search of new safe antibiotics for marine uses. For this reason, the hydrazone **H** was examined for their bactericidal effect towards tow marine pathogenic bacteria; *Vibrio anguillarum* and *Photobacterium damselae*. The results displayed in **Fig.2.** indicate an excellent bactericidal effect of **H** against *Vibrio anguillarum* with inhibition percentages of 98.38% and 98.65% at 500 µg/mL and 1000 µg/mL respectively. Also, **IH** exhibited good effect against *Photobacterium damselae* with inhibition percentages of 97.79% and 97.74% at the same concentrations respectively.

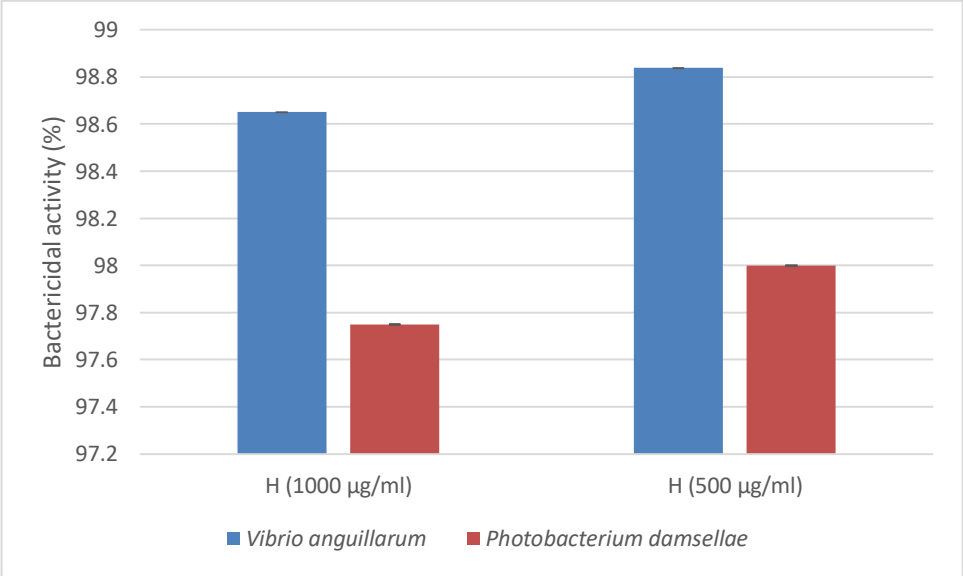


Fig.2: bactericidal effect of H

In silico anti-tubercular activity evaluation

According to World Health Organization statistics, tuberculosis is the principle infectious disease responsible of mortality in the word[13], which leads to search of potential anti-tubercular drugs[14]. In this context, the computational study is the essential step for the designing of new efficient drugs. The hydrazone **H** was investigated for these effects. The predicted results (**Fig.3**) were compared with the anti-tubercular drug **IZD**, and indicated excellent effects of this hydrazone with probabilities of anti-tubercular effects of 84.6, while the standard anti-tubercular drug **IZD** has the probability of 81.3 %. These results showed that **H** has higher effect than **IZD**. These results are in good agreement with the literature precedent investigations, which showed that hydrazones derived from **IZD** have a similar or improved effect than the drug itself[15].

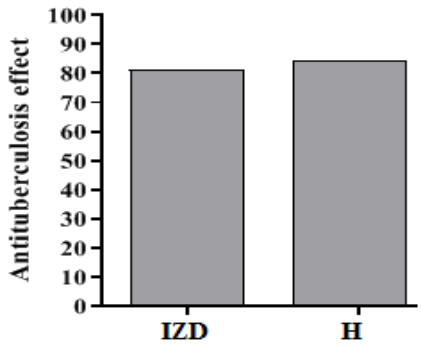


Fig.3: Anti-tubercular activity prediction of H and IZD

Molecular docking

Molecular docking study was performed to predict the possible binding modes and to rationalize the observed biological activity[16]. In order to comprehend the mechanism of action of the new hydrazones, the active molecules were subjected to molecular docking studies against InhA which is considered as a promising target for the discovery of novel anti-tubercular drugs[17]. The molecular docking studies of active compounds were accomplished against the active site of InhA enzyme structure (PDB code: 4DRE) using SwissDock server. FullFitness and Gibbs free energy (ΔG) were evaluated. Favorable binding modes were scored based on FullFitness and cluster formation. Ranking of the cluster was accomplished using the value of FullFitness. As shown in **Table 5,** the clustering results obtained from the docking of the investigated hydrazones into InhA enzyme. **H** showed FullFitness values of –1084.565 kcal/mol and estimated ΔG of –7.187 kcal/mol, for the most favorable interaction. While, **IZD** showed FullFitness of –1088.536 kcal/mol and ΔG value of 6.458 kcal/mol. These results indicate that **H** had the highest anti-tubercular activity with the lowest energy ΔG.

The best poses obtained using SwissDock server for the synthesized compounds binding to enoyl ACP reductase (InhA) enzyme are presented in **Fig. 4**, which showed different interactions traduced by H-bonds (green color). It's clear that **H** possess two centers of interaction; O of the amide and O of the nitro group. The standard drug **IZD** has two interaction centers of H-bonds, O of amide and N of hydrazine function.

Table 5: Clustering results obtained from the docking of IZD and H InhA.

Compounds	Cluster rank	Fullfitness kcal/mol	Estimated ΔG kcal/mol
H	1	−1084.565	−7.187
	2	−1084.455	−7.141
	3	−1071.394	−7.142
	4	−1068.268	−7.138
	5	−1068.057	−6.999
IZD	1	−1088.536	−6.458
	2	−1086.227	−6.157
	3	−1083.790	−6.073
	4	−1083.735	−6.068
	5	−1079.096	−6.104

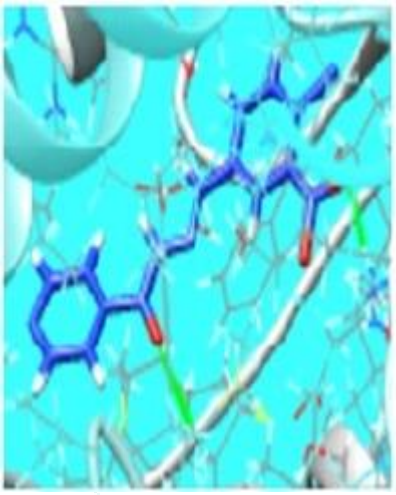


Fig. 4: Visualization of binding of H to enoyl ACP reductase (InhA) enzyme (PDB code: 4DRE), Visualization is performed using UCSF Chimera.

CONCLUSION

In silico toxicological studies were undertaken using online software programs; from toxicophores, acute toxicity, and mutagenicity; these studies showed low toxic effect of **H** with predicted LD_{50}values of 1816 mg/kg. Additionally, reduction in the mutagenic effect was observed with probability value of 29%. The anti-tubercular effect was predicted, results demonstrated the persistence of the effect in the investigated compound with predicted value of 84.6 %. Results of *in vitro* bactericidal effect evaluation using microorganism (*Vibrio anguillarum* and *Photobacterium damselae*) indicated an excellent effect of this compound at the doses of 1000 and 500 µg/mL. We conclude that the hydrazone can be a marine antibiotic.

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