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Isonicotinohydrazones as Inhibitors of Alkaline Phosphatase and Ecto-5'-nucleotidase

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Running Title:
Isonicotinohydrazones as TNAP and e5'NT Inhibitors.

Abstract:

A series of isonicotinohydrazides derivatives were synthesized and tested against recombinant human and rat ecto-5'-nucleotidases (h-e5'NT & r-e5'NT) and alkaline phosphatase isozymes including both bovine tissue-nonspecific alkaline phosphatase (*b*-TNAP) and tissue specific calf intestinal alkaline phosphatase (*c*-IAP). These enzymes are implicated in vascular calcifications, hypophosphatasia, solid tumors and cancers, such as colon, lung, breast, pancreas and ovary. All tested compounds were active against both enzymes. The most potent inhibitor of h-e5'NT was derivative (E)-N'-(1-(3-(4-fluorophenyl)-5-phenyl-4,5-dihydro-1*H*-pyrazol-1-yl)ethylidene)isonicotinohydrazide (**3j**) whereas, derivative (*E*)-*N*'-(4-hydroxy-3-methoxybenzylidene)isonicotinohydrazide (**3g**) exhibited significant inhibitory activity against r-e5'NT**.** In addition, the derivative (E)-N'-(4′-Chlorobenzylidene)isonicotinohydrazide (**3a**) was most potent inhibitor against calf Intestinal alkaline phosphatase and the derivative (*E*)-*N*'-(4-hydroxy-3-methoxybenzylidene)isonicotinohydrazide (**3g**) was found to be most potent inhibitor of bovine tissue non-specific alkaline phosphatase. Furthermore, after assay binding modes of the most compounds against e5'NT (human and rat e5'NT) and AP (including *b*-TNAP and *c*-IAP) was determined computationally.

Introduction:

All cells have the ability to release ATP, an extracellular nucleotide, either intrinsically or under a pathological condition. These molecules have an important role in regulation of a variety of physiological functions, such as inflammation, blood clotting, pain perception, immune reactions, smooth muscle contraction, cell proliferation, and cancer (1, 2). Ecto-nucleotidases are

surface-located enzymes, particularly regulating the availability of the important signaling molecules (nucleotides) in extracellular environment and are thus vital for maintaining normal cell functioning (3).

Ecto-nucleotidases enzymes are categorized into four main groups: the alkaline phosphatases (APs), the ecto-5′-nucleotidases (e5′NT), the ecto-nucleoside-5′-triphosphate diphosphohydrolases (NTPDases) and the ecto-nucleotide pyrophosphatases (NPPs)(4). Ecto-5'-nucleotidase (e5′NT, EC 3.1.3.5) is widely distributed in the body, i.e. brain, liver, intestine and lymphocyte membrane (5). The enzyme attached to the extracellular surface membrane by glycosyl phosphatidyl inositol linkage with its catalytic site containing divalent cation *i.e.* Zn faces the extracellular surface (6). Eukaryotic ecto-5'-nucleotidase consist of two structural domain, *i.e.* C–terminal and N-terminal. Both terminals are connected with small hinge region that allows them to display greater domain movement and change their conformation *i.e.* open or closed (7). Ecto-5'-nucleotidase catalytic activity involves the dephosphorylation of nucleoside monophosphate into its respective nucleoside.

Previous studies described the non-enzymatic function of e5'NT (CD73) in the body such as cell–cell, cell–matrix interactions (8-11), in drug resistance (12) and tumor-promoting functions (13). The expression of CD73 was associated with tumor metastasis, invasion and neovascularization. CD73 exhibited broad substrate specificity, but adenosine 5-monophosphate is highly preferable. There is a positive correlation of higher adenosine level with tumor progression and invasion (9). ADP and ATP are the physiological antagonist of ecto-5'-nucleotidase with Ki values in the low micro molar range, even though these nucleotides shows instability as they can be easily hydrolyzed by other ectonucleotidases such as NTPDases, APs and NPPs (7). The structural analog of ADP has been synthesized. The potential role of e5'NT in various cancers has been elaborated in previous studies, therefore, e5'NT inhibitors are important pharmaceutical targets cancer and inflammatory diseases (14, 15) Until now, only few inhibitors have been discovered such as sulfonamides (16), anthraquinones (17) and polyoxometalates (POMs) (18) and various polyphenols (19). Sulphonamides and anthraquinones inhibit the catalytic site of enzyme by showing the competitive mechanism, whereas POMs and the polyphenol display noncompetitive mechanism of inhibition. Due to upregulation of e5'NTin various carcinomas, there is a need to synthesize novel inhibitors of e5'NT having less toxicity, more selectivity and potency towards its target.

Alkaline phosphatase (AP; EC 3.1.3.1), a metal containing enzyme, is involved in catalyses of phosphoryl transfer reaction. There is only one alkaline phosphatase found in *Escherichia coli* (ECAP), but four types of alkaline phosphatase isoenzymes have been isolated from humans. In humans, three isozymes are classified as tissue-specific: first one is placental (PLAP), second is germ cell (GCAP), and the third is present in intestine (IAP) (3). The fourth one tissue-nonspecific (TNAP), 50% alike to the other three isozymes, is present in bone, liver, and kidney (20-22). TNAP gene is present on chromosome number 1p34–36 (23) and mutations in the TNAP gene resulted in hypophosphatasia, a hereditary disorder, characterized by defective bone mineralization (24). Evidences showed that the major role of TNAP in bones is hydrolysis of PPi, a potent inhibitor of mineralization, thus ensuring normal bone growth (25, 26).

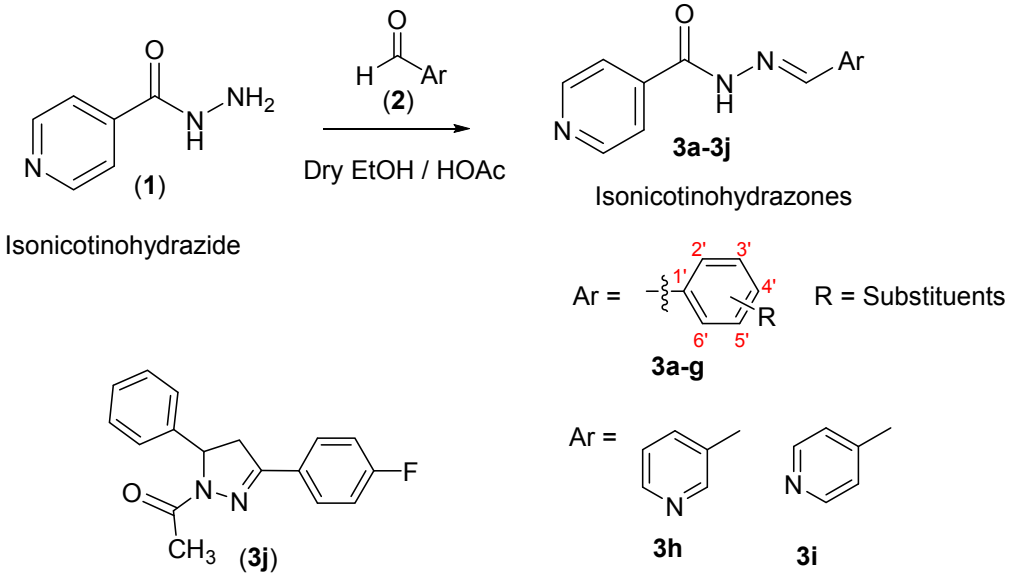
Different pathological conditions have been ensued due to high levels of APs (3). Consequently, it necessitates to design small, potent inhibitors of APs having ability to look at the causative mechanisms, or cure the diseases. Up till now, very few selective inhibitors of APs have been described due to high structural similarity between two isozymes; tissue-specific IAP and TNAP. AP is inhibited by various compounds on the basis of mechanisms of inhibition. L-phenylalanine, imidazole, histamine and theophylline are uncompetitive inhibitors of alkaline phosphatase while phosphate, phosphoethanolamine and phenylphosphonate inhibit alkaline phosphatase competitively (27-29). Levamisole derivatives have also been found selective inhibitors of TNAP (30) while selective inhibitors of IAP such as ML260 (N-(2,5-dimethylphenyl)-2-(2-oxo-2,3-dihydro-1,3-benzoxazole-6-sulphonamide acetamide) have also been reported (31).

To determine the accurate mechanisms and the related consequences due to increased levels of nucleotides it is requisite for the screening of ecto-5'-nucleotidase inhibitors for the management of cancer, central nervous system and cardiovascular disorders. (3, 32). Previously isonicotinohydrazide derivatives have been synthesized and screened for *In vitro* potential of the compounds against *Mycobacterium tuberculosis* (33). Herein, we synthesized different isonicotinohydrazides derivatives and evaluated their AP and ecto-5'-nucleotidase inhibitory potential for the possible management of the ailments discussed above.

Results and Discussion

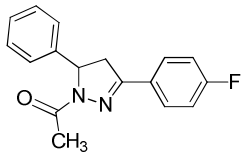
Chemistry

The synthetic layout of the aimed isonicotinohydrazones (**3a-3j**) is presented in the Scheme 1. Isoniazid **1** was condensed with a range of substituted aromatic and heteroaromatic aldehydes in dry ethanol, catalyzed by acetic acid to afford the desired hydrazones (**3a-3j**) in good yields (Scheme 1, Table 1). The structures of hydrazones (**3a-3j**) were established with 1H NMR spectroscopy. A broad singlet belongs to –NH- group of synthesized hydrazones was observed in the range of 12.26 to 11.87 (δ in ppm). Two sets of protons (H2′H6′ and H3′H5′) of 4-substituted pyridine ring appeared as two pseudoquartets or multiplets at δ 8.75 and 7.74 ppm. The characteristic singlet for –N=CH appeared between 7.1-7.7 ppm. The compounds were obtained in 65-90% yields depend upon the nature of substituents on aromatic ring and type of aldehyde (Table 1).



Scheme 1: Synthetic layout of isonicotinohydrazones (**3a-3j**)

Table 1: Synthesized isonicotinohydrazones (**3a-3j**)

Compound	Ar	Yield (%)
3a	4′-Chlorophenyl	80
3b	4′-bromophenyl	85
3c	4′-Fluorophenyl	78
3d	4′-Methoxyphenyl	88
3e	2′-hydroxyphenyl	70
3f	3′-Nitrophenyl	79
3g	4′-Hydroxy-3′-methoxyphenyl	90
3h	4′-pyridyl	68
3i	3′-pyridyl	65
3j		70

Alkaline Phosphatase and Ecto-5'-nucleotidase Activity and SAR:

A series of *N*-ethylidene isonicotinohydrazide derivatives were synthesized and their biological potential was evaluated by performing in-vitro assays on both ecto-5'-nucleotidase (human and rat) and alkaline phosphatase isoenzymes (b-TNAP and c-IAP). The derivatives were tested at concentration of 200 µM for APs and 100 µM for e5′NT. These derivatives exhibited more inhibition for e5′NT as compared to alkaline phosphatase even in lower micro molar range. All the derivatives exhibited initibion of h-e5′NT wth IC50±SEM in the range of 0.21±0.05 to 13.8±0.12 µM and of r-e5′NT IC_{50}±SEM = 0.21±0.05 to 7.13±0.63 µM. The most potent inhibitor of h- e5′NT was 3j having IC_{50}±SEM = 0.19±0.02 exhibiting ≈1.9 fold selectivity over r- e5′NT. This compound also exhibited ≈222 fold improvement in inhibition as compared to reference standard i.e sulfamic acid (IC_{50}±SEM = 42.1±7.8 µM). The detailed structural comparison of this derivative with other compounds suggested that the activity of this compound might be due to the presence of pyrazol ring. The most potent inhibitor of r-e5′NT was 3g having inhibitory value of IC_{50}±SEM = 0.14±0.001 exhibiting ≈4.8 fold selectivity over h-e5′NT. This

compound also exhibited ≈552 fold improvement in inhibition as comared to reference standard i.e. sulfamic acid (IC_{50}±SEM = 77.3±7.0 µM). The comprehensive study of its structure in comparison with the other derivatives of the series suggested that the activity of this compound might be due to the presence of electron donating group on the phenyl ring i.e MeO at 4 position making the ring stable by electronic cloud. This compound exhibited ≈3.2 fold improvement in inhibition as compared to compound 3e (IC_{50}±SEM = 0.45±0.03 µM) in which the phenyl ring was un-substituted. Similarly, ≈2.2 fold maximum inhibition was seen in 3g, its inhibitory potential was compared with 3d (IC_{50}±SEM = 0.31±0.01 µM) in which the MeO was substituted to phenyl ring.

When the activity of other derivatives was observed it can be suggested that the presence of electronegative substititution exhibited maximum inhibition of r-e5′NT as compared to h- e5′NT. The effect was interestingly observed in case of compounds 3a, 3b and 3c. Among these three, compound 3c exhibited maximum inhibitory value i.e IC_{50}±SEM = 0.44± 0.02 µM and compound 3b the least inhibition i.e IC_{50}±SEM = 7.13± 0.63 µM, having F and Br substitution respectively. The reverse effect was observed for h-e5′NT i.e. the compounds having less electronegative substitution exhibited more inhibition of h-e5′NT e.g compound 3b exhibited ~31 fold selectivity over r-e5′NT.

As for as alkaline phosphatase activity is concerned, the compounds were screened against two isozymes of APs i.e. *b*-TNAP, *c*-IAP. All synthetic compounds containing isonicotinohydrazide nucleus, showed potent inhibition against *c*- IAP, though, some were found to be potent inhibitors of *b*-TNAP comparable to their respective reference standards used in the study.

It is evident from Table-2 that compounds **3b, 3c, 3d, 3f, 3g, 3i and 3j** showed potent *b*-TNAP inhibition with the inhibitory values (IC_{50}) in the range of 0.35±0.01 to 4.13±0.91 μM as compared to standard inhibitor i.e. Levamisole with IC_{50} values 19.21±0.001 μM. The compound **3g** exhibited maximum inhibition potential against TNAP with IC_{50} values 0.35±0.01 μM. While the whole series (10 compounds) was found to be active against *c*-IAP with the inhibitory values of IC_{50} 0.67±0.02 to 17.5±1.01 μM and showed more potency than the familiar reference standard used in this study i.e. L-Phenylalanine with IC_{50} values 80.21±0.001μM. Maximum inhibition activity against c-IAP with IC_{50} values 0.67±0.02 μM was exhibited by the compound **3a**.

Whereas the derivatives **3b, 3c, 3d, 3f, 3g, 3i and 3j** showed dual inhibition against both the isoenzymes. All these compounds showed inhibitory values in the range of 0.35±0.01 to 4.13±0.91 μM and 0.67±0.02 to 17.5±1.01 μM, against *b*-TNAP & *c*-IAP, respectively. Similarly the derivatives **3a**, **3e**, and **3h** showed selective and potent inhibition of *c*-IAP with IC_{50} values in the range of 0.67±0.02 to 2.46±0.87 μM. Through a comprehensive study of the structure of the active compound it is clear that potent activity against *b*-TNAP was due to the substitution at C-3 and C-4 of the benzene ring of hydrazide derivative with electron donating groups (-OH and $–OCH_3$). The compound **3g** showed potent inhibition against *b*-TNAP due to the presence of hydroxyl and methoxy groups on the benzene ring, thus promoting its activity. While the presence of electrophilic substitution (bromine) at the para position of the same nucleus (**3b**) reduces its activity against b-TNAP as compared to compound **3g**. Electron donating groups (-OH and $-OCH_3$) present at C-4 and C-3 of the benzene ring, enhancing its nucleophilic character (more responsive towards electrophiles) thus, making it more reactive. It resulted in carbocation formation due to stabilization of the transition state of the benzene ring system, leading to faster reaction rate of electrophilic attack. This phenomenon bettered solubility of the compound in polar solvent (water) because anions are extremely solvated by H-bonding.

While the isonicotinohydrazide derivative (**3a**) showed potent inhibition of *c*-IAP with IC_{50} values 0.67±0.02 μM, due to substitution on the benzene ring with chlorine, an electron withdrawing group. Since the electron withdrawing group (chlorine) is present at para position of the benzene ring, hence boosting its reaction rate and lessening nucleophilic property of the compound (less reactive towards electrophiles). Due to electronegativity, chlorine withdraws electron density from the benzene ring system, deactivates it weakly, develops an inductive withdrawal effect, the consequence of which is a slower rate of reaction. By donating electrons to the benzene ring system, chlorine also displays the resonance effect, but this effect is suppressed by inductive effect. Due to this attribute, **3a** showed increased inhibition potential against *c*-IAP.

Table 2. Ecto-5′-nucleotidase and Alkaline Phosphatase inhibition activity by the synthesized compounds

	Ecto-5-nucleotidase		Alkaline Phosphatase	
Compound	**h- e5'NT IC_{50} (μM) ±SEM**	**r- e5'NT IC_{50} (μM) ±SEM**	***b*-TNAP IC_{50} (μM) ±SEM**	***c*-IAP IC_{50} (μM) ±SEM**
3a	1.67±0.58	1.08±0.24	48.95%	0.67±0.02
3b	0.23±0.02	7.13±0.63	4.13±0.91	9.72± 1.51
3c	6.76±0.16	0.44±0.02	2.26±0.56	17.5±1.01
3d	13.78±0.12	0.31±0.01	0.56±0.06	1.74±0.16
3e	1.34±0.26	0.45±0.03	45.14%	1.44±0.05
3f	1.90±0.01	0.21±0.05	1.82±0.63	7.34±0.93
3g	0.68±0.01	0.14±0.001	0.35±0.01	1.92±0.03
3h	2.12±0.15	2.89±0.41	44.74%	2.46±0.87
3i	0.21±0.05	3.01±0.12	2.43±0.86	1.46±0.06
3j	0.19±0.02	0.36±0.01	1.06±0.26	3.98±0.96
Sulfamic acid	42.1±7.8	77.3±7.0		
Levamisole			19.2±.001	-
L-Phenylalanine			-	80.2±0.001

The IC_{50} value is the concentration at which 50% of the enzymatic activity is inhibited. Ecto-5-nucleotidase (human and rat) activity was performed at final concentration of 100 μM while alkaline phosphatase (*b*-TNAP, *c*-IAP) activity was done at the final concentration of 200 μM.

Homology Modeling

From the Protein Data Bank, the X-ray structure of the closed form (PDB ID 4H2I) of human e5'NT was taken as structural templates for modeling of rat e5'NT (89 % sequence identity). Homology modeling was carried out using Molecular Operating Environment (MOE) 2014, 09 (34). The active site region of the human and rat enzyme is highly conserved, with only one residue replacement, Phe500 (human) vs. Tyr502 (rat). The superposition of the human template structure and rat model of the closed (active) form yielded a Cα RMSD values of less than 0.5 Å. Ramachandran diagrams confirming high stereochemical quality of the models are provided as Supplementary Information.

Molecular Docking

To model a putative binding mode of synthesized compounds, docking studies were carried using the crystal structure of the closed (active) form human e5'NT and the corresponding rat e5'NT homology model as well as previously reported models of bovine tissue non-specific and intestinal alkaline phosphatase..

Molecular docking of the most potent compound **3j** was performed using the human e5'NT crystal structure, while docking of compound **3g** was carried out using the closed form of the rat e5'NT homology model. Compound **3j** in the docking model with the human e5'NT closed form is involved in hydrogen bonding interactions with residue Asn390 and Asp506. These interactions are similar to those formed by an adenosine moiety (35). In the model, the dihydro pyrazole ring of the compound **3j** was sandwiched between residue Phe417 and Phe500. The fluorobenzene ring of compound **3j** also formed putative hydrogen bonding interactions with Thr420 and Asp524. Docking of compound **3g** into the rat e5'NT model resulted in putative hydrogen bonding interactions with residues Arg356, Asn247 and His245. Such interactions have also been reported in case of adenosine (35) as well as sulfonic acid derivatives (36). The pyridine ring of the compound formed stacking interaction with residue Phe419 (corresponding to residue Phe417 of human e5'NT) and Tyr502 (Phe500 residue of human e5'NT). The modeled binding modes of compound **3j** and compound **3g** are shown in Figure 1.

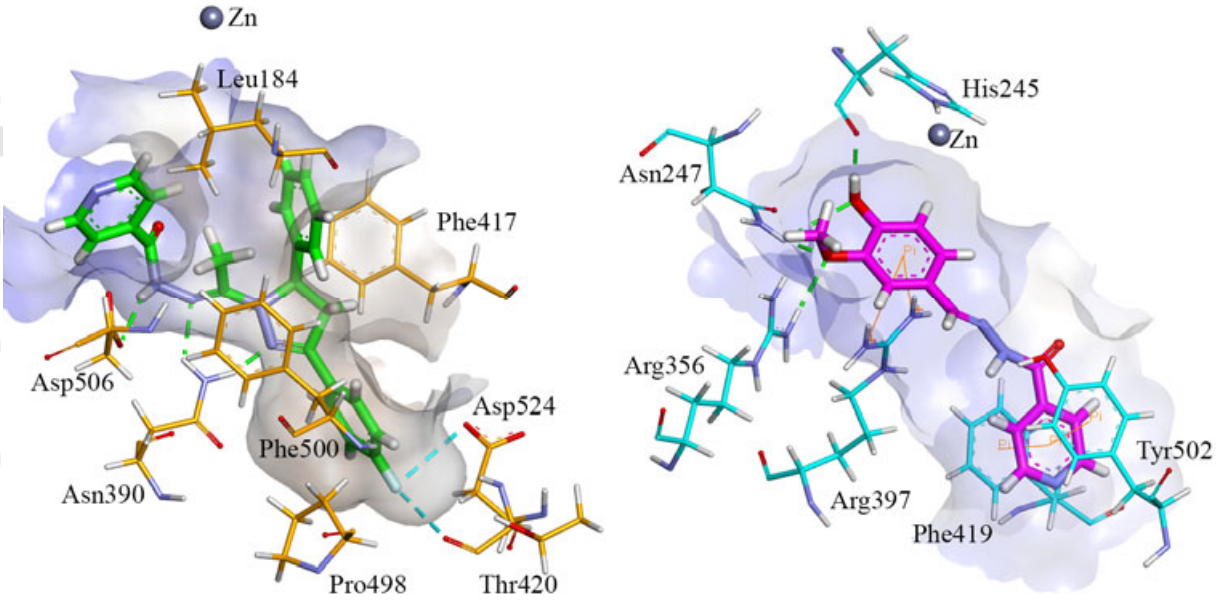


Fig. 1 Putative binding mode of compound **3j** (colored green) in the human e5'NT crystal structure (brown) and compound **3g** (colored magenta) in the rat e5'NT homology model (cyan). Hydrogen bonding interactions are shown as dashed green lines, pi-pi interactions as solid yellow lines, and interaction with fluorine interactions as dashed blue lines.

In the docking model with bovine tissue non-specific alkaline phosphatase, the most potent compound **3g** formed hydrogen bonding interactions with the catalytic Ser110 and with residue His338. Residue Arg184 and His451 also formed hydrogen bonding interactions with the inhibitor, consistent with previously observed interactions (37). Residue His451 interacted with the pyridine ring of the compound. In the case of calf intestinal alkaline, the most potent compound **3a** formed putative hydrogen bonding interactions with residue Ser111 and Arg185 and additional interactions were observed with His172. Figure 2 shows the putative binding mode of compound **3g** and **3a** in the models of bovine tissue non-specific and intestine specific alkaline phosphatase, respectively.

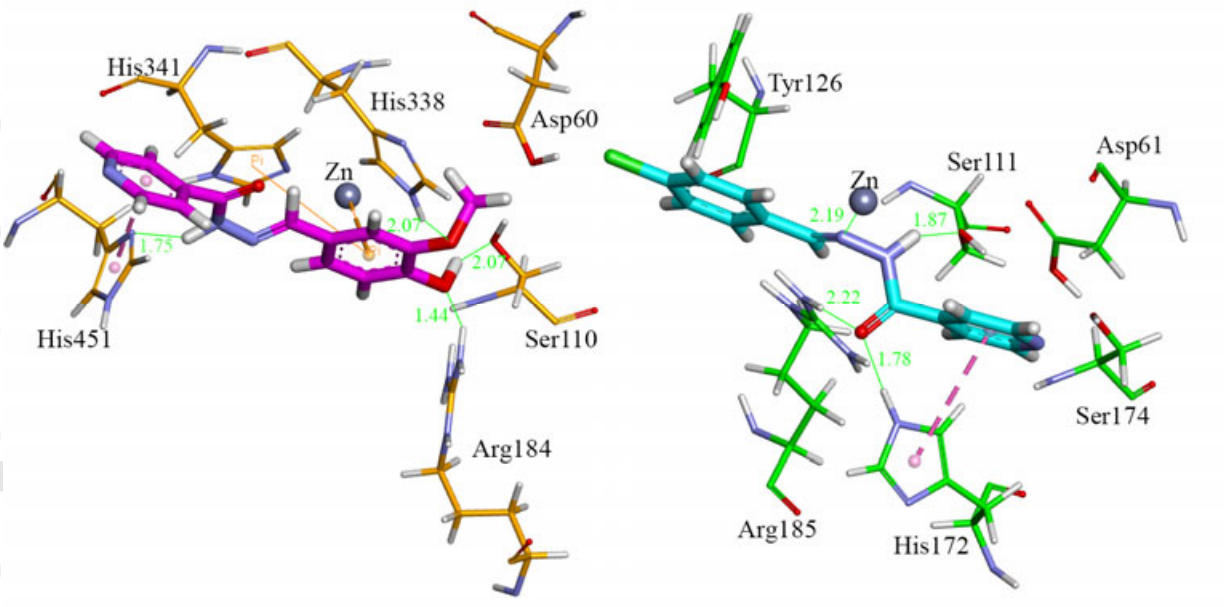


Fig. 2 Putative binding mode of compound **3g** (colored magenta) in the model of bovine tissue non-specific alkaline phosphatase (brown) and compound **3a** (colored cyan) in calf intestinal alkaline phosphatase (green). Putative hydrogen bonding interactions are shown as green lines, stacking interactions as solid yellow lines, and pi-pi t-shaped interactions as dashed purple dashed lines.

Conclusion

The bioassay results of the synthesized series and their modelling and docking studies suggested that the derivatives of Isonicotinohydrazones possess significant activity against APs and e5'NT. The compound **3j** was found to be most active against human e5'NT with an IC_{50} value of 0.19±0.02. Compound **3a** was determined to be most potent against *c*-IAP with an IC_{50} value of 0.67±0.02 and compound **3g** was found to be most active against rat e5'NT with an IC_{50} value of 0.14±0.00, as well as potent against *b*-TNAP with an IC_{50} value of 0.35±0.01. These newly synthesized class of compounds can be used to develop novel drug candidates against defective bone mineralization, solid tumors and cancers of different organs.

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