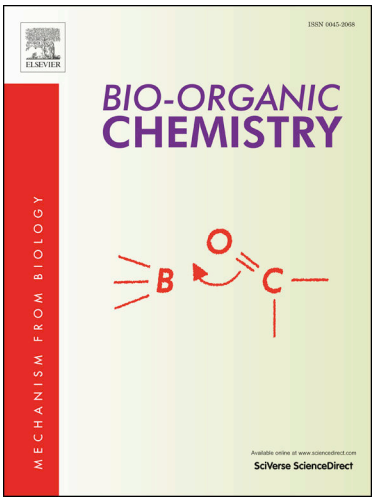
Journal Pre-proofs



Bisthioureas of pimelic acid and 4-methylsalicylic acid derivatives as selective inhibitors of tissue-nonspecific alkaline phosphatase (TNAP) and intestinal alkaline phosphatase (IAP): Synthesis and molecular docking studies

Amara Mumtaz, Kiran Saeed, Abid Mahmood, Sumera Zaib, Aamer Saeed, Julie Pelletier, Jean Sévigny, Jamshed Iqbal

PII: S0045-2068(20)31293-1
DOI: https://doi.org/10.1016/j.bioorg.2020.103996
Reference: YBIOO 103996

To appear in: *Bioorganic Chemistry*

Received Date: 18 April 2020
Revised Date: 27 May 2020
Accepted Date: 2 June 2020

Please cite this article as: A. Mumtaz, K. Saeed, A. Mahmood, S. Zaib, A. Saeed, J. Pelletier, J. Sévigny, J. Iqbal, Bisthioureas of pimelic acid and 4-methylsalicylic acid derivatives as selective inhibitors of tissue-nonspecific alkaline phosphatase (TNAP) and intestinal alkaline phosphatase (IAP): Synthesis and molecular docking studies, *Bioorganic Chemistry* (2020), doi: https://doi.org/10.1016/j.bioorg.2020.103996

Bisthioureas of pimelic acid and 4-methylsalicylic acid derivatives as selective inhibitors of tissue-nonspecific alkaline phosphatase (TNAP) and intestinal alkaline phosphatase (IAP): Synthesis and molecular docking studies

Amara Mumtaz[1#*], Kiran Saeed[1#], Abid Mahmood[2], Sumera Zaib[2], Aamer Saeed[3], Julie Pelletier[4], Jean Sévigny[4,5], Jamshed Iqbal[2*]

[1]*Department of Chemistry, COMSATS University Islamabad, Abbottabad Campus, Abbottabad-22060, Pakistan.*
[2]*Center for Advanced Drug Research, COMSATS University Islamabad, Abbottabad Campus, Abbottabad-22060, Pakistan*
[3]*Department of Chemistry, Quaid-i-Azam University Islamabad, Pakistan.*
[4]*Centre de Recherche du CHU de Québec – Université Laval, Québec, QC, G1V 4G2, Canada.*
[5]*Département de microbiologie-infectiologie et d'immunologie, Faculté de Médecine, Université Laval, Québec, QC, G1V 0A6, Canada.*

[*]Corresponding author: Dr. Amara Mumtaz, Department of Chemistry, COMSATS University, Abbottabad Campus Ph No. +923005316570, email: amaramumtaz@ciit.net.pk. Prof. Dr. Jamshed Iqbal, Centre for Advanced Drug Research, COMSATS University, Abbottabad Campus, 22060, Pakistan, Ph No. +923365101651, Email: drjamshed@cuiatd.edu.pk; jamshediqb@gmail.com. [#]equal contribution: Dr Amara Mumtaz, KiranSaeed, Department of Chemistry, COMSATS University, Abbottabad Campus Ph No. +923125091628, email: kiransaeed8310@gmail.com

Bisthioureas of pimelic acid and 4-methylsalicylic acid derivatives as selective inhibitors of tissue-nonspecific alkaline phosphatase (TNAP) and intestinal alkaline phosphatase (IAP): Synthesis and molecular docking studies

Amara Mumtaz[1*], Kiran Saeed[1], Abid Mahmood[2], Sumera Zaib[2], Aamer Saeed[3], Julie Pelletier[4], Jean Sévigny[4,5], Jamshed Iqbal[2*]

[1]*Department of Chemistry, COMSATS University Islamabad, Abbottabad Campus, Abbottabad-22060, Pakistan.*
[2]*Center for Advanced Drug Research, COMSATS University Islamabad, Abbottabad Campus, Abbottabad-22060, Pakistan*
[3]*Department of Chemistry, Quaid-i-Azam University Islamabad, Pakistan.*
[4]*Centre de Recherche du CHU de Québec – Université Laval, Québec, QC, G1V 4G2, Canada.*
[5]*Département de microbiologie-infectiologie et d'immunologie, Faculté de Médecine, Université Laval, Québec, QC, G1V 0A6, Canada.*

Abstract:

Alkaline phosphatases (ALPs) are membrane bound metalloenzymes, distributed all over the body. Recent studies have revealed that by targeting ALPs can lead towards the treatment of many deadliest diseases including cardiac, cancerous and brain diseases. Thioureas and their derivatives are of considerable significance and are privileged scaffolds in medicinal chemistry. They show a wide range of pharmacological activities such as antibacterial, antiparasitic, anti-inflammatory and antioxidants *etc.* On the other hand, salicylic acid and its derivatives are known for its broad spectrum of activities. The work presented comprises of synthesis of *N*-acyl-*N*′-aryl substituted bisthioureas of pimelic acid (1-7) and 3,5-dimethyl pyrazole (11), 1-aroyl-3-aryl thiourea (12) and 1,3,4-oxadiazole (13) derivatives of 4-methyl salicylic acid. Structures of all the synthesized compounds were characterized by FT-IR and ^{1}H NMR spectroscopic analysis. Synthesized compounds were evaluated for their alkaline phosphatases inhibition potential and exhibited high potency as well as selectivity towards *h*-TNAP and *h*-IAP. Compound 7 and 12 which were the bisthiourea derivative of pimmelic acid and thiourea derivative of 4-methyl salicylic acid, respectively, showed excellent selectivity against *h*-TNAP and *h*-IAP, respectively.

Key Words: Thiourea, pimelic acid, salicylic acid, alkaline phosphatase inhibitors

1. Introduction:

Alkaline phosphatases (ALPs) are membrane bound metalloenzymes and found in most of the living organisms including prokaryotes and eukaryotes. Mammalian ALPs have low sequence similarities with the *E. Coli*, but the residues involved in the catalytic activity of active sites were found to be identical. Therefore, catalytic mechanism of both the enzymes is similar [1]. Mammalian ALPs are classified based on distribution in different tissues and organs. In humans, expression of some ALPs is specifically confined to certain tissue or organs and designated as tissue specific alkaline phosphatases [2]. Intestinal ALPs (IAP), placental ALPs (PALP) and germ cells ALPs (GCAP) are tissue specific isozymes of ALPs. Tissue specific ALPs have different heat stabilities and competitive mode of inhibition. Tissue specific isozymes of ALPs differ based on level of post-translational glycosylation which affect their catalytic efficacy [3]. A fourth isozyme is ubiquitous, expressed in liver, bones and kidneys, known to be tissue non-specific ALP (TNAP) [4]. ALPs catalyze the nucleotides and liberate inorganic phosphates (P*i*). Such cleavage of nucleotides results in generation of adenosine, an agonist for P1 receptors in purinergic signaling pathways. Purinergic signaling is known to involve in various physiological and patho-physiological conditions [5]. Alkaline pH is essential for optimal activity of isozymes. ALPs exhibit wide range of substrate specificity including inorganic polyphosphates, phosphatidates, glucose-phosphates and *p*-nitrophenyl phosphates [6]. TNAP is the most abundant isozyme, comprising about more than 90% of all other circulating ALPs isozymes. The highest levels of TNAP has been found in bones, liver and kidneys. Active pocket of TNAP possesses two zinc ions and a magnesium ion as cofactors as well as a calcium ion in specific calcium binding sites, thus regulating the activity of enzyme [7]. All isozymes of ALPs are ectoenzymes and anchor to the cell membrane through glycosylphosphatidylinositol (GPI) moiety. Only the circulation level of ALPs defines the activity level of enzyme. It has been reported that GPI-specific phospholipase-D release the anchored enzyme into serum. TNAP causes bone mineralization by providing inorganic phosphate (P*i*) and inactivation of calcification inhibitors [8]. TNAP and IAP detoxify the circulating endotoxins through dephosphorylation. Under-expression of TNAP results in genetic disorder, like rickets/osteomalacia, known as hypophosphatasia. Level of circulating TNAP has been reported to increase in bone diseases, cardiovascular abnormalities, kidney

dysfunctions and genetic disorders [9]. TNAP seem to be a novel target for treatment of vascular calcification and diabetes mellitus type 2 and chronic kidney disease (CKD). Intestinal AP prevents the accumulation of unwanted compounds from small intestine by the absorption of lipopolysaccharide (LPS). Excess level of IAP may leads to inflammatory bowel disease (IBD) [10]. Variety of salicylic acid derivatives are known as COX inhibitors and have been reported for treatment of IBD [11]. Salicylic acid derivatives of thiourea and sulfur containing heterocyclic compounds have been investigated for biological activities and known to possess good antimycobacterial activity [12]. Levamisole and theophylline are well known inhibitors of TNAP with *Ki* values of 16 µM and 82 µM, respectively [13]. Thiourea derivatives have been well reported for broad range of biological activities in pharmaceutical industry including antibacterial, antiparasitic, antifungal, antioxidant, anticancer, anti-HIV activities, urease inhibition, carbonic anhydrase inhibition, butyrylcholinesterase and acetylcholinesterase inhibition [14]. Several thiourea derivatives have been synthesized and characterized for their biological activities while bis(thiourea) are relatively less reported for their biological potential. Incorporation of alkyl chain in thiourea derivatives was reported to possess enhanced biological activities due to increased lipophilicity [15]. Presence of free N-H allows the further derivatization of thiourea compounds with heterocycles. Carbonyl and thiocarbonyl moieties provide hard and soft donors for metal complexes formation [14].

In the present study, bis(thiourea) derivatives of pimmelic acid (1-7) and 3,5-dimethyl pyrazole (11), 1-aroyl-3-aryl thiourea (12) and 1,3,4-oxadiazole (13) derivatives of 4-methyl salicylic acid were synthesized and evaluated for selective inhibitory potential for *h*-TNAP and *h*-IAP. Moreover, molecular docking studies were carried out to investigate the role of selective compounds at catalytic site of enzymes.

2. Results & Discussion:

Chemistry

The work presented describes the synthesis of bis(thiourea) derivatives of pimmelic acid (1-7), 3,5-dimethyl pyrazole (11), 1-aroyl-3-aryl thiourea (12) and 1,3,4-oxadiazole (13) derivatives of 4-methyl salicylic acid were synthesized and evaluated for selective inhibitory potential for *h*-TNAP and *h*-IAP. For the synthesis of bisthioureas (1-7) pimelic acid was first converted into its

respective acid chloride by reaction with thionyl chloride which was further reacted with solution of potassium isothiocyanate in dry acetone followed by reaction with different substituted anilines for appropriate time till completion of reaction. Progress of reaction was monitored by using TLC. Thioureas were obtained by adding cold water to reaction mixture and purified using column chromatography. FT-IR spectra of compounds **s**howed characteristic stretching band for NH around 3200-3300 cm^{-1}, band for C=O from 1650-1695 cm^{-1}, C=S stretching band at around 1220-1254 cm^{-1} and aliphatic C-H stretching band around 2900 cm^{-1} confirmed the synthesis of compounds (1-7). In the ^{1}HNMR spectrum of compounds 1-7 and 12 appearance of two characteristic singlets at 12.60 ppm (CONH) and 11.46 ppm (CSNH) confirmed the presence of two NH groups which are the characteristics of thiourea [14]. 3,5-Dimethylpyrazole (11) and 1,3,4-oxadiazole (13) derivatives of 4-methylsalicylic acid were synthesized starting from the common precursor hydrazide (9). Condensation of 9 with acetyl acetone led toward the compound 11 while reaction of 9 with carbondisulfide, potassium hydroxide followed by acidic workup finished on compound 13. FT-IR spectrum of compound (11) showed characteristic stretching band for C-O at 1299 cm^{-1}, appearance of characteristic band of C=O at 1650 cm^{-1} and =C-H stretching band at 3000 cm^{-1} confirmed the synthesis of 3,5-dimethyl-1*H*-pyrazol-1-yl)(4-methyl-2-(4nitro benzyl)oxy)phenyl)methanone (11), while in the ^{1}HNMR spectrum appearance of a singlet at 6.30 ppm and two singlets at 2.35 ppm and 2.51 due to methyl groups attached to pyrazole ring confirmed the synthesis of the 3,5-dimethyl-1*H*-pyrazol-1-yl)(4-methyl-2-(4nitro benzyl)oxy)phenyl)methanone (11) [16]. In the FT-IR spectrum of 5-(4-methyl-2-(4-nitrobenzyl)oxy)phenyl)-1,3,4-oxadiazole-2(3*H*)-thione (13) absorption band at 3327 cm^{-1} isthe characteristic band for N-H stretching while the appearance of C=N bands at 1605 cm^{-1}, C=S stretching band at 1170 cm^{-1} and Ar-CH absorption band at 3000 cm^{-1} confirmed the synthesis of compound. In the ^{1}HNMR spectrum, broad singlet at 14.9 ppm confirmed the synthesis of compound (13)**.** Doublets appearing at 8.24 ppm and 7.86 ppm showed the presence of protons attached to benzene ring [17].

Structure-activity relationship (SAR):

Thioureas also known as thiocarbanides are rich source of nitrogen. Such derivatives have wide range of application in medicinal chemistry. These compounds are known to possess antibacterial, antifungal, anticancer, antioxidant, anti-diabetics as well as DNA binding properties [18]. The data

presented in the Table 1 provide an overview about the inhibitory potential as well as selectivity of synthesized compounds. Compounds were found to show selective inhibitory activities for *h*-TNAP and *h*-IAP. Among bis(thiourea) derivatives, compound **7** exhibited higher inhibitory activity for *h*-TNAP, represented by IC_{50} value of 4.63±0.31 µM which is about five times more than positive control levamisole (IC_{50} value: 20.2±1.90). Compound **7**was also demonstrated inhibition for *h*-IAP with an IC_{50} value of 6.72±0.94 µM. Presence of 4-nitrophenyl moiety at the both ends of compound **7**conferred this potency as well as dual inhibition. Compounds **3, 4**and**5** demonstrated higher selective inhibitory activity for *h*-TNAP with an IC_{50} values of 15.4±0.75 µM, 5.28±0.51 µM and 15.9±0.31 µM, respectively. These compounds possess dichlorophenyl moieties at their terminal ends that might be responsible potency as well as selectivity for *h*-TNAP. It is evident from the structures of **3, 4** and **5** compounds, change in position of substitution of chloro group on terminal benzene has no influence on the selectivity of compounds towards *h*-TNAP. Even reducing the di-substitution to mono-substitution of chloro group has no effect on the selectivity of these compounds. Thus chloro group substitution on terminal benzene rings confer a selectivity towards *h*-TNAP. Bis(thiourea) derivatives containing dimethoxyphenyl (compound **1**) and methoxyphenyl (compound **2)** moieties were expressed selectivity towards *h*-IAP with IC_{50} value of 1.01±0.11 µM and 9.46±1.23 µM, respectively. Compound **1** was most potent and selective compound for *h*-IAP among all the tested compounds that indicate the importance of number of methoxy substitutions on phenyl rings. *p*-tolyl group in bis(thiourea) derivative (**6**) was also proved to play significant role to confer selectivity towards *h*-IAP isozyme. As replacement of methoxy group in **1** and **2** compounds with methyl moieties diminish the selectivity of both compounds. IC_{50} values of compound **6** were 11.6±0.55 µM and 2.80±0.94 µM for *h*-TNAP and *h*-IAP, respectively. 4-methyl salicylic acid derivatives were also expressed selectivity and potency for both isozymes of alkaline phosphatase. Among salicylic acid derivatives, 4-methyl-2-((4-nitrobenzyl) oxy)-*N*-(o-tolylcarbamothioyl)benzamide (**12**) was found to possess highest potency and selectivity for *h*-IAP with IC_{50} value of 1.50±0.24 µM, in comparison to positive control (L-Phenylalanine: 100±3.15 µM). Chemical structure of compound **12** possesses all those features that made it selective for *h*-IAP, including the presence of tolyl-group, 4-nitrophenyl group and carbamothioylbenzamide moiety. 5-(4-Methyl-2-((4-nitrobenzyl) oxy) phenyl)-1,3,4-oxadiazole-2(3*H*)-thione (**13**) exhibited inhibition for *h*-TNAP with IC_{50} value of 4.89±0.84 which is comparable to inhibitory potential of standard inhibitor levamisole. This

selectivity of **13**might be due to 1,3,4-oxadiazole-2(3*H*)-thione substitution, based on literature available. 1,3,4-oxadiazole-2(3*H*)-thione derivatives were found to possess wide range of biological application including potent inhibitors of various enzymes. These derivatives have the ability to inhibit carbonic anhydrase, lipoxygenase, succinate dehydrogenase, monoamine oxidase and cyclooxygenase [19]. Rest of the synthesized derivatives of 4-methyl salicylic acid were exhibited less than 50 percent of inhibitory activities for both isozymes of alkaline phosphatase.

Table 1. Alkaline phosphatase inhibition and docking scores of pimelic acid and 4-methylsalicylic acid derivatives.

Compound	*h*-TNAP	*h*-IAP	*h*-TNAP	*h*-IAP
	(IC_{50}±SEM)[a]/ %Inhibition		docking score by FlexX for top pose	
1	21.6%	1.01 ± 0.11	–11.29	–28.54
2	32.5%	9.46 ± 0.34	–13.32	–21.19
3	15.4 ± 0.75	36.3%	–21.57	–12.65
4	5.28 ± 0.51	28.8%	–27.88	–11.38
5	15.9 ± 0.31	37.4%	–20.16	–10.24
6	11.6 ± 0.55	2.80 ± 0.03	–24.64	–24.57
7	4.63 ± 0.31	6.72 ± 0.02	–30.25	–22.80
8	27.6%	21.6%	–10.09	–9.82
9	30.2%	23.4%	–10.92	–14.25
10	25.4%	26.7%	–9.27	–12.56
11	34.8%	30.3%	–13.21	–11.23
12	38.3%	1.50 ± 0.24	–12.86	–27.34
13	4.89 ± 0.84	40.23%	–26.37	–13.61
Levamisole	20.2 ± 1.90	-		
L-phenylalanine	-	100 ± 3.15		

[a] IC_{50} represents concentration at which the 50% of the enzyme activity was inhibited. All the values were expressed as IC_{50} ± SEM (standard error mean), n=3.

Molecular modeling investigation

For further detailed insight into the activities of synthetic compounds (**1-13**) for human alkaline phosphatases (tissue non-specific and intestinal), molecular modeling was performed for selective and most active analogues. LeadIT software was used for carrying out the modeling analysis of selected compounds. The crystal structures of the target proteins were unavailable at RCSB protein databank, hence previously reported homology models were used.

Levamisole ((*S*)-6-phenyl-2,3,5,6-tetrahydroimidazo[2,1-*b*]thiazole) was used as positive control in the biological assay and was docked inside the binding site of tissue non-specific APs (Figure 1). The resultant interactions involve conventional hydrogen bonding (1.86 Å) by nitrogen of imidazole ring with His437 and π-alkyl linkage of the same residue (3.85 Å) with thiazole ring. Val90 was at alkyl linkage (4.61 Å) with the thiazole ring. Three additional carbon H-bonds were noticed by Glu108 (2.60 Å), Pro91 (2.76 Å) and Thr436 (2.45 Å). Compound **4**, the most potent and selective inhibitor exhibited several interactions within the binding pocket of *h*-TNAP as represented in Figure 1a. The compound contain bis(2,6-dichlorophenyl)carbamothioylheptanediamide and shown several hydrogen bonds with important residues of the active site of human tissue non-specific alkaline phosphatase. Hydrogen bonds were formed by His434 (2.75 Å) with oxygen, His437 (1.64 Å) by amide group, His437 (2.86 Å) and Thr436 (3.36 Å) with oxygen of carbamothioylheptanediamide moiety. Moreover, His437 was also involved in π-lone pair interaction with oxygen (2.97 Å) and π-alkyl linkage with one of the chloro group (4.29 Å). Additionally, Asp320 showed pi-anion interactions with phenyl ring (4.57 Å). His324 formed 2 π-alkyl linkage with (3.85 Å and 4.96 Å) with both the chloro groups of same phenyl ring. Amino acid His434 in addition to hydrogen bond, was found to involve in π-sulfur interaction (5.20 Å) and 2 π-alkyl interactions one with chloro group (4.29 Å) at one phenyl ring, while second π-alkyl linkage with chloro group (4.48 Å) of another phenyl ring. When the interaction analysis of compound **13** was performed, it was noted that His324 (4.23 Å) was at π-π stacked interaction with nitrobenzyl ring and π-π T-shaped interactions at 5.93 Å with 4-methyl phenyl ring. Moreover, His434 (3.37 Å) also formed π-πstacked with 4-methyl phenyl ring. 2 hydrogen bonds were formed by Ser93 (3.18 Å and 2.46 Å) with both the oxygens of nitro group. Similarly, Arg167 (2.39 Å) was making H bond with oxygen of nitro group. In addition to these interactions, metal interactions were noted by nitrobenzyl ring Zn ions (2.89 Å and 3.92 Å) and withMg ion (4.67 Å).In case of compound **7**, His437 exhibited π-π T-shaped 4.24 Å with 1 of the nirophenyl group and H bond at a distance of 2.63 Å with oxygen atom of the carbamothioyl)heptanediamide. Similarly, hydrogen bonds were noticed by His434 (1.75 Å), Arg151 (2.84 Å) and Thr436 (2.50 Å) with oxygen atom of the carbamothioyl)heptanediamide. Moreover, π-π T-shaped interactions (5.71 Å) were observed by His321 with 1 of the phenyl rings. Ser93 was at distance of 2.50 Å making H bond with oxygen atom of other nitrophenyl

232 ring. π-π stacked interaction was noticed by phenyl ring with His324 (5.03 Å). Additionally,
233 metal interactions were found with Mg (4.56 Å) and both the Zn ions (3.96 Å and 3.26 Å). The
234 docking analysis revealed that the most active inhibitor exhibits the important interactions
235 within the binding pocket of TNAP and maybe responsible for the inhibitory activity of the
236 compound towards the enzyme. Table 1 showed the docking scores of all the compounds
237 against TNAP and IAP.

238

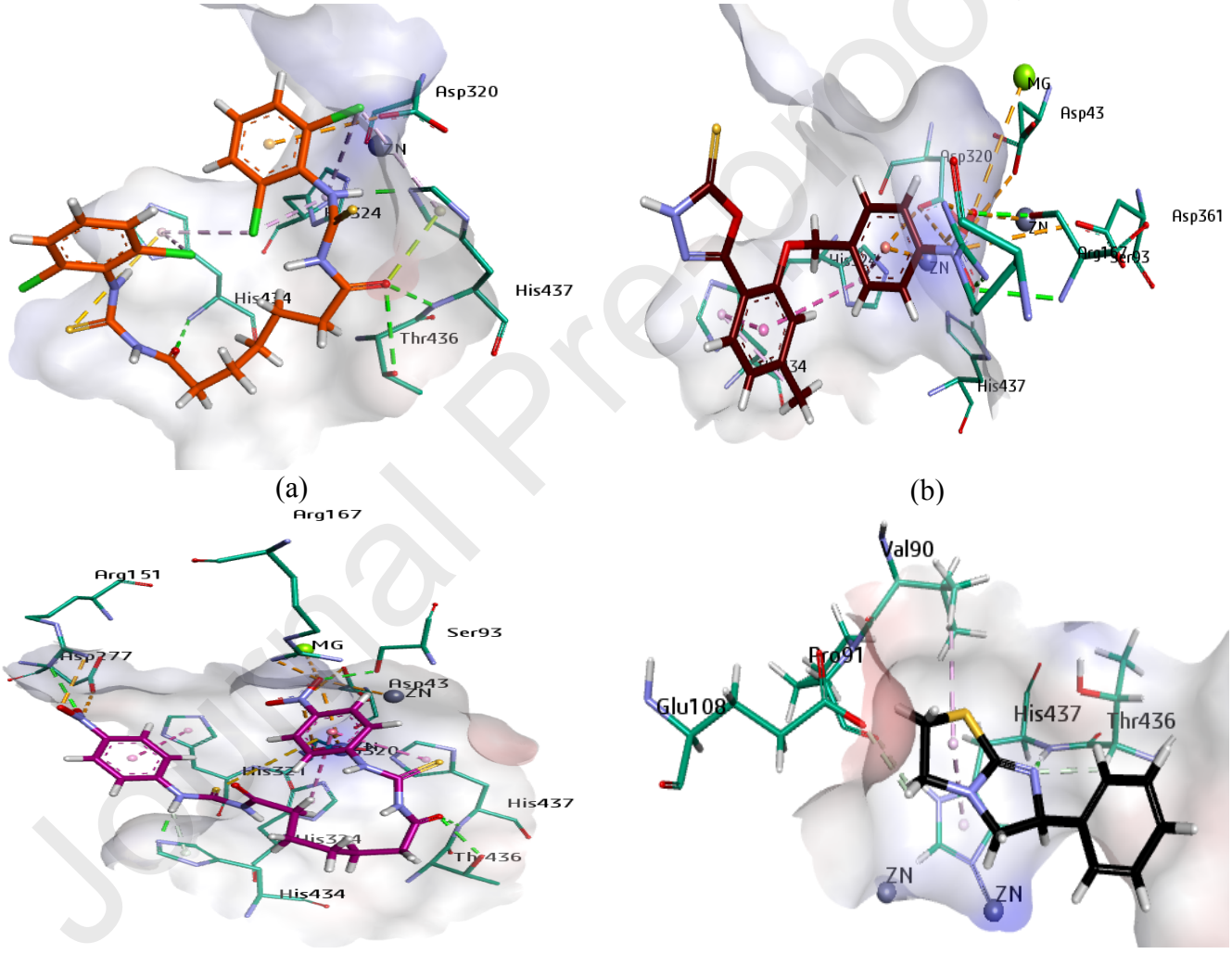


Figure 1. Plausible binding modes of compounds **4** (a); **13** (b); **7** (c) and **Levamisole** (d) inside tissue non-specific alkaline phosphatase model

239

Molecular docking analysis of L-phenylalanine inside the binding pocket of human intestinal alkaline phosphatase revealed that π-π stacked interactions were shown by phenyl ring with His320 (4.32 Å) and π-π T shaped with His432 (5.55 Å). His358 showed a hydrogen bond (2.77 Å) and a carbon H bond (2.18 Å) with phenylalanine. Amino acid Ser92 made a hydrogen bond (2.16 Å) with oxygen of the alanine group and the same oxygen was involved in a metal acceptor interaction with one of the Zinc ion (2.39 Å). Compound **1** (the most active and selective inhibitor of *h*-IAP) exhibited extensive network of hydrogen bonds like in one of the 3,4-dimethoxyphenyl ring formed H bond with Arg150 (3.09 Å and 2.90 Å), and the other one showed H-bonding with Arg314 (3.06 Å) by one methoxy group while with His279 (2.98 Å) by second methoxy group. However, carbamothioylheptanediamide moiety formed 3 hydrogen bonds one with Tyr276 (2.62 Å), second with Glu321 (1.96 Å) and third by sulfur of thiol group with Arg166 (3.79 Å).Other important interactions were π-sulfur bonds of thiol group with His317 (5.26 Å) and His153 (4.87 Å). Both the phenyl rings showed π-π T shaped interactions with amino acid His153 (4.74 Å) and π-π stacked interactions with His279 (5.39 Å). Moreover, metal acceptor interactions were also shown by oxygen (2.40Å) and (3.21 Å) sulfur atoms of carbamothioyl)heptanediamide moiety with Zinc ion. Moreover, a metal acceptor interaction was observed with zinc ion. Compound **12** showed significant number of hydrogen bonds by Arg150 (2.87 Å and 2.95 Å) with benzamide group and by Ser92 (2.99 Å and 3.09 Å) with 4-nitrobenzyl group. Moreover, other important interactions like π-π stacked with His317 (4.02 Å) and π-πT-shaped with His153 (5.80 Å) were also observed. In case of metal interactions, compound 12 showed interaction with both the Zn ions (3.12 Å and 3.98 Å). For compound **7**showed hydrogen bond by Glu321 (2.16 Å) with heptanediamide moiety, Ser92 (3.16 Å) with nitro phenyl group, Tyr276 (2.57 Å) and Arg150 (3.04 Å) with oxygen atom. π-sulfur interactions were noticed by His153 (4.81 Å) sulfur atom in the compound. Moreover, Tyr276 (5.76 Å) showed π-π T-shaped interactions. Additionally, metal interactions were noticed by Zn ions (3.25 Å and 3.84 Å). The structure-activity relationship and the docking studies of identified potent inhibitors provide an outstanding platform for further development of alkaline phosphatase inhibitors. The results of docking studies for the selected compounds were descriptive of the *in-vitro* enzyme inhibitory activity results, and the plausible binding poses elucidated the binding modes of these analogues.

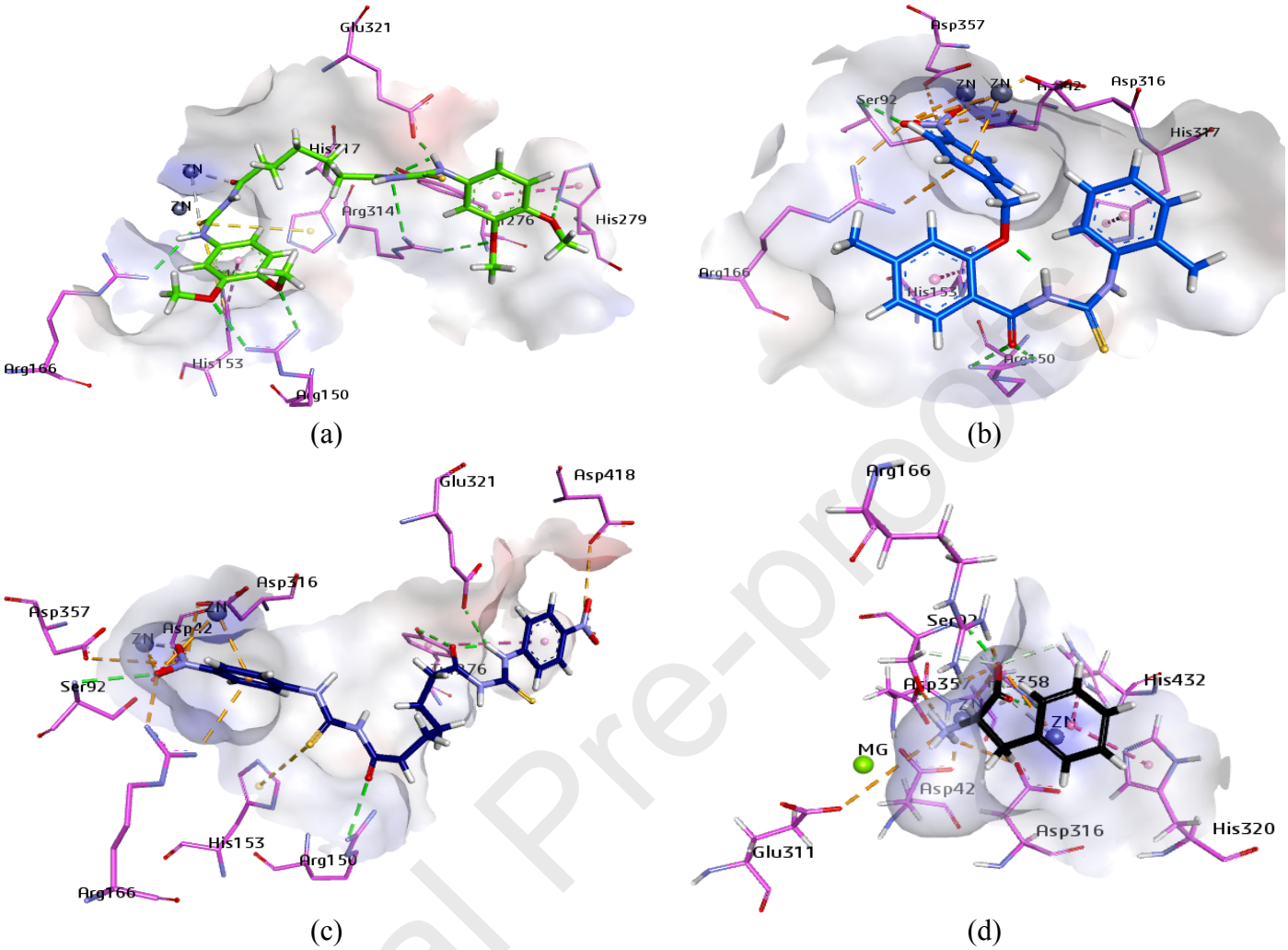


Figure 2. Plausible binding modes of compounds **1**(a); **12** (b); **7**(c) and **L-phenylalanine** (d) inside intestinal alkaline phosphatase model

3. Conclusions:

Synthesis of bis(thiourea) derivatives of pimmelic acid (**1-7**), and 3,5-dimethyl pyrazole (**11**), 1-aroyl-3-aryl thiourea (**12**) and 1,3,4-oxadiazole (**13**) derivatives of 4-methyl salicylic acid were synthesized and evaluated for selective inhibitory potential for *h*-TNAP and *h*-IAP. Among bis(thiourea) derivatives, **7** exhibited higher inhibitory activity for *h*-TNAP, represented by IC_{50} value of 4.63±0.31 µM which is about four times more than positive control levamisole (IC_{50} value: 19.2±0.01. Compounds **3, 4** and **5** demonstrated higher selective inhibitory activity for *h*-TNAP with IC_{50} values of 15.4±0.75 µM, 5.28±0.51 µM and 15.9±0.31 µM, respectively. Among salicylic acid derivatives, 4-methyl-2-((4-nitrobenzyl) oxy)-*N*-(o-

tolylcarbamothioyl)benzamide(**12**) was found to possess highest potency and selectivity for *h*-IAP with IC_{50} value of 1.50±0.24 μM, in comparison to positive control (L-phenylalanine: 80.1±0.01 μM). Compound (**13**), 1,3,4-oxadiazole-2-thione derivative of salicylic acid exhibited inhibition for *h*-TNAP with IC_{50} value of 4.89±0.84 which is comparable to inhibitory potential of standard inhibitor levamisole. Further insight of these selective and potent compounds were investigated by molecular docking analysis and it was observed that selected compounds exhibit key interactions with important residues of active site in the structures of enzymes.

4. Experimental:

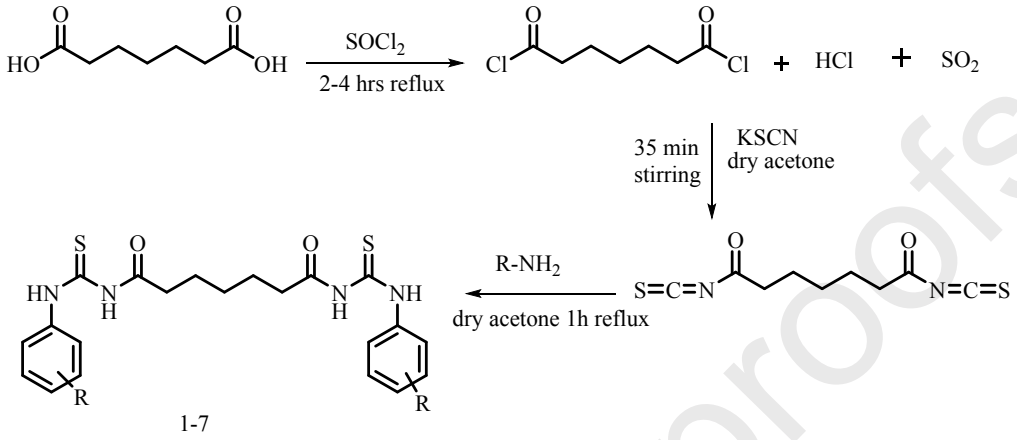
Material & Method:

Pimelic acid, thionyl chloride, potassium thiocyanate, *p*-methoxy aniline, 3,4 dimethoxy aniline, *p*-chloroaniline, 2,6-dichloroaniline, 2,5-dichloroaniline, *p*-toluidine, *p*-nitroaniline, 4-methyl salicylic acid, *p*-nitrobenzyl bromide, potassium carbonate, sodium bicarbonate, sodium hydroxide were purchased from sigma Aldrich. Acetone, ethyl acetate, *n*-Hexane, methanol, acetonitrile, ethanol, dichloromethane, carbon disulphide were brands of Riedel-de-Haen. All solvents were distilled before use. The progress of reaction was observed with the help of chromatography technique using TLC silica gel 60 F_{254} (coated on aluminum sheet) produced by MERCK. For development of chromatogram different solvent systems were used including *n*-Hexane, ethyl acetate and methanol in different ratios. Melting point was observed in organic and biological lab COMSATS University Islamabad, Abbottabad campus on SMP 20 digital Melting point apparatus OE/Digi(08-09)169/1 DMPA 09-01. Infrared spectra of all the samples were recorded on Hitachi Infrared spectrometer model 270 in KBr pellets Quaid-e-Azam University Islamabad. ^{1}H NMR δ (ppm) spectra were recorded in $CDCl_3$ and DMSO- d_6 by using Bruker AM-400 NMR spectrometer at 400 MHz in COMSATS University Islamabad, Abbottabad Campus.

Synthesis of *N*-aryl-*N′*-acyl-*bis*thioureas of pimelic acid (1-7)

The acid chloride was prepared by treating pimelic acid (3 mmol, 0.5 g) with $SOCl_2$ (9 mmol, 0.7 mL) on gentle heating for 3-4 hours. The pimeloyl chloride was reacted drop by drop with the solution of potassium thiocyanate (6 mmol, 0.6 g) in dry acetone. The resulting mixture was subjected to stirring for 45 mins accompanied by the addition of solution of substituted anilines which led to conversion into thioureas on heating under reflux. Upon completion, small amount

of ice-cold water was poured to precipitate out the product. Purification of all the products was carried out by using coloum chromatography. Synthesized compounds were obtained in yield (55-80%) [14].



1-7

Scheme 1. Synthesis of *N*-acyl-*N'*-aroyl-thioureas of pimelic acid

N^1,N^7-*bis*(3,4-dimethoxyphenyl)carbamothioyl)heptanediamide (1)

Yield: 60 %; R_f=0.3 (*n*-Hexane/EtOAc/MeOH, 2:7:1); m.p: 200-204 ^oC; FT-IR (KBr pellets) ($\bar{\upsilon}$, cm^{-1}): 3273 (NH stretch), 2941 (CH stretch), 1651 (C=O Stretch), 1233 (C=S), 1171 (CN); (^1H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.60 (s, 1H, NH), 11.69 (s, 1H, OH), 11.46 (s, 1H, NH), 7.12 (d, *J* = 8.7 Hz, 1H, Ar-H-5), 6.80 (dd, *J* = 8.6, 2.2 Hz, 1H, Ar-H-6), 6.67 (d, *J* = 2.1 Hz, 1H, Ar-H-2), 3.80 (s, 3H, OCH_3), 3.69 (s, 3H, OCH_3), 2.45 (t, *J* = 7.6 Hz, 2H, H-2"), 2.20 (t, *J* = 7.3 Hz, 2H, H-6"), 1.49-1.58 (m, 4H, $2CH_2$,H-3", H-5"), 1.30-1.27 (m, 2H, H-4").

N^1,N^7-*bis*(4-methoxyphenyl)carbamothioyl)heptanediamide (2)

Yield: 55 %; R_f =0.3 (*n*-Hexane/EtOAc/MeOH = 2:7:1); m.p: 182-185 ^oC; FT-IR (KBr pellets) ($\bar{\upsilon}$, cm^{-1}): 3273 (NH stretch), 2941 (CH stretch), 1651 (C=O Stretch), 1233 (C=S), 1171 (CN); ^1H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.35 (s, 2H, NH), 11.38 (s, 2H, NH), 7.50 (d, *J* = 8.6 Hz, 4H, Ar-H-2, Ar-H-6), 6.49 (d, *J* = 8.6 Hz, 4H, Ar-H-3, Ar-H-5), 3.37 (s, 6H, $2OCH_3$), 2.21 (t, *J* = 7.3 Hz, 4H, $2CH_2$, H-1"), 1.50-1.58 (m, 4H, $2CH_2$, H-2"), 1.30-1.27 (m, 2H, CH_2, H-3").

N^1,N^7-*bis* (2,5 dichlorophenyl)carbamothioyl)heptanediamide) (3)

Yield: 80 %; R_f = 0.5 (*n*-Hexane/EtOAc, 7:3); m.p: 176-179 ^oC; FT-IR (KBr pellets) ($\bar{\upsilon}$, cm^{-1}): 3188 (NH stretch), 2948 (CH stretch), 1649 (C=O Stretch), 1242 (C=S), 1171 (CN); ^1H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.46 (s, 2H, NH), 11.64 (s, 2H, NH), 7.89 (s, 2H, Ar-H-6), 7.48 (d, *J* = 8.0 Hz, 2H, Ar-H-3), 7.22 (d, *J* = 8.0 Hz, 2H, Ar-H-4), 2.29 (t, *J* = 7.8 Hz, 4H, $2CH_2$, H-1"), 1.53-1.58 (m, 4H, $2CH_2$, H-2"), 1.23-1.29 (m, 2H, CH_2, H-3").

N^1,N^7-*bis* (2,6 dichlorophenyl)carbamothioyl)heptanediamide (4)

Yield: 65 %; R_f = 0.5 (*n*-Hexane/EtOAc = 7:3); m.p: 175-178 ^oC; FT-IR (KBr pellets) ($\bar{\upsilon}$, cm^{-1}): 3273 (NH stretch), 2941 (CH stretch), 1651 (C=O Stretch), 1233 (C=S), 1171 (CN); ^1H NMR (400 MHz, DMSO-d_6) δ (ppm): 11.80 (s, 2H, NH), 11.60 (s, 2H, NH), 7.50 (d, *J* = 8.1 Hz, 4H, Ar-H-3 & Ar-H-5), 7.33 (t, *J* = 8.0 Hz, 2H, Ar-H-4), 2.21 (t, *J* = 7.28, Hz, 4H, $2CH_2$, H-1"), 1.58-1.51 (m, 4H, $2CH_2$, H-2"), 1.29-1.35 (m,2H, CH_2, H-3").

N^1,N^7-*bis*((4-chlorophenyl)carbamothioyl)heptanediamide (5)

Yield: 65 %; R_f = 0.4 (*n*-Hexane/EtOAc = 7:3); m.p: 203-206 ^oC; FT-IR (KBr pellets) ($\bar{\upsilon}$, cm^{-1}): 3300 (NH stretch), 2919 (CH stretch), 1665 (C=O Stretch), 1246 (C=S), 1171 (CN); ^1H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.64 (s, 2H, NH), 11.40 (s, 2H, NH), 7.69 (d, *J* = 8.0 Hz, 4H ,Ar-H-3, Ar-H-5), 7.37 (d, *J* = 8.0 Hz, 4H, Ar-H-2, Ar-H-6), 2.29 (t, *J* = 7.8 Hz, 4H, $2CH_2$, H-1"), 1.48-1.53 (m, 4H, $2CH_2$, H-2"), 1.29-1.23 (m ,2H, CH_2, H-3").

N^1,N^7-*bis*(*p*-tolylcarbamothioyl)heptanediamide (6)

Yield: 60 %; R_f = 0.5 (*n*-Hexane/EtOAc = 7:3); m.p: 212-214 ^oC; FT-IR (KBr pellets), ($\bar{\upsilon}$, cm^{-1}): 3304 (NH stretch), 2939, (CH stretch), 1694 (C=O Stretch), 1254 (C=S), 1171 (CN); ^1H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.25 (s, 2H, NH), 10.01 (s, 2H, NH), 7.72 (d, *J* = 8.2 Hz, 4H, Ar-H-2 & Ar-H-6), 7.34 (d, *J* = 8.1, 4H, Ar-H-3 & Ar-H-5), 2.53 (s, 6H, $2CH_3$), 2.40 (t, *J* = 7.3 Hz, 4H, $2CH_2$, H-1"), 1.74-1.87 (m, 4H, $2CH_2$, H-2"), 1.59-1.51 (m, 2H, CH_2, H-3").

N^1,N^7-*bis*(*p*-nitrocarbamothioyl)heptanediamide(7)

Yield: 69 %; R_f = 0.4 (*n*-Hexane/EtOAc = 7:3); m.p: blacken at 222 ^oC; FT-IR (KBr pellets), ($\bar{\upsilon}$, cm^{-1}): 3185 (NH stretch), 2968, (CH stretch), 1692 (C=O Stretch), 1592, 1429, 1264 (C=S), 1171 (CN).

Synthesis of ester from 4-methyl salicylic acid

Ester of 4-methyl salicylic acid was prepared by refluxing methanolic solution of salicylic acid (6.57 mmol, 0.5g) with catalytic amount of concentrated sulphuric acid for 12 hours. Excess acid was quenched using sodium bicarbonate solution (10 %). Ethyl acetate (20 mL × 3) was used to extract the ester from aqueous layer. Anhydrous $MgSO_4$ was added to remove water from organic layer. Filtration was performed to remove $MgSO_4$ and filtrate was evaporated to get ester (57) as an oily product with 85 % yield [20].

Yield: 85 %; R_f = 0.8 (*n*-Hexane/EtOAc = 7:3); m.p (°C):Oil.

Alkylation of OH group of 4-methyl salicylic acid(8)

p-Nitrobenzyl bromide (3 mmol, 0.648 g) was dissolved in 10 ml of acetonitrile. 4-Methyl salicylate (3 mmol, 0.5 g) and potassium carbonate (3.3 mmol, 0.453 g) was added to this solution. Stirring the mixture for 1.5 hours and then heating at 60 ℃ for 16 hours led to the formation of product followed by filtration to remove impurities. To remove the salt formed during the reaction filtration was performed. Filtrate was concentrated to get protected ester (8). Purification of the products 8 was carried out by using coloum chromatography to get 72 % yield [20].

Yield: 72 %; R_f =0.5 (*n*-Hexane/EtOAc = 7:3); m.p: 135-138°C; FT-IR (KBr pellets) ($\bar{\upsilon}$, cm^{-1}): 2950 (CH stretch), 1692 (C=O stretch), 1539 (NO_2 stretch), 1299 (C-O);^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.31 (d, *J* = 8.8 Hz, 2H, Ar-H-3', Ar-H-5'), 7.80 (d, *J* = 8.8 Hz, 2H, Ar-H-2', H-6'), 7.67 (d, *J* = 7.8 Hz, 1H, Ar-H-6), 7.09 (s, 1H, H-3), 6.90 (d, *J* = 7.8 Hz, 1H, H-5), 5.35 (s, 2H, H-7'), 3.82 (s, 3H, OCH_3), 2.35 (s, 3H, CH_3).

Synthesis of 2-(4-nitrobenzyl)oxy)benzohydrazide (9)

O-Protected methyl salicylate (8) (0.7 mmol, 0.2 g,) & $NH_2NH_2.H_2O$ (1.3 mmol, 0.1mL) were dissolved in ethanol and subjected to heating under reflux for 4 hours. Ethanol was evaporated on rotavapor and solid product (9) was rinsed with cold water, filtered, dried and was purified by using coloum chromatography to yield 78 % pure compound [20].

Yield: 78 %; R_f = 0.2 (*n*-Hexane/EtOAc = 6:4); m.p: 156-158 °C; FT-IR (KBr pellets) ($\bar{\upsilon}$, cm^{-1}): 3350 (NHstretch), 2990 (Ar CH stretch), 1604 (C=O stretch), 1299 (C-O); ^{1}H NMR: (400 MHz,

DMSO-d_6) δ (ppm): 9.20 (s, 1H, NH), 8.28 (d, *J* = 8.7 Hz, 2H Ar-H-3', Ar-H-5'), 7.77 (d, *J* = 8.7 Hz, 2H, Ar-H-2', Ar-H-6'), 7.50 (d, *J* = 7.8 Hz, 1H, Ar-H-6), 6.98 (s,1H, H-3), 6.78 (d, *J* = 7.8 Hz, 1H, H-5), 5.38 (s, 2H, H-7), 4.52 (s, 2H, NH_2), 2.35 (s, 3H, CH_3).

Saponification process (10)

O-Protected ester (9) (0.664 mmol, 0.2 g) was dissolved in 10 mL of methanol. To this solution was added sodium hydroxide (1.5 equiv, 20 % aqueous solution). Solution was heated at 65 ℃ for 8 hours. Progress of reaction was followed using TLC. Addition of 5M solution of HCl to the reaction mixture on completion of reaction decreased pH = 2. Acid (10) was precipitated out and precipitates were collected by filtration and washed with water and petroleum ether to remove the unreacted ester. Purification of the compounds was done by recrystallization using methanol.A yellow colored product with 78 % yield was obtained [20].

Yield: 78 %; R_f =0.3 (*n*-Hexane/EtOAc = 6:4); m.p: 175-178 ^oC; FT-IR (KBr pellets) ($\bar{\upsilon}$, cm^{-1}): 3050 (Ar CH stretch), 1650 (C=O stretch), 1539 (NO_2 stretch), 1243 (C-O), ^{1}HNMR (400 MHz, DMSO-d_6) δ (ppm): 11.20 (s, 1H, OH), 8.39 (d, *J* = 8.0 Hz, 2H, Ar-H-5', H-3'), 8.15 (d, *J* = 8.0 Hz, 2H, Ar-H-6', Ar-H-2'), 7.87 (d, *J* = 8.2 Hz, 1H, H-6), 7.67 (s, 1H, H-3), 6.75 (d, *J* = 8.2 Hz, 1H, H-5), 5.35 (s, 2H, H-7'), 2.08 (s, 3H, CH_3).

Synthesis of (3,5-dimethyl-1*H*-pyrazol-1-yl)(4-methyl-2-(*4*nitro benzyl)oxy)phenyl)methanone (11)

Hydrazide (11) (0.83 mmol, 0.2508 g) & acetyl acetone (0.83 mmol, 0.085 mL) were dissolved in 5 mL of MeOH and concentrated HCl was used to catalyse the reaction. Heating of reactants were carried out at 56^{o}C for 12 hours to achieve the formation of products. By using rotary excess solvent was removed and solid residue was washed with chilled ethanol to get product (62) in 70 % yield [16].

Yield: 70 %; R_f =0.6 (*n*-Hexane/EtOAc = 6:4); m.p: 105-107 ^oC; FT-IR (KBr pellets) ($\bar{\upsilon}$, cm^{-1}): 2990 (Ar CH stretch), 1650 (C=O Stretch), 1299 (C-O); ^{1}H NMR: (400 MHz, DMSO-d_6) δ (ppm): 8.24 (d, *J* = 8.7 Hz, 2H, Ar-H-3', Ar-H-5'), 7.80 (d, *J* = 8.7 Hz, 2H, Ar-H-2', H-6'), 7.70 (d, *J* = 8.0 Hz, 1H, H-6), 7.19 (s, 1H, H-3), 7.01 (d, *J* = 7.8 Hz, 1H, H-5), 6.30 (s, 1H, H-4"), 5.44 (s, 2H, H-7'), 2.50 (s, 3H, H-6"), 2.35 (s, 3H, H-7"), 2.19 (s, 3H, H-7).

Synthesis of 1-aroyl-3-arylthiourea of 4-methyl salicylic acid (12)

The acid chloride was prepared by dissolving 4-methyl-2-((4-nitrobenzyl)oxy)benzoic acid (10) (0.348 mmol, 0.1 g) in toluene. $SOCl_2$ (0.45 mmol, 0.04 mL) was added to convert acid into halide. Synthesis of acid chloride involved the heating of reactants for 4 hrs. Conversion of acid chloride into isothiocyanate was achieved by dropwise addition of it to the potassium thiocyanate solution in dry acetonitrile. The reaction mixture was subjected to stirring for 1.5 hours leading to the formation of isothiocyanate which was attacked by *p*-toluidine in dry acetonitrile. Upon completion of reaction small amount of distilled ice-cold water was poured in reaction mixture to precipitate out thiourea (12). Purification of the compounds was carried out by recrystallization using methanol to get 70 % yield [20].

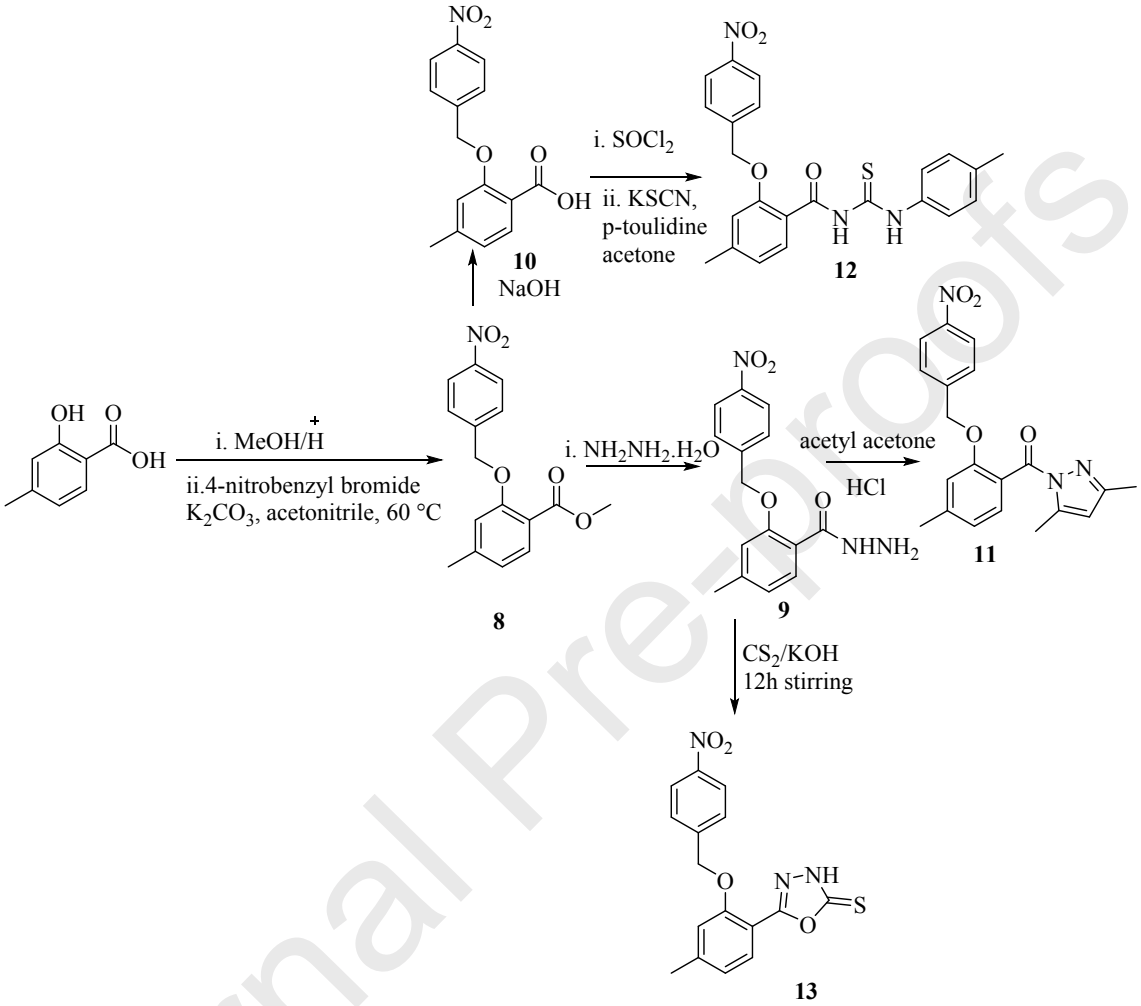
Yield: 70 %; R_f =0.6 (*n*-Hexane/EtOAc = 6:4); m.p: 135 ^oC; FT-IR (KBr pellets) ($\bar{\upsilon}$, cm^{-1}): 3300 (NH stretch), 2990 (Ar CH stretch), 1650 (C=O stretch), 1239 (C=S), 1171 (CN); ^1H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.54 (s, 1H, NH), 11.10 (s, 1H, NH), 8.23 (d, *J* = 8.6 Hz, 2H, Ar-H-3', Ar-H-5'), 7.91 (d, *J* = 8.6, 2H, Ar-H-2', Ar-H-6'), 7.80 (d, *J* = 7.8 Hz, 1H, H-6), 7.68 (s, 1H, H-3), 7.53 (d, *J* = 7.8 Hz ,1H, H-5), 7.20 (d, *J* = 8.5 Hz, 2H, Ar- H-2", Ar-H-6"), 6.93 (d, *J* = 8.5 Hz, 2H, Ar -H-3", Ar-H-5"), 5.47 (s, 2H, CH_2, H-7'), 2.40 (s, 3H, CH_3, H-7), 2.31 (s, 3H, CH_3, H-7").

Synthesis of 5-(4-methyl-2-(4-nitrobenzyl)oxy)phenyl)-1,3,4-oxadiazole-2(*3H*)thione (13)

2-(4-Nitrobenzyl)oxy)benzohydrazide (9) (0.3322 mmol, 0.1 g) was dissolved in ethanol (5 mL), carbon disulfide (0.495 mmol, 0.03 mL,) and potassium hydroxide (0.3322 mmol, 0.002 g) was added to it. Mixture was refluxed till the required product was obtained. After the reaction was completed, solid residue obtained by evaporation of solvent was dissolved in water to separate the water insoluble impurities. Conc. HCl was added to acidify the filtrate. Product (13) was precipitated out which was washed with water and purified by colum chromatography to obtain in 65 % yield [17].

Yield: 65 %; R_f =0.3 (*n*-Hexane/EtOAc = 6:4); m.p: 200-204 ^oC; FT-IR (KBr pellets) ($\bar{\upsilon}$, cm^{-1}): 3327 (NHstretch), 3000 (Ar CH stretch), 1605 (C=O stretch), 1299 (C-O), 1170 (C=S); ^1H NMR (400 MHz, DMSO-d_6) δ (ppm): 14.9 (s, 1H, NH), 8.24 (d, *J* = 8.7 Hz, 2H, Ar-H-3', Ar-H-5'), 7.86

(d, J = 8.7 Hz, 2H, Ar-H-2 & H-6'), 7.70 (d, J = 7.8 Hz, 1H, H-6), 7.19 (s, 1H, H-3), 7.00 (d, J = 7.8 Hz, 1H, H-5), 5.40 (s, 2H, H-7'), 2.38 (s, 3H, H-7).



Scheme 2. Synthesis of 4-methyl salicylic acid derivatives

Biological Evaluation:

Transfection to express alkaline phosphatases

Expression of ALPs (*h*-TNAP and *h*-IAP) in COS-7 cells was carried out in 10cm^2 plates through lipofectamine, as previously reported protocol [21]. The plasmids expressing human TNAP and IAP were already described [22, 23]. Transfection was performed when cells became confluent. Cells were incubated in serum free DMEM/F-12 containing 24µL of lipofectamine reagent and 6

µL of plasmid DNA. After 24 hr, media was replaced with DMAM/F-12 containing 20% fetal bovine serum (FBS). Transfected cells were harvested after 40-72 hr from the addition of plasmid and transfecting reagent mixture.

Extraction of protein content:

Desired protein was extracted from the cells as previously reported [21]. Transfected cells were washed with Tris-saline buffer and were removed from the plate through harvesting buffer (0.1 mM phenylmethylsulfonyl fluoride (PMSF), 95 mM NaCl and 45 mM Tris-buffer, pH 7.5). Harvested cells were washed twice by centrifugation at 4 ^{o}C and 300×g for 5 min. Cells were re-suspended and sonicated in harvesting buffer containing aprotinin (10 µg/mL). Subsequently, cellular debris was removed by centrifugation for 10 min at 4 ^{o}C and 300×g. Glycerol was added to the supernatant at a final concentration 7.5% and store at -80 ^{o}C. Before use in the assay, protein concentration was determined by Bradford microplate assay [22].

Enzyme inhibition assay for alkaline phosphatases:

CDP-Star®, a chemiluminescent substrate, was used to determine the inhibitory potential of synthesized compounds after slight modification in previously reported protocol [24]. Assay buffer containing 250 mM diethanolamine (DEA), 2.5 mM $MgCl_2$, 0.5 mM $ZnCl_2$ at a pH 9.5 was used. *h*-TNAP and *h*-IAP were added in each well of white 384 wells microplate at a concentration of 47 ng and 56 ng protein/well, respectively. Test compounds were analyzed at a final concentration of 200 µM with subsequent addition of CDP-Star® substrate. Change in luminescence was recorded by microplate reader (BioTek FLx800, Instruments, Inc. USA) in reference to positive control as well as blank. Levamisole is the known inhibitor of *h*-TNAP and L-phenylalanine was used as positive control for *h*-IAP. Data was analyzed PRISM 5.0 (GraphPad, San Diego, California, USA). IC_{50} values for those compounds were determined that exhibited inhibitory potential more than 50%.

Molecular docking studies

To justify the inhibition caused by potent inhibitors, most plausible binding modes were predicted using molecular docking studies. Because of unavailability of x-ray crystallographic structure of human alkaline phosphatases, homology models generated previously by our research group were used for docking studies [23]. Structures of the tested compounds were drawn by MOE builder

tool [25] and optimization was achieved using MMFF94x forcefield [26]. Afterwards the energy minimization of the target proteins was carried out by Molecular Operating Environment [25]. LeadIT (BioSolveIT GmbH, Germany) [27] was used to perform docking analysis of the prepared ligands inside the respective receptors. Load Receptor Utility of the LeadIT software was used to load the receptor and the metallic ions were selected as part of the protein. Active pocket of the protein for docking analysis was identified by keeping the amino acid residues in 10.0 Å radius around zinc ions. Values of the amino acid flips, metal co-ordinates and water handling were kept as by default. Once docking analysis was completed, the possible interactions of ligands with receptor proteins were inspected for studying the possible interactions using HYDE assessment [28]. Discovery Studio Visualizer was used to perform visualize the interactions of ligand and receptors [29].

Conflict of Interest:

The author(s) declare that they have no conflict of interests.

Acknowledgements:

A.M. is thankful to CUI for providing startup research grant under CIIT. J.I. is thankful to the Higher Education Commission of Pakistan for the financial support through Project No.Ph-V-MG-3/Peridot/R&D/HEC/2019. J.S. received support from the Natural Sciences and Engineering Research Council of Canada (NSERC; RGPIN-2016-05867) and was the recipient of a "Chercheur National" Scholarship from the *Fonds de Recherche du Québec – Santé* (FRQS).

References:

1. M.H.Le Du, et al., Crystal structure of alkaline phosphatase from human placenta at 1.8Å resolution implication for a substrate specificity. J. Biolo. Chem., 276(12) (2001) 9158-9165.
2. J. Iqbal, et al., Tricyclic coumarin sulphonate derivatives with alkaline phosphatase inhibitory effects: in vitro and docking studies. J Enzyme Inhib Med Chem, 33(1) (2018), 479-484.
3. C.H. Linder, et al., Glycosylation differences contribute to distinct catalytic properties among bone alkaline phosphatase isoforms. Bone,. 45(5) (2009)987-993.
4. M.-H. Le Du, J.L. Millán, Structural evidence of functional divergence in human alkaline phosphatases. J. biolo. Chem., 277(51) (2002) 49808-49814.

5. G. Burnstock, The therapeutic potential of purinergic signalling. Biochemical pharmacology, 151 (2018) 157-165.
6. D. M. Zaher, et al., Recent advances with alkaline phosphatase isoenzymes and their inhibitors. Arch Pharm, 353(5) (2020), e2000011.
7. M. Haarhaus, et al., Alkaline phosphatase: a novel treatment target for cardiovascular disease in CKD. Nat. Rev. Nephrol., 13(7) (2017) 429.
8. U.Sharma, D. Pal, R. Prasad, Alkaline phosphatase: an overview. Indian J. Clinical Biochem., 29(3) (2014) 269-278.
9. J.L. Millán, M.P. Whyte, Alkaline phosphatase and hypophosphatasia. Calcif. Tissue Int., 98(4) (2016) 398-416.
10. J.Fawley, D.M. Gourlay, Intestinal alkaline phosphatase: a summary of its role in clinical disease. J. Surg. Res., 202(1) (2016) 225-234.
11. D. Ekinci, M. Şentürk, Ö.İ. Küfrevioğlu, Salicylic acid derivatives: synthesis, features and usage as therapeutic tools. Expert Opin. Ther. Pat.21(12) (2011) 1831-1841.
12. M.S.I.Makki, et al., Synthesis of substituted thioureas and their sulfur heterocyclic systems of p-amino salicylic acid as antimycobacterial agents. J. Chem., 2012. 2013.
13. A.Kozlenkov, et al., Residues determining the binding specificity of uncompetitive inhibitors to tissue-nonspecific alkaline phosphatase. J. Bone Miner. Res., 19(11) (2004) 1862-1872.
14. A.Saeed, et al., Synthesis of sulfadiazinyl acyl/aryl thiourea derivatives as calf intestinal alkaline phosphatase inhibitors, pharmacokinetic properties, lead optimization, Lineweaver-Burk plot evaluation and binding analysis. Bioorg. & Med. Chem., 26(12) (2018) 3707-3715.
15. A. Halim, A. Nadiah, Z. Ngaini, Synthesis and bacteriostatic activities of bis (thiourea) derivatives with variable chain length. J. Chem., 2016 (2016).
16. A. Saeed, A. Mumtaz, Microwave-accelerated Synthesis of Some 1-Aroyl-3,5-dimethylpyrazoles, Chinese Chemical Lett., 19 (2008) 1305-1308,.
17. A. Mumtaz, A. Saeed, I. Malik, Synthesis, characterization and anti-microbial activities of mercaptoxazole, mercaptotriazole and their derivatives, J. ChemicalSoc. Pak., 36(5)(2014).
18. S. Aaeem, et al., Thiourea derivatives in drug design and medicinal chemistry: A short review. Journal of Drug Design and Medicinal Chemistry, 2(1) (2016), 10.
19. K.M.Khan, et al., 1, 3, 4-Oxadiazole-2 (3H)-thione and its analogues: A new class of non-competitive nucleotide pyrophosphatases/phosphodiesterases 1 inhibitors. Bioorg. & Med. Chem., 17(22)(2009) 7816-7822.
20. L.Shaowei, C.Xiufang, C.Changshui, K.Shaoyong, Novel salicylic acid-oriented thiourea-type receptors as colorimetric chemosensor: Synthesis, characterizations and selective naked-eye recognition properties, Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 96 (2012) 18–23.
21. M.Wink, et al., Nucleoside triphosphate diphosphohydrolase-2 (NTPDase2/CD39L1) is the dominant ectonucleotidase expressed by rat astrocytes. Neuroscience, 138(2) (2006) 421-432.
22. T.Kiffer-Moreira, et al., Catalytic signature of a heat-stable, chimeric human alkaline phosphatase with therapeutic potential. PLoS One, 9(2) (2014) 89374.

23. I.Khan, , et al., Investigation of quinoline-4-carboxylic acid as a highly potent scaffold for the development of alkaline phosphatase inhibitors: synthesis, SAR analysis and molecular modelling studies. RSC Advances, 5(79) (2015) 64404-64413.
24. S. Narisawa, et al., High throughput screening and identification of novel alkaline phosphatase inhibitors-A druggable target for the treatment of vascular calcification. J. Bone Miner. Res, 22 (2007) 1700-1710.
25. MOE (Molecular Operating Environment) Version 2016.01. Chemical Computing Group, (CCG). http://www.chemcomp.com/MOE Molecular_Operating_Environment.htm.
26. P. Labute, Protonate 3D: Assignment of Macromolecular Protonation State and Geometry, Chemical Computing Group, 2007. http://www.chemcomp.com/journal/proton.htm.
27. LeadIT version 2.3.2; BioSolveIT GmbH, Sankt Augustin, Germany, 2017, www.biosolveit.de/LeadIT
28. N. Schneider, G. Lange, S. Hindle, R. Klein, M. A. Rarey, A consistent description of HYdrogenbond and DEhydration energies in protein–ligand complexes: methods behind the HYDE scoring function, J. Comp. aided. Mol. Des. 27 (2013) 15-29.
29. Dassault Systèmes BIOVIA, Discovery Studio Modeling Environment, Release 2017, San Diego: Dassault Systèmes, 2016.

Bisthioureas of pimelic acid and 4-methylsalicylic acid derivatives as selective inhibitors of tissue-nonspecific alkaline phosphatase (TNAP) and intestinal alkaline phosphatase (IAP): Synthesis and molecular docking studies

Amara Mumtaz[1*], Kiran Saeed[1], Abid Mahmood[2], Sumera Zaib[2], Aamer Saeed[3], Julie Pelletier[4], Jean Sévigny[4,5], Jamshed Iqbal[2*]

[1]*Department of Chemistry, COMSATS University Islamabad, Abbottabad Campus, Abbottabad-22060, Pakistan.*
[2]*Center for Advanced Drug Research, COMSATS University Islamabad, Abbottabad Campus, Abbottabad-22060, Pakistan*
[3]*Department of Chemistry, Quaid-i-Azam University Islamabad, Pakistan.*
[4]*Centre de Recherche du CHU de Québec – Université Laval, Québec, QC, G1V 4G2, Canada.*
[5]*Département de microbiologie-infectiologie et d'immunologie, Faculté de Médecine, Université Laval, Québec, QC, G1V 0A6, Canada.*

Abstract:

Alkaline phosphatases (ALPs) are membrane bound metalloenzymes, distributed all over the body. Recent studies have revealed that by targeting ALPs can lead towards the treatment of many deadliest diseases including cardiac, cancerous and brain diseases. Thioureas and their derivatives are of considerable significance and are privileged scaffolds in medicinal chemistry. They show a wide range of pharmacological activities such as antibacterial, antiparasitic, anti-inflammatory and antioxidants *etc.* On the other hand, salicylic acid and its derivatives are known for its broad

spectrum of activities. The work presented comprises of synthesis of *N*-acyl-*N′*-aryl substituted bisthioureas of pimelic acid (1-7) and 3,5-dimethyl pyrazole (11), 1-aroyl-3-aryl thiourea (12) and 1,3,4-oxadiazole (13) derivatives of 4-methyl salicylic acid. Structures of all the synthesized compounds were characterized by FT-IR and ^{1}H NMR spectroscopic analysis. Synthesized compounds were evaluated for their alkaline phosphatases inhibition potential and exhibited high potency as well as selectivity towards *h*-TNAP and *h*-IAP. Compound 7 and 12 which were the bisthiourea derivative of pimmelic acid and thiourea derivative of 4-methyl salicylic acid, respectively, showed excellent selectivity against *h*-TNAP and *h*-IAP, respectively.

Bis thioureas of pimelic acid and 4-methylsalicylic acid derivatives as selective inhibitors of tissue-nonspecific alkaline phosphatase (TNAP) and Intestinal alkaline phosphatase (IAP) inhibitors: Synthesis and molecular docking studies

Amara Mumtaz[1#*], Kiran Saeed[1#], Abid Mahmood[2], Sumera Zaib[2], Aamer Saeed[3], Julie Pelletier[4], Jean Sévigny[4,5], Jamshed Iqbal[2*]

[1]*Department of Chemistry, COMSATS University Islamabad, Abbottabad Campus, Abbottabad-22060, Pakistan.*
[2]*Center for Advance Drug Research, COMSATS University Islamabad, Abbottabad Campus, Abbottabad-22060, Pakistan*
[3]*Department of Chemistry, Quaid-i-Azam University Islamabad, Pakistan.*
[4]*Centre de Recherche du CHU de Québec – Université Laval, Québec, QC, G1V 4G2, Canada.*
[5]*Département de microbiologie-infectiologie et d'immunologie, Faculté de Médecine, Université Laval, Québec, QC, G1V 0A6, Canada.*

Conflict of Interest:

The author(s) declare that they have no conflict of interests.

Bis thioureas of pimelic acid and 4-methylsalicylic acid derivatives as selective inhibitors of tissue-nonspecific alkaline phosphatase (TNAP) and intestinal alkaline phosphatase (IAP): Synthesis and molecular docking studies

Amara Mumtaz[1#*], Kiran Saeed[1#], Abid Mahmood[2], Sumera Zaib[2], Aamer Saeed[3], Julie Pelletier[4], Jean Sévigny[4,5], Jamshed Iqbal[2*]

[1]*Department of Chemistry, COMSATS University Islamabad, Abbottabad Campus, Abbottabad-22060, Pakistan.*
[2]*Center for Advance Drug Research, COMSATS University Islamabad, Abbottabad Campus, Abbottabad-22060, Pakistan*
[3]*Department of Chemistry, Quaid-i-Azam University Islamabad, Pakistan.*
[4]*Centre de Recherche du CHU de Québec – Université Laval, Québec, QC, G1V 4G2, Canada.*
[5]*Département de microbiologie-infectiologie et d'immunologie, Faculté de Médecine, Université Laval, Québec, QC, G1V 0A6, Canada.*

Research Highlights

1. Synthesis of Pimelic acid and 4-methylsalicylic acid derivatives
2. Identification of selective alkaline phosphatases inhibitors
3. Molecular docking studies