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ORIGINAL ARTICLE / ÖZGÜN MAKALE

SYNTHESIS, *IN SILICO* AND *IN VITRO* ANTI-PROLIFERATIVE STUDIES OF SOME NOVEL BENZAMIDO SUBSTITUTED IMIDAZO[1,2-*b*]PYRIDAZIN-2-ONES

BAZI YENİ BENZAMİDO SÜBSTİTÜE İMİDAZO[1,2-*b*]PİRİDAZİN-2-ON BİLEŞİKLERİNİN SENTEZİ, *İN SİLİKO* VE *İN VİTRO* ANTİ-PROLİFERATİF ÇALIŞMALARI

Kanna SRUTHI[1]*, Mogili SUMAKANTH[1], Kumar CB MAHENDRA[2], Kondapalli NARESH[3]

[1]Department of Pharmaceutical Chemistry R.B.V.R.R Women's College of Pharmacy, Osmania University, Hyderabad, India
[2]Department of Pharmaceutical Analysis, St. Mary's College of Pharmacy, Secunderabad, India
[3]Department of Pharmaceutical Chemistry, G. Pulla Reddy College of Pharmacy, Osmania University, Hyderabad, India

SUMMARY

*A novel series of Benzamido substituted imidazo[1,2-b]pyridazin-2-ones were designed ,synthesized and tested for their possible anti-proliferative activity. The title compounds were accomplished by the reaction of various 4-Arylidine-2-Phenyl-Oxazol-5-Ones with 3-Aminopyridazine in the presence of ethylene glycol. The chemical structures of the synthesized compounds was characterised by IR, ^{1}H NMR, ^{13}C NMR, Mass spectra and elemental analysis. The synthesized compounds were evaluated for their possible anti-proliferative activity on human cancer cell lines A375 and Colo-205 using MTT assay. Among the tested compounds, compound **6m** and **6n** showed potent anti-proliferative activity on both cell lines and compounds **6i, 6g** and **6d** also demonstrated significant activity. Molecular properties and toxicity were predicted for compounds **6a-6n** with OSIRIS property explorer. All the tested compounds were proved to be druggable candidates and free from toxicity and teratogenecity. The molecular docking studies of synthesized compounds **6a-6n** on B-Raf V600E kinase (PDB ID: 3IDP) further revealed that the active compounds bound to the active site and interacting with Cys532, Phe595, Lys483 residues are comparable with the interactions of Dabrafenib. These studies broadened the scope of imidazo[1,2-b]pyridazine benzamides as promising antiproliferative agents.*

Keywords: *anti-proliferative agents; benzamides; B-Raf V600E kinase; imidazopyridazines*

* **Corresponding Author / Sorumlu Yazar:** Kanna SRUTHI
e-mail: sruthik.207@gmail.com

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ÖZET

Yeni bir seri benzamido sübstitüe imidazo[1,2-b]piridazin-2-on bileşikleri tasarlanmış, sentezlenmiş ve olası anti-proliferatif etkisi test edilmiştir. Bu bileşikler, çeşitli 4-arilidin-2-feniloksazol-5-on bileşiklerinin etilen glikol varlığında 3-aminopiridazin ile reaksiyona sokulmasıyla gerçekleştirilmiştir. Sentezlenen bileşiklerin kimyasal yapıları IR, ^{1}H NMR, ^{13}C NMR, Kütle spektrumu ve elemental analiz ile karakterize edilmiştir. Sentezlenen bileşikler, insan kanser hücre hatları A375 ve Colo-205 üzerinde olası anti-proliferatif etkileri açısından MTT testi ile değerlendirilmiştir. Test edilen bileşikler arasında bileşik ***6m*** *ve* ***6n*** *her iki hücre hattı üzerinde güçlü anti-proliferatif etki göstermiş ve bileşik* ***6i, 6g*** *ve* ***6d*** *de önemli bir etki göstermiştir. OSIRIS Property Explorer programı ile bileşik* ***6a-6n*** *için moleküler özellikleri ve toksisitesi tahmin edilmiştir. Test edilen tüm bileşiklerin, ilaç olmaya aday olduğu kanıtlanmış ve toksisite ve teratojeniteden arındırılmıştır. Sentezlenen* ***6a-6n*** *bileşiklerinin B-Raf V600E kinaz (PDB ID: 3IDP) üzerine yapılan moleküler doking çalışmaları, aktif bölgeye bağlanan aktif bileşiklerin Cys532, Phe595, Lys483 aminoasit kalıntılarıyla etkileşimleri ile Dabrafenib'in etkileşimlerinin karşılaştırılabilir olduğunu ortaya koymuştur. Bu çalışmalar, imidazo[1,2-b]piridazin benzamitlerin umut verici anti-proliferatif ajanlar kapsamını genişletmiştir.*

Anahtar kelimeler: *anti-proliferatif ajanlar; B-Raf V600E kinase; benzamidler; imidazopiridazinler*

INTRODUCTION

Mitogen-activated protein kinases (MAPKs) constitute an important signalling pathway in cells, and is involved in various processes like controlling gene expression, cell division, cell survival, apoptosis and cell differentiation. The most significant upstream activators of this pathway are the Raf proteins (A-Raf, B-Raf or c-Raf) which are the key mediators of response to growth factors (EGF, FGF, PDGF, etc.) [1-4]. The apparent association between the presence of B-Raf and RAS mutations in various cancer types is due to the upregulation of the RAS–RAF–MAPkinase-(MEK)–ERK pathway. Interruption of this mechanism can have a profound influence in inhibiting the abnormal signalling of these mutated tyrosine kinases upon ligand binding. Mutation occurs most frequently in B-Raf oncogenic protein kinase. Inhibition of V600E [5-7] which is a mutated strain of B-RAF kinase represents a promising avenue for melanoma treatment.

Vemurafenib (PLX4032, RG7204), PLX-4720, Dabrafenib (GSK2118436) (Fig.1.) demonstrated clinical benefits as Raf kinase inhibitors [8,9,10] in the treatment of various types of tumours with manageable side effects. The inhibitory activity of these drugs on B-Raf kinase [11,12] revealed that the substituted benzene sulphonamido group is important for inhibitory activity. On the other hand the molecular entities possessing Heterocyclic/ non heterocyclic and substituted/unsubstituted benzamides have been well investigated to possess antimicrobial [13-20], analgesic, anti-inflammatory [21,22], anticonvulsant [23-28], anticancer [29-33], and other biological activities [34-41] and imidazopyridazines being structural analogues of purines were also reported to be promising scaffolds exhibiting a wide range of biological activities [42-46]. In view of these reports, our interest moved to develop new benzamido substituted scaffolds which are isosteric analogs of sulphonamido substituted marketed drugs as possible B-Raf Kinase inhibitors and are expected to acquire different selectivities

and novelties. Our strategy is to develop a novel series imidazo[1,2-*b*]pyridazines as possible anti-proliferative agents, occupying di-substitution at C-3 position (aryl and benzamido). This was achieved by the ring opening of 4- arylidine-2-phenyl-oxazol-5-one with 3-aminopyridazine.

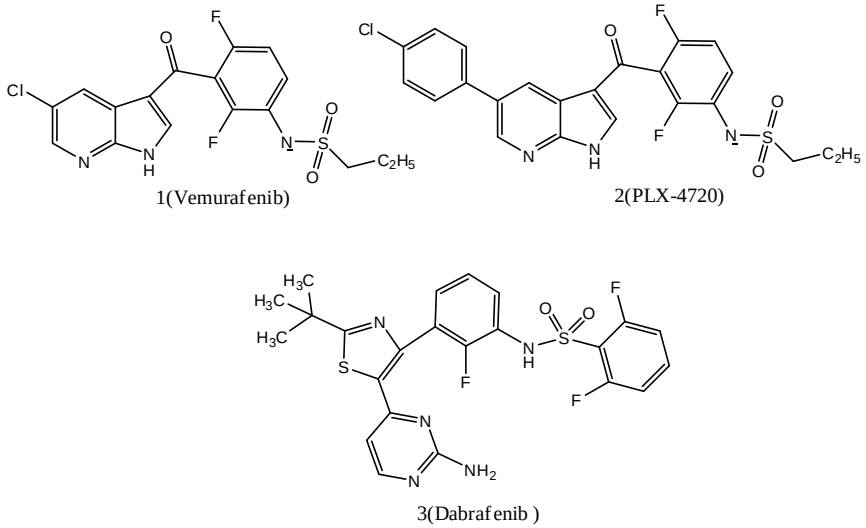

Figure 1. Vemurafenib (**1**), PLX-4720 (**2**), Dabrafenib (**3**)

MATERIALS AND METHODS

Synthetic grade reagents and solvents were obtained from SD fine chemicals, Sigma Aldrich and Merck. The progress of the reactions was monitored by using Silica Gel coated TLC plates visualised under UV light or in iodine chamber. The synthesized compounds were purified by recrystallization and purity was determined by measuring melting point in Kofler hot stage melting point apparatus and are uncorrected. The IR spectrums were recorded on Shimadzu FTIR spectrophotometer (1% KBr discs). ^{1}H NMR at 400MHz and ^{13}C NMR 100MHz was recorded on Bruker Avance II 400 MHz NMR spectrophotometer with TMS as internal standard mass spectrum was recorded using Agilent 1100. Elemental analysis was determined in Carlo Erba 1108 elemental analyzer and compared with the calculated data.

Experimental methods

Synthesis

General procedure for the synthesis of 4-arylidine-2-phenyl oxazole-5-one (5a-5n)

Synthesis of 4-arylidine-2-phenyl oxazole-5-one (**5a-5n**) carried out as per the procedure described in our earlier publications [12]. Equimolar amount of (0.25 mol), benzoyl glycine and aromatic/hetero aromatic aldehyde taken along with acetic anhydride (0.75 mol) and anhydrous sodium acetate (0.25 mol) in a 100ml conical flask and heated on electrical hot plate with constant shaking. The contents of the reaction were transferred to a RB flask and refluxed for 2 hrs. Then 100 ml of ethyl

alcohol was added to the contents of the flask, and the mixture was allowed to stand overnight. The product obtained was collected through suction, and washed with ice-cold alcohol followed by of boiling water and dried.

General procedure for the synthesis of imidazo[1,2-*b*]pyridazine benzamide (6a-6n)

Each of the 0.001 mole of 4-arylidine-2-phenyl oxazole-5-one (**5a-5n**) were allowed to react with 0.001 mole of 3-amino pyridazine in ethylene glycol at 120°C for 6-8 hrs. The reactions were mentored by TLC. After completion of the reaction the contents were cooled to room temperature and then poured into beaker containing 100ml water. The crude product was collected by filtration under suction, dried and recrystallized from ethanol.

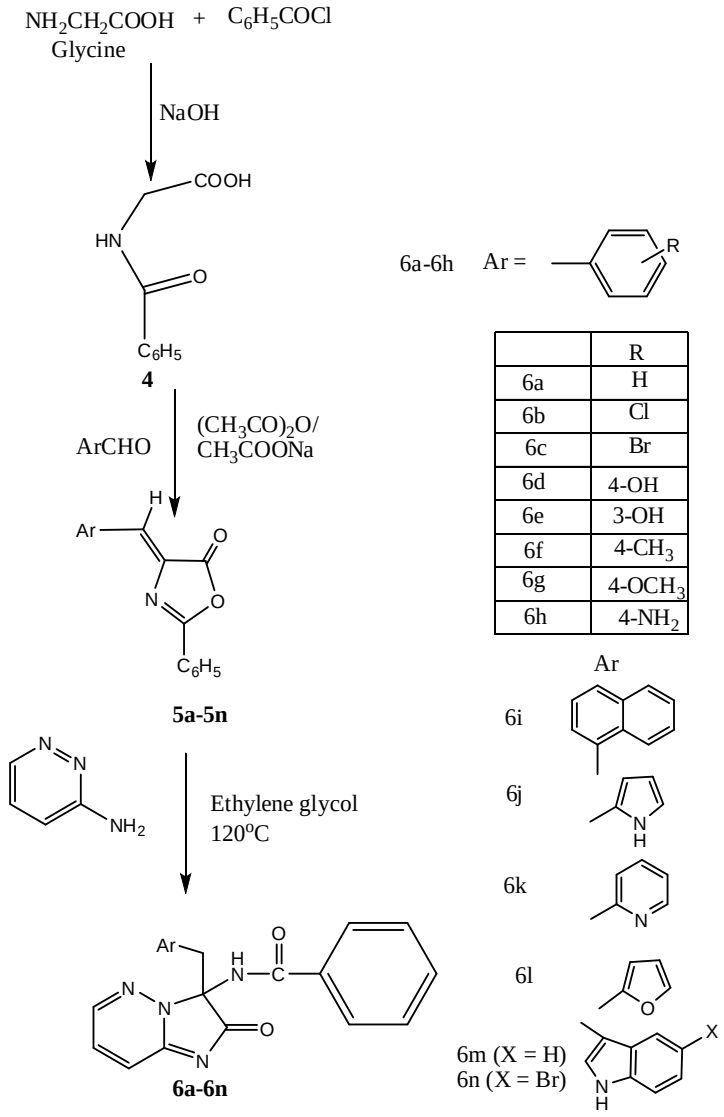

Figure 2. Synthetic route for the benzamido substituted imidazo[1,2-*b*]pyridazine-2-ones

***N*-(3-Benzyl-2-oxo-2,3-dihydroimidazo[1,2-*b*]pyridazin-3-yl)benzamide (6a)**

% Yield: 78%, mp(°C): 216-218, IR(KBr) ν (cm^{-1}): 3328(NH), 2928(CH), 1681(C=O), ^{1}H NMR (DMSO) δ (ppm): 9.71(s,1H,NH), 7.95(d, 1H, IPArH) , 7.56-7.88 (m, 4H, ArH),7.26-7.38 (m, 6H, ArH), 7.24(t, 2H, IPArH), 3.48(s, 2H,-CH_2), ^{13}C NMR (DMSO-d_6) δ (ppm): 174.2, 169.3, 162.1, 153.3, 141.5, 138.3, 134.1, 132.1, 131.4, 129.1, 128.6, 127.3, 122.2, 56.8, 48.3; MS m/z: 344(M+), Anal. calcd. (%) for: $C_{20}H_{16}N_4O_2$: C, 69.76; H, 4.68; N, 16.27; O, 9.29, found: C, 69.72; H, 4.66; N, 16.30; O, 9.32

***N*-(3-(4-Chlorobenzyl)-2-oxo-2,3-dihydroimidazo[1,2-*b*]pyridazin-3-yl)benzamide (6b)**

% Yield: 74%, mp(°C): 210-212, IR(KBr) ν (cm^{-1}): 3330(NH); 2924(CH), 1681(C=O), ^{1}H NMR (DMSO) δ (ppm): 9.68 (s,1H,NH), 8.12(d, 1H, IPArH), 7.56-7.67(m, 5H, ArH), 7.22-7.45(m, 6H, ArH, IPArH), 3.48(s, 2H,-CH_2), ^{13}C NMR (DMSO-d_6) δ (ppm): 173.9, 168.3, 162.2, 152.4, 141.3, 136.0, 134.1, 133.5, 1318, 129.2, 128.6, 126.9, 122.8, 57.4, 49.6, MS m/z: 378(M+), Anal. calcd. (%) for: $C_{20}H_{15}ClN_4O_2$: C, 63.41; H, 3.99; Cl, 9.36; N, 14.79; O, 8.45, found: 63.45; H, 3.95; Cl, 9.40; N, 14.77; O, 8.43

***N*-(3-(4-Bromobenzyl)-2-oxo-2,3-dihydroimidazo[1,2-*b*]pyridazin-3-yl)benzamide (6c)**

% Yield: 72 %, mp(°C):244-246, IR(KBr) ν (cm^{-1}): 3326(NH); 2926(CH), 1682(C=O), ^{1}H NMR (DMSO) δ (ppm): 9.71(s,1H,NH), 8.06(d, 1H, IPArH), 7.48-7.58(m, 5H, ArH), 7.14-7.35(m, 6H, ArH, IPArH), 3.38(s, 2H,-CH_2), ^{13}C NMR (DMSO-d_6) δ (ppm): 172.5, 167.9, 162.3, 152.1, 142.3, 136.3, 134.1, 133.7, 130.4, 128.6, 127.1, 126.4, 55.0, 44.4. MS m/z: 422(M+), Anal. calcd. (%) for: $C_{20}H_{15}BrN_4O_2$: C, 56.75; H, 3.57; Br, 18.88; N, 13.24; O, 7.56, found C, C, 56.71; H, 3.59; Br, 18.90; N, 13.28; O, 7.52

***N*-(3-(4-Hydroxybenzyl)-2-oxo-2,3-dihydroimidazo[1,2-*b*]pyridazin-3-yl)benzamide (6d)**

% Yield: 78 %, mp(°C): 250-252, IR(KBr) ν (cm^{-1}): 3329(NH); 2930(CH), 1681(C=O), ^{1}H NMR (DMSO) δ (ppm): 9.71(s, 1H, NH), 7.95(d, 1H, IPArH), 7.38-7.58(m, 5H, ArH), 7.11-7.28(m, 6H, ArH, IPArH), (4.96s, 1H, OH), 3.32(s, 2H,-CH_2), ^{13}C NMR (DMSO-d_6) δ (ppm): 174.1, 166.8, 162.5. 152.4, 155.1, 142.2, 136.7, 134.5, 132.2, 130.1, 129.5, 127.6, 126.4, 59.1, 47.8, MS m/z: 360(M+), Anal. calcd. (%) for: $C_{20}H_{16}N_4O_3$: C, 66.66; H, 4.48; N, 15.55; O, 13.32, found C, 66.69; H, 4.50; N, 15.52; O, 13.30

***N*-(3-(3-Hydroxybenzyl)-2-oxo-2,3-dihydroimidazo[1,2-*b*]pyridazin-3-yl)benzamide (6e)**

% Yield: 72%, mp(°C): 198-200, IR(KBr) ν (cm^{-1}): 3329(NH); 2930(CH), 1681(C=O), ^{1}H NMR (DMSO) δ (ppm): 9.84(s,1H,NH), 7.98(d, 1H, IPArH), 7.28-7.68(m, 6H, ArH), 7.16-7.20(m, 5H, IPArH), (4.59s, 1H, OH), 3.45(s, 2H,-CH_2), ^{13}C NMR (DMSO-d_6) δ (ppm): 173.1, 168.2, 162.2, 156.2, 151.9, 144.3, 136.8, 134.3, 132.1, 130.2, 129.8, 127.6, 126.4, 58.9, 47.5, MS m/z: 360(M+), Anal. calcd. (%) for: $C_{20}H_{16}N_4O_3$: C, 66.66, H, 4.48, N, 15.55, O, 13.32, found C, 66.68; H, 4.46; N, 15.51; O, 13.28

***N*-(3-(4-Methylbenzyl)-2-oxo-2,3-dihydroimidazo[1,2-*b*]pyridazin-3-yl)benzamide (6f)**

% Yield: 85%, mp(°C): 212-214, IR(KBr) ν (cm^{-1}): 3328(NH); 2928(CH), 1680(C=O), ^{1}H NMR (DMSO) δ (ppm): 9.71(s,1H,NH), 7.95(d, 1H, IPArH) , 7.38-7.58(m, 5H, ArH),7.10-7.28 (m, 6H, ArH, IPArH), 3.48(s, 2H,-CH_2), 1.78 (s,3H,-CH_3), ^{13}C NMR (DMSO-d_6) δ (ppm): 17.36, 167.9, 163.2, 152.3, 141.4, 138.6, 136.2, 124.5, 132.1, 130.3, 128.1, 127.8, 122.3, 57.4, 46.9, 25.4, MS m/z: 358(M+), Anal. calcd. (%) for: $C_{21}H_{18}N_4O_2$: C, 70.38; H, 5.06; N, 15.63; O, 8.93, found C, 70.35; H, 5.05; N, 15.66; O, 8.94

***N*-(3-(4-Methoxybenzyl)-2-oxo-2,3-dihydroimidazo[1,2-*b*]pyridazin-3-yl)benzamide (6g)**

% Yield: 74%, mp(°C): 226-228, IR(KBr) ν (cm^{-1}): 3330(NH); 2928(CH), 1681(C=O), ^{1}H NMR (DMSO) δ (ppm): 9.82(s,1H, NH), 7.96(d, 1H, IPArH), 7.45-7.67(m, 5H, ArH),7.19-7.38 (m, 6H, ArH, IPArH), 3.41(s, 2H,-CH_2), 2.92(s, 3H,-OCH_3), ^{13}C NMR (DMSO-d_6) δ (ppm): 174.3, 168.9, 162.4, 153.3, 145.8, 141.2, 137.9, 134.6, 131.4, 129.6, 128.4, 127.5, 119.2, 68.5, 58.6, 41.5, MS m/z: 374(M+), Anal. calcd. (%) for: $C_{21}H_{18}N_4O_3$: C, 67.37; H, 4.85; N, 14.96; O, 12.82, found 67.33; H, 4.83; N, 14.99; O, 12.85

***N*-(3-(4-Aminobenzyl)-2-oxo-2,3-dihydroimidazo[1,2-*b*]pyridazin-3-yl)benzamide (6h)**

% Yield: 65%, mp: 302-304, IR(KBr) ν (cm^{-1}): 3332(NH), 3329(NH); 2930(CH), 1681(C=O), ^{1}H NMR (DMSO) δ (ppm): 9.78(s,1H,NH), 8.02(d, 1H, IPArH), 7.42-7.82(m, 6H, ArH), 7.22-7.38 (m, 5H, ArH, IPArH), 5.24(s, 2H,-NH_2), 3.44(s, 2H,-CH_2), ^{13}C NMR (DMSO-d_6) δ (ppm): 173.9, 168.8, 162.3, 153.4, 146.8, 141.9, 138.6, 134.1, 131.2, 129.7, 128.6, 121.8, 120.1,55.9, 44.6, MS m/z: 359 (M+), Anal. calcd. (%) for: $C_{20}H_{17}N_5O_2$: C, 66.84; H, 4.77; N, 19.49; O, 8.90, found C, 66.88; H, 4.79; N, 19.46; O, 8.87.

***N*-(3-(Naphthalen-1-ylmethyl)-2-oxo-2,3-dihydroimidazo[1,2-*b*]pyridazin-3-yl) benzamide (6i)**

% Yield: 69%, mp(°C): 304-306, IR(KBr) ν (cm^{-1}): 3329(NH), 2930(CH), 1681(C=O), ^{1}H NMR (DMSO) δ (ppm): 9.74(s,1H,NH), 7.81-8.01(m, 6H, ArH), 7.35-7.62(m, 6H, ArH),7.18(t, 2H, IPArH), 7.06(d, 1H, ArH), 3.44(s, 2H,-CH_2), ^{13}C NMR (DMSO-d_6) δ (ppm): 173.6, 168.9, 162.4, 153.5, 142.2, 138.4, 135.6, 132.1, 131.8, 129.1,128.4, 127.6, 126.4,125.3, 122.4, 121.2, 56.4, 45.6, MS m/z: 394(M+), Anal. calcd. (%) for: $C_{24}H_{18}N_4O_2$: C, 73.08; H, 4.60; N, 14.20; O, 8.11, found C, 73.05; H, 4.56; N, 14.24; O, 8.15

***N*-(3-((1*H*-Pyrrol-3-yl)methyl)-2-oxo-2,3-dihydroimidazo[1,2-*b*]pyridazin-3-yl) benzamide (6j)**

% Yield: 74%, mp(°C): 224-226, IR(KBr) ν (cm^{-1}): 3330(NH), 3328(NH), 2929(CH), 1682(C=O), ^{1}H NMR (DMSO) δ (ppm): 9.78(s,1H,NH), 7.91(d, 1H, IPArH), 7.44(t, 2H, IPArH), 7.24-7.36(m, 4H, ArH), 7.22(d, 2H, ArH), 7.18(d, 1H, ArH), 7.08(d, 1H, ArH), 4.9(s,1H,NH), 3.45(s, 2H,-CH_2), ^{13}C NMR (DMSO-d_6) δ (ppm): 174.1,169.4, 162.3, 153.3, 141.2, 138.2, 134.3, 132.1, 131.2, 128.2, 123.4, 122.1, 120.5, 118.3, 116.4, 55.6, 48.9, MS m/z: 333 (M+), Anal. calcd. (%) for: $C_{18}H_{15}N_5O_2$: C, 64.86; H, 4.54; N, 21.01; O, 9.60, found C, 64.81; H, 4.55; N, 21.03; O, 9.61.

***N*-(2-Oxo-3-(pyridin-3-ylmethyl)-2,3-dihydroimidazo[1,2-*b*]pyridazin-3-yl)benzamide (6k)**

% Yield: 77%, mp(°C): 230-234, IR(KBr) ν (cm^{-1}): 3330(NH), 29229(CH), 1681(C=O), ^{1}H NMR (DMSO) δ (ppm): 9.78(s,1H,NH), 7.92(d, 1H, IPArH), 7.65-7.86(m, 3H, ArH), 7.36-7.57(m, 4H, ArH), 7.28(dd, 2H, ArH), 7.21(t, 2H, IPArH), 3.41(s, 2H,-CH_2), ^{13}C NMR (DMSO-d_6) δ (ppm): 174.2, 169.1, 162.2, 153.2, 148.3, 146.7, 142.2, 139.2, 138.2, 136.4, 134.2, 132.5, 129.2, 126.2, 122.4, 54.6, 48n.3. MS m/z: 345 (M+), Anal. calcd. (%) for: $C_{19}H_{15}N_5O_2$: C, 66.08; H, 4.38; N, 20.28; O, 9.27, found C, 66.01; H, 4.40; N, 20.29; O, 9.30

***N*-(3-(Furan-3-ylmethyl)-2-oxo-2,3-dihydroimidazo[1,2-*b*]pyridazin-3-yl)benzamide (6l)**

% Yield: 71%, mp(°C): 286-288, IR(KBr) ν (cm^{-1}): 3330(NH), 2928(CH), 1681(C=O), 1152(C-O), ^{1}H NMR (DMSO) δ (ppm): 9.72(s,1H,NH), 7.81(d, 1H, IPArH), 7.38-51(m, 5H, ArH), 7.28-7.35(m, 4H, IPArH), 7.15(t, 1H, ArH), 3.48(s, 2H,-CH_2), ^{13}C NMR (DMSO-d_6) δ (ppm): 174.1,169.4, 162.3, 153.3, 142.2, 141.2, 140.7, 138.2, 134.3, 132.1, 131.2, 128.2, 122.1, 118.9, 116.6, 55.6, 48.9,MS m/z: 334 (M+), Anal. calcd. (%) for: $C_{18}H_{14}N_4O_3$: C, 64.66; H, 4.22; N, 16.76; O, 14.36, found C, 64.63; H, 4.25; N, 16.75; O, 14.37

***N*-(3-((1*H*-Indol-3-yl)methyl)-2-oxo-2,3-dihydroimidazo[1,2-*b*]pyridazin-3-yl)benzamide (6m)**

% Yield: 70%, mp(°C): 306-310, IR(KBr) ν (cm^{-1}): 3340(indole NH); 3232(NH); 2932(CH), 1681(C=O), ^{1}H NMR (DMSO) δ (ppm): 9.78(s,1H,NH), 7.94(d, 1H, IPArH), 7.38-7.52(m, 6H, ArH), 7.24-7.30(m, 3H, ArH), 7.21(t, 2H, IPArH), 7.05(d, 1H, ArH), 4.91(s,1H, NH), 3.48(s, 2H,-CH_2), ^{13}C NMR (DMSO-d_6) δ (ppm): 174.2, 169.1 , 162.3, 153.4, 141.2, 138.5, 134.3, 132.8, 132.0, 129.2, 125.2, 122.4, 121.4, 119.5, 118.4, 117.3, 55.8, 42.5, MS m/z: 383 (M+), Anal. calcd. (%) for: $C_{22}H_{17}N_5O_2$: C, 68.92; H, 4.47; N, 18.27; O, 8.35, found; C, 68.90; H, 4.49; N, 18.30; O, 8.31.

***N*-(3-((5-Bromo-1*H*-indol-3-yl)methyl)-2-oxo-2,3-dihydroimidazo[1,2-*b*]pyridazin-3-yl) benzamide (6n)**

% Yield: 76%, mp(°C): 298-302, IR(KBr) ν (cm^{-1}): 3331(indole NH); 3329(NH); 2929(CH), 1681(C=O), ^{1}H NMR (DMSO) δ (ppm): 9.79(s, 1H, NH), 7.85(d, 1H, IPArH), 7.36-7.61(m, 5H, ArH), 7.25-7.30(m, 3H, ArH), 7.18(t, 2H, IPArH), 7.08(d, 1H, ArH), 4.94(s, 1H, NH), 3.44(s, 2H,-CH_2), NMR (DMSO-d_6) δ (ppm): 174.4, 169.4 , 162.4, 153.5, 141.3, 139.8, 138.2, 134.3, 133.5, 132.8, 129.2, 125.6, 121.5, 120.8, 119.5, 118.2, 117.5, 55.4, 48.6, MS m/z: 461 (M+), Anal. calcd. (%) for: $C_{22}H_{16}BrN_5O_2$: C, 57.16; H, 3.49; Br, 17.28; N, 15.15; O, 6.92, found C, 57.10; H, 3.51; Br, 17.30; N, 15.17; O, 6.92.

Anti-proliferative studies

The detailed procedure for anti-proliferative activity was described in our earlier publication [12]. ATCC type A375 and colo-205 cell lines were used and the viability and cell number was determined by Trypan blue exclusion dye method. The cells were seeded at a density of $1x10^5$ per each well in 100µl

of DMEM along with 10% fetal bovine serum. After 12 hrs seeding, above media was replaced with fresh media. Each of the test compound and standard were diluted to get the concentrations from 0.1nM to 10μM. Then 10 μl of sample was added to test wells for each concentration in triplicate and DMSO was added to control wells. The above wells were incubated for 48 hours at 37°C supplemented with 5% CO_2. After 48 hours incubation, the above media was replaced with 100 μl of fresh DMEM and to this 10 μ l of MTT was added and incubated for 3 hours at 37°C with 5% CO_2. Then media was extracted with pipette and 200 μl of DMSO was added to each well and again incubated at 37°C for 15 minutes. Finally, the plates were read at 570 nm using plate reader and IC_{50} values were calculated from the absorbance of test and control wells.

Molecular Docking

Molecular docking was carried out by using AUTODOCK 4.2.6 on Linux system [12,47]. The 3D structure of protein was obtained from RCSB protein data bank [48] PDBID 3IDP. The inhibitor molecule from PDB was removed and protein PDB was prepared using Autodock tools as per the procedure described in our recent work. The ligand structures were prepared using chemdraw and converted into 3D structures in pdb format using Prodrg server [49]. PDBQT files were prepared using Autodock from the pdb structures and used for docking. Docking procedure [47] was validated by re-docking with the inhibitor molecule (L1E) of the co-crystal structure (3IDP) in the active site and the same validated procedure was used for docking of the test and standard dabrafenib.

RESULTS AND DISCUSSION

Chemistry

In continuation to our earlier research on design and synthesis of heterocycles possessing anticancer activity and the importance of benzamido functionality in marketed and approved anticancer agents, we herein report the incorporation of benzamido functionality onto the imidazo[1,2-*b*]pyridazine leading to the development of hitherto unreported imidazo[1,2-*b*]pyridazine benzamides and their anti-proliferative activity on cell lines.

The preparation of 4-arylidine-2-phenyl oxazole-5-ones **5a-5n** was the integral part of the synthesis and was obtained from the reaction of benzoyl glycine with various aromatic hetero aromatic aldehydes. The attack of carbonyl of aromatic aldehydes and subsequent cylization constitute the formation of oxazole. This intermediate turned into title compounds **6a-6n** when treated with 3-amino pyridazine in ethylene glycol at 120°C. This reaction involves the ring opening of arylidine oxazole followed by condensation with 3 amino pyridazine.

The IR spectrum of compound **5a** showed absorption at 1155 cm^{-1} corresponds to C-O-C also the disappearance of NH absorption band at 3340 cm^{-1} and disappearance of absorption band at 3400 cm^{-1} confirmed the formation of compound **5a**. It is further confirmed by the disappearance of NH proton peak in ^{1}H NMR spectrum at δ 9.89 ppm and the absence of another proton peak δ 9.56 ppm corresponds to COOH confirms the formation of **5a**.

The title compounds **6a-6n** obtained by refluxing each of the 4-arylidine-2-phenyl oxazole-5-one **5a-5n** with equimolar concentration of 3-amino pyridazine in ethylene glycol at 120°C. The progress of the reaction and formation of compound was monitored by TLC. Initial ring opening of oxazole was facilitated by the attack of 3-amino pyridazine followed by cyclization gave the respective compounds. The FTIR spectra of compound **6a** exhibited a characteristic absorption band at 3334 cm^{-1} correspond to NH and another band at 1680 cm^{-1} corresponds to carbonyl of benzamide confirmed the formation of compound **6a**. Further, the appearance of prominent singlet at δ 9.68 ppm corresponds to NH proton and another at δ 3.41 ppm corresponds two methylene protons in ^{1}H NMR spectra confirmed the formation of compounds. The appearance of characteristic signals in ^{13}C NMR of compound **6a** δ 174.2, 169.3, 162.1, 153.3, 141.5, 138.3, 134.1, 132.1, 131.4, 129.1, 128.6, 127.3, 122.2, 56.8, 48.3 and molecular ion peak at m/z 344(M+) in the mass spectra characterize the compound **6a**. All the synthesized compounds characterized in the same way from their NMR, mass, FTIR spectra and elemental analysis data.

Anti-proliferative studies

The anti-proliferative ability of the synthesized compounds was studied by employing MTT assay on A 375 and Colo-205 human cancer cell lines. Initially the viability of the cells is determined by Trypan Blue dye exclusion method and maintained in DMEM and FBS. IC_{50} values were calculated from the absorbance read from test wells and control wells at 570nm. Dabrafenib was taken as the standard drug which is a B Raf V600E kinase inhibitor in order to compare the anti-proliferative activity of title compounds on B Raf V600E kinase expressed cell lines. Control wells also maintained in DMSO to compare the growth inhibition of test compounds. All the tests were carried out in triplicate and results are presented in terms of $IC_{50} \pm$ SD in table 1. The results of the anti-proliferative studies showed that most of the *N*-substituted imidazo[1,2-*b*]pyridazine benzamides were found remarkable activity in nanomolar range on A375 and colo-205 cell lines and compared with standard drug (Dabrafenib IC_{50} 5nM on A375 and 8nM on colo-205). In case of A375 cell lines compounds **6m, 6n, 6i** and **6g** exhibited highest activity with IC_{50} values 14 nM, 16nM, 20 nM & 35 nM respectively. Whereas, compounds containing pyridyl (**6k**), *p*-hydroxy phenyl **(6d**) and *p*-amino phenyl (**6h**) connected to $–CH_2$ exhibited excellent activity with IC_{50} values 44 nM, 46 nM and 76 nM respectively.

On Colo-205 cell lines, compounds carrying indole substituted imidazo[1,2-*b*]pyridazine benzamide exhibited potent inhibition with IC_{50} values of 21 nM & 20 nM. Compound bearing naphthyl substitution also demonstrated highest growth inhibition. Compounds **6d**, **6g**, **6h** and **6k** showed remarkable activity with IC_{50} values 58 nM, 48 nM, 45 nM and 52 nM respectively.

Table 1. Antiproliferative activity results of *N*-(3-substituted-2-oxo-2,3-dihydroimidazo[1,2-*b*] pyridazine)acetamides **6a-6n**

Compound	IC $_{50}$ value (nM) ± SD on A375 cell line	IC $_{50}$ value (nM)± SD on Colo-205 cell line
6a	425±5.1	590±4.2
6b	625±4.2	>1000
6c	188±3.5	356±3.4
6d	46±4.3	58±1.5
6e	505±2.8	895±5.6
6f	180±5.8	221±4.1
6g	35±2.9	48±2.2
6h	76±4.7	45±3.5
6i	20±1.9	28±1.7
6j	506±2.8	485±2.4
6k	44±2.1	52±2.2
6l	86±3.4	105±4.3
6m	14±1.1	21±1.4
6n	16±1.2	20±1.3
Dabrafenib	5±1.5	8±1.4

Molecular properties and toxicity prediction

The structures of synthesized benzamido substituted imidazo[1,2-*b*]pyridazines **6a-6n** were subjected to predict important molecular properties and the toxicity using OSIRIS molecular properties explorer. Properties such as cLogP, solubility, H-bond donors, H-bond acceptor, number of rotatable bonds and polar surface area were calculated. From these properties, it was observed that all the synthesized compounds could be druggable candidates as their properties are in accordance with Lipinski rule. Further, all the synthesized compounds were predicted to be free from mutagenicity.

Table 2. Molecular properties, toxicity prediction by OSIRIS explorer and docking score

Molecule Name	cLogP	cLogS	H-bond Acceptors	H-bond Donors	Polar Surface Area	Drug likeness	Binding Energy (Kcal/mol) [Autodock]
6a	1.8985	-3.48	6	1	74.13	6.5143	-8.68
6b	2.5045	-4.216	6	1	74.13	6.5484	-8.73
6c	2.6237	-4.314	6	1	74.13	4.7243	-9.15
6d	1.5528	-3.184	7	2	94.36	6.5109	-9.24
6e	1.5528	-3.184	7	2	94.36	6.5109	-8.39
6f	2.2424	-3.824	6	1	74.13	6.4656	-9.12
6g	1.8285	-3.498	7	1	83.36	6.5086	-9.07
6h	1.2212	-3.556	7	2	100.15	6.4891	-8.94
6i	3.0929	-5.086	6	1	74.13	6.5143	-10.18
6j	0.621	-2.504	7	2	89.92	6.5921	-8.45
6k	0.8976	-2.685	7	1	87.02	6.5143	-9.24
6l	1.0332	-3.138	7	1	87.27	6.2314	-8.75
6m	1.9379	-4.005	7	2	89.92	6.5921	-9.94
6n	2.6631	-4.839	7	2	89.92	4.8021	-9.76

Molecular Docking studies

The molecular docking methodology adopted for the present investigation was validated appropriately. In this docking validation protocol the inhibitor molecule (L1E) that lies in the co-crystal structure of BRafv600E (3IDP) was removed from the protein and re-docked into the active site of the same protein. The root mean square deviation (1.012) of the L1E docked conformation with that of the co-crystal conformation proven the validity of the method. This validated method was further used for the docking of imidazo pyridazine benzamides(**6a-6n**) and standard drug Dabrafenib. In further docking analysis, for each of the docked ligand, least binding energy (Kcal/mol) conformation (Table 2) was taken from the top ten conformations and ligand binding interactions with the BRafv600E kinase were studied and compared with Dabrafenib (-9.85 Kcal/mol).

The molecular docking studies of the structures of synthesized imidazo pyridazine benzamides (**6a-6n**) on BRafV600E kinase revealed that the ligand **6m** that exhibited highest anti-proliferative activity in the *in vitro* studies, bound to protein with binding energy -9.94 Kcal/mol. The indole NH of the ligand **6m** involved in H bond with the Gln 530 (2.141 Å) and the indole ring is closely associate with Cys 532, Trp 531 (Fig. 3a). The benzimido portion is in interaction with Phe 595, Asp 594, Gly 593. However, the imidazopyridazine ring is in close contact with Lys 483.

Another potent compound containing indole substitution **6n** aligned into the active site with a binding energy -9.76 Kcal/mol. In this compound imidazo pyridazine ring flips between the Phe 595, Leu 514 and involved in hydrophobic interactions (Fig. 3b). However, the indole ring is in close contact with the Cys 532 and Thr 529. The benzimido portion is positioned at the opening of the active site and associated with Leu597. Ligand **6i** (Fig. 3c) showed highest binding affinity than the standard dabrafenib (Fig. 3d) with binding energy of -10.18 Kcal/mol. The N1 of imidazopyridazine forms H bond (2.008 Å) with Lys 483. The benzimido group lies at the entrance of active site by interacting with Leu 505. However, the naphtyl ring interacted hydrophobically with Phe 595, Val 471 and the connecting methyl group interacts with Asp 594.

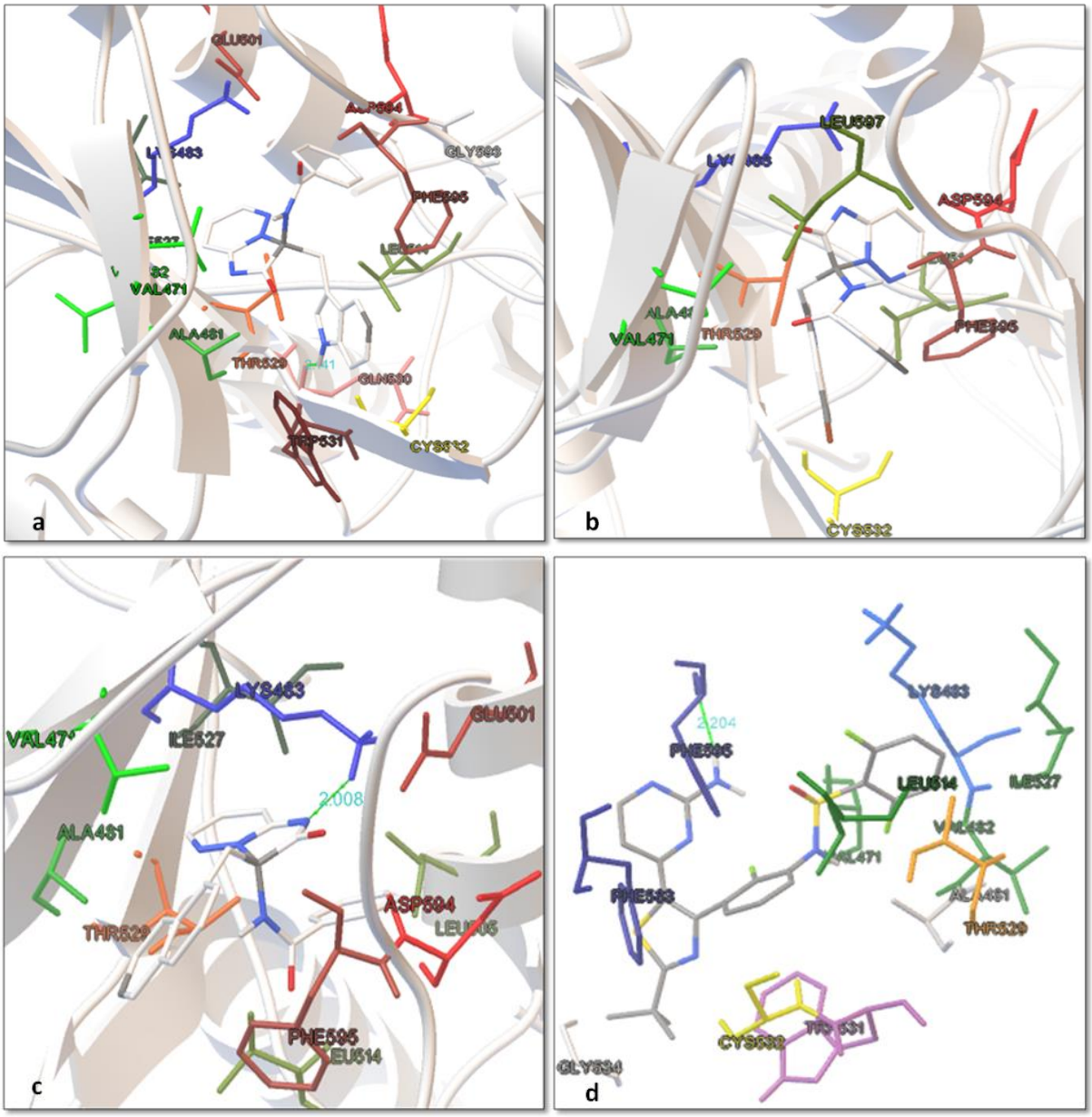

Figure 3. Compounds **6m** (a), **6n** (b), **6i** (c) and Dabrafenib (d) interactions with BrafV600E kinase (**PDBID: 3IDP**)

The above results suggest that the activity of the compounds correlated with the binding alignment and the interactions with the amino acid residues Phe 595, Gly 593 Phe 583, Lys 483, Val 471, Glu 501, Leu 505, Leu 514, Cys 532, Thr 529 and Val 471 within the active site of BrafV600E kinase is important for the inhibition when compared with the standard Dabrafenib.

CONCLUSION

In the present investigation fourteen benzamides incorporated into imidazine[1,2-*b*]pyridazine were prepared from the reaction of 4-arylidine-5-phenyl oxazole-2-ones **5a-5n** with 3-aminopyridazines. Compounds **6a-6n** were screened for possible anti-proliferative activity on A375 and colo-205 cancer cell lines, among the compounds, **6m** and **6n** exhibited highest anti-proliferative activity. Molecular docking studies of these compounds on BRaf V600E kinase revealed that the binding affinities and interactions of these molecules are in good agreement with the standard BRaf V600E kinase inhibitor (Dabrafenib). This work suggests that the synthesized compounds are potential to develop into new anti-proliferative agents and can be used in rational design of BRaf V600E kinase inhibitors for melanoma and colorectal cancers.

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