Chem. Res. Chin. Univ., 2017, 33(1), 61—69 doi: 10.1007/s40242-017-6330-8

Synthesis, Preliminary Structure-activity Relationships and Biological Evaluation of Pyridinyl-4,5-2*H*-isoxazole Derivatives as Potent Antitumor Agents

YANG Hongliang[1*], XU Guoxing[2] and PEI Yazhong[2]

1. College of Veterinary Medicine, Northeast Agricultural University, Harbin 150030, P. R. China;

2. School of Pharmaceutical Sciences, Jilin University, Changchun 130021, P. R. China

Abstract A series of novel pyridinyl-4,5-2*H*-isoxazole derivatives was synthesized and their chemical structures were characterized by ^{1}H NMR, ^{13}C NMR as well as MS spectroscopic methods, their melting points were also determined. The inhibitory effects of them against breast cancer cell line(MCF-7) were evaluated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide(MTT) procedure *in vitro*. Most of them possesed potent anti-proliferative activities, among which compounds **11**c and **11**j exhibited half maximal inhibitory concentrations(IC_{50}) of 1.9 and 1.5 μmol/L, respectively. These compounds also exhibited potent anti-proliferative activities against both human hepatoma cell line(HepG2) and cervical cancer cell line(HeLa). Preliminary structure-activity relationship (SAR) information from these compounds can be used to guide further exploration of new compounds with better potency as molecular probes. Further study on the mechanism-of-action of these compounds is under investigation.

Keywords Pyridine; Isoxazole; Anticancer activity; Chemotherapeutic agent

1 Introduction

Cancer is considered as the second most cause of death after cardiovascular diseases which results from uncontrolled cell division[1]. Several strategies have been discovered for the treatment of cancer progression, which includes surgery[2], radiation therapy[3], chemotherapy[4,5] and so on. Although different kinds of strategies have been developed for anticancer therapy, chemotherapy continues to be an indispensable approach for inoperable or metastatic cancers[6—8]. However, the use of available chemotherapeutics is often limited due to their poor inhibitory potency or side effects such as myelosuppression, gastrointestinal side effects(nausea and vomiting), hair loss(alopecia) and the development of clinical resistance[9—15]. Thus, it is highly desirable to discover and develop novel cancer inhibitors with high efficacy and selectivity, low toxicity, and minimum side effects[16—18]. Recently, great attention has been paid to develop the multifunctional anticancer chemotherapeutic agents that possess a broad range of antitumor activity and can destroy the tumor mass without affecting the normal tissue[19,20]. The medicinal chemists worldwide have paid great attention to the development of novel lead molecules over the past years[21].

Over the past few years, we have been principally engrossed in the synthesis and biological evaluation of novel pyridine-incorporating compounds as novel chemotherapeutic agents, among which compound **1**(Fig.1) exhibited moderate inhibition against the human breast cancer cell line MCF-7[half maximal inhibitory concentration(IC_{50})=7.3 μmol/L] by 3-(4,5-climethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide(MTT) assay[22]. To optimize the activity of compound **1**, a series of novel compounds **13**a—**13**v was synthesized, and their anticancer activities were tested *in vitro*. Fig.1 illustrates three regions of interest for structure-activity relationship(SAR) evaluation of screening hit compound **1**. For Region 3, we replaced isoxazole with isoxazoline to increase the flexibility of the whole molecule. Then various substituent groups in Region 1 and Region 2 were examined.

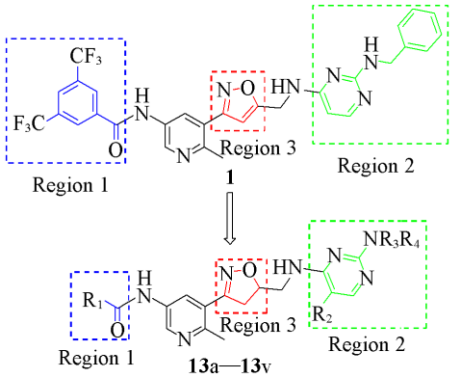


Fig.1 Optimization strategy for compound 1

2 Experimental

2.1 Chemsitry

Unless otherwise noted, all the chemicals and solvents

*Corresponding author. E-mail: hongl_yang@126.com

Received July 29, 2016; accepted September 7, 2016.

Supported by the Youth Natural Science Foundation of Heilongjiang Province, China(No.QC2016025).

were analytical reagents and used directly without further purification. Solvents were dried according to standard procedures. Column chromatography was performed with silica gel(300—400 mesh) using different ratios of petroleum ether(PE) and ethyl acetate(EA), which are commercially available. The melting points were measured on an X-4 electrothermal digital melting point apparatus and uncorrected. Mass spectra data were obtained using an LCQ Deca XP Plus mass spectrometer system(Thermo Finnigan, USA). ^{1}H NMR spectra(300 MHz) were recorded on a Bruker Advance spectrometer with tetramethylsilane(TMS) as internal standard and $CDCl_3$ or DMSO-d_6 as the solvent. ^{13}C NMR spectra(75 MHz) were determined with complete proton decoupling.

*2.1.1 Synthesis of Methyl 2-Methyl-5-nitronicotinate(**3**)*

A solution of compound **2**(12.89 g, 50 mmol) in ethanol (50 mL) was slowly added to a solution of sodium nitrite (13.8 g, 200 mmol) in water(50 mL) cooled with ice-bath. After the addition was complete, the resulting mixture was warmed to 60 °C for 30 min. Stirring was continued for additional 60 min without external heating. The reaction mixture was then placed at 0 °C overnight. After that, methyl 3-aminocrotonate (8.05 g, 70 mmol) and glacial acetic acid(2.38 mL, 75 mmol) were added to the mixture. The resulting mixture was stirred at 50 °C overnight, and then concentrated under vacuo. The residue was dissolved in dichloromethane(DCM, 200 mL) and filtered. The filtrate was concentrated and the residue was purified by column chromatography[*V*(EA):*V*(PE)= 1:10] to give compound **3** as a yellow solid. Yield 23%; m. p. 49—51 °C; ^{1}H NMR(300 MHz, $CDCl_3$), δ: 9.41(d, *J*=2.7 Hz, 1H, H6 pyridine), 8.96(d, *J*=2.7 Hz, 1H, H4 pyridine), 3.99(s, 3H, OCH_3), 2.97(s, 3H, CH_3); LC-MS, *m*/*z*: 196.9$[M+H]^+$.

*2.1.2 Synthesis of Methyl 5-Amino-2-methylnicotinate(**4**)*

To a stirred solution of compound **3**(1.96 g, 10 mmol) in methanol (20 mL) under hydrogen atmosphere was added Pd/C(196 mg). The mixture was stirred at room temperature for 24 h and filtered through a pad of celite. The filtrate was concentrated *in vacuo* to give compound **4** as a yellow solid. Yield 92%; m. p. 166—168 °C; ^{1}H NMR($CDCl_3$), δ: 8.13(d, *J*=3.0 Hz, 1H, H6 pyridine), 7.52(d, *J*=2.7 Hz, 1H, H4 pyridine), 3.90(s, 3H, OCH_3), 3.65(s, 2H, NH_2 exchangeable), 2.70(s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$), δ: 167.9, 148.9, 140.3, 139.2, 124.8 , 123.6, 52.0, 23.4; LC-MS, *m*/*z*: 167.2$[M+H]^+$.

*2.1.3 Synthesis of 2-(5-Hydroxymethyl-6-methylpyridin-3-yl)isoindoline-1,3-dione(**5**)*

To a stirred solution of compound **4**(3.44 g, 20.75 mmol) in anhydrous tetrahydrofuran(THF, 25 mL) at 0 °C was added $LiAlH_4$(1.577 g, 41.5 mmol) over 30 min. The mixture was stirred at 0 °C for 2 h and the remaining hydride was carefully quenched by dropwise addition of water(6.6 mL) and then 10%(mass fraction) NaOH(aq., 1.6 mL). The white precipitate was filtered off through a pad of celite. The filtrate was concentrated *in vacuo*. Then the residue, *o*-phthalic anhydride(2.917 g, 19.71 mmol) and molecular sieve(6.0 g) were added to anhydrous dioxane(30 mL). The mixture was stirred at room temperature overnight and then heated to reflux for 12 h. After that, *p*-toluenesulfonic acid(6.78 g, 39.4 mmol) was added and the mixture was stirred overnight at room temperature and then filtered through a pad of celite. The filtrate was concentrated *in vacuo*. The residue was extracted with DCM(50 mL×3), and the combined organic layer was washed with brine(30 mL×2) and dried over anhydrous Na_2SO_4 and concentrated under vacuum. The resulting residue was purified on a silica gel column [*V*(PE):*V*(EA)=3:1] to give compound **5** as a white solid. Yield 81%; m. p. 215—217 °C; ^{1}H NMR(300 MHz, DMSO-d_6), δ: 8.40(s, 1H, H6 pyridine), 7.98—7.96(m, 2H, Ar-H), 7.95—7.93 (m, 2H, Ar-H), 7.83(s, 1H, H4 pyridine), 5.44(s, 1H, OH, exchangeable), 4.59(s, 2H, OCH_2), 2.47(s, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6), δ: 166.9, 154.9, 144.9, 135.9, 134.7, 132.4, 131.6, 126.7, 123.4, 59.7, 21.2; LC-MS, *m*/*z*: 269.3 $[M+H]^+$.

*2.1.4 Synthesis of 5-(1,3-Dioxoisoindolin-2-yl)-2-methylnicotinaldehyde(**6**)*

To a stirred solution of compound **5**(1.49 g, 5.56 mmol) in a mixed solvent of anhydrous DCM/DMSO(15 mL, volume ratio 3/2) were added triethylamine(TEA, 6.18 mL, 44.5 mmol) and sulfur trioxide pyridine complex(5.33 g, 33.3 mmol) over 30 min. The mixture was stirred at room temperature for 3 h and then concentrated *in vacuo*. The residue was extracted with 10%(mass fraction) $KHSO_4$ solution and DCM(30 mL×3). The combined organic layer was washed with brine(30 mL×2), dried over anhydrous Na_2SO_4 and concentrated under vacuum. The resulting residue was purified on a silica gel column [*V*(PE):*V*(EA)=3:1] to give compound **6** as a white solid. Yield 90%; m. p. 199—201 °C; ^{1}H NMR(300 MHz, $CDCl_3$), δ: 10.36(s, 1H, CHO), 8.87(d, *J*= 2.7 Hz, 1H, H6 pyridine), 8.27(d, *J*=2.4 Hz, 1H, H4 pyridine), 8.01—7.99(m, 2H, Ar-H), 7.86—7.84(m, 2H, Ar-H), 2.95(s, 3H, CH_3); ^{13}C NMR(75 MHz, DMSO-d_6), δ: 191.9, 166.6, 159.0, 151.0, 135.0, 134.9, 131.6, 129.1, 127.4, 123.6, 21.7.

*2.1.5 Synthesis of 5-(1,3-Dioxoisoindolin-2-yl)-2-methylnicotinaldehyde Oxime(**7**)*

To a stirred solution of compound **6**(191.5 mg, 0.72 mmol) in EtOH(5 mL) was added hydroxylamine hydrochloride (100 mg, 1.44 mmol). The mixture was stirred at 60 °C for 3 h, then cooled to room temperature. The white precipitate was filtered off to give a *cis*/*trans* mixture of compound **7** as a white solid. Yield 70%; m. p. 239—241 °C; ^{1}H NMR(300 MHz, DMSO-d_6), δ: 11.68(s, 1H, OH, exchangeable), 8.55(d, *J*=2.1 Hz, 1H, H6 pyridine), 8.38(s, 1H, CH, —N=CH—), 8.12(d, *J*=1.8 Hz, 1H, H4 pyridine), 8.00—7.97(m, 2H, Ar-H), 7.93—7.91(m, 2H, Ar-H), 2.63(s, 3H, CH_3); ^{13}C NMR(75 MHz, DMSO-d_6), δ: 166.2, 152.3, 143.5, 141.1, 135.5, 135.1, 131.4, 130.1, 128.4, 123.8, 19.1; LC-MS, *m*/*z*: 282.3$[M+H]^+$.

*2.1.6 Synthesis of 5-(1,3-Dioxoisoindolin-2-yl)-2-methylnicotinaldehyde Oxime(**8**)*

To a stirred solution of compound **7**(989 mg, 3.52 mmol) in anhydrous *N*,*N*-dimethylformamide(DMF, 10 mL) at 0 °C was added *N*-chlorosuccinimide(520 mg, 3.877 mmol) over 30 min. The resultant solution was warmed to 50 °C and stirred for 6 h, then cooled at 0 °C with an ice bath and *tert*-butyl

allylcarbamate(1.091 g, 7.04 mmol), anhydrous triethylamine (0.59 mL, 4.224 mmol) and anhydrous DCM(10 mL) were added. The solution was stirred at ambient temperature overnight. The residue was extracted with DCM(30 mL×3), and the combined organic layer was washed with brine(30 mL×2), dried over anhydrous Na_2SO_4 and concentrated under vacuum. The resulting residue was purified on a silica gel column [*V*(PE):*V*(EA)=3:1] to give compound **8** as a yellow solid. Yield 52%; m. p. 101—103 °C; ^{1}H NMR(300 MHz, $CDCl_3$), *δ*: 8.70(d, *J*=2.4 Hz, 1H, H6 pyridine), 8.00—7.97(m, 2H, Ar-H), 7.86—7.83(m, 2H, Ar-H), 7.79(d, *J*=2.4 Hz, 1H, H4 pyridine), 4.98(t, *J*=6.6 Hz, 1H, NH, exchangeable), 4.95—4.82(m, 1H, CH, isoxazoline), 3.50—3.47(m, 1H, CH_2, isoxazoline), 3.46—3.37 (m, 2H, CH_2), 3.21(m, 1H, CH_2, isoxazoline), 2.87(s, 3H, CH_3, pyridine), 1.41(s, 9H, CH_3); ^{13}C NMR(75 MHz, DMSO-d_6), *δ*: 179.6, 166.7, 155.9, 155.5, 147.1, 135.0, 134.9, 131.6, 126.7, 124.6, 123.6, 79.2, 77.9, 43.1, 29.5, 27.7, 25.3; LC-MS, *m*/*z*: 437.0[M+H]$^+$.

*2.1.7 Synthesis of tert-Butyl{[3-(5-Amino-2-methylpyridin-3-yl)-4,5-dihydroisoxazol-5-yl]methyl}carbamate(**9**)*

Compound **8**(1.08 g, 2.48 mmol) was added to methylamine alcohol(5 mL). The mixture was stirred at 60 °C for 3 h and then concentrated *in vacuo*. The residue was extracted with DCM(15 mL×3), and the combined organic layer was washed with brine(15 mL×2), dried over anhydrous Na_2SO_4 and concentrated under vacuum. The resulting residue was purified on a silica gel column[*V*(DCM):*V*(MeOH)=30:1] to give compound **9** as a yellow solid. Yield 99%; m. p. 58—60 °C; ^{1}H NMR(300 MHz, $CDCl_3$), *δ*: 8.03(d, *J*=2.7 Hz, 1H, H6 pyridine), 6.97(d, *J*=2.7 Hz, 1H, H4 pyridine), 4.96(t, *J*=6.6 Hz, 1H, NH, exchangeable), 4.87—4.78(m, 1H, CH, isoxazoline), 3.71(s, 2H, NH_2, exchangeable), 3.45—3.41(m, 1H, CH_2, isoxazoline) 3.38—3.32(m, 2H, CH_2), 3.19—3.11(m, 1H, CH_2, isoxazoline), 2.63(s, 3H, CH_3, pyridine), 1.41(s, 9H, CH_3); ^{13}C NMR(75 MHz, $CDCl_3$), *δ*: 156.3, 146.4, 140.2, 136.7, 129.9, 124.1, 121.9, 79.4, 43.5, 39.5, 28.1, 24.0; LC-MS, *m*/*z*: 307.0[M+H]$^+$.

*2.1.8 General Procedure for the Synthesis of tert-Butyl {[3-(5-Amide-2-methylpyridin-3-yl)-4,5-dihydroisoxazol-5-yl]methyl}carbamate(**10**)*

To a stirred solution of compound **9**(3.06 g, 10 mmol) in DCM(25 mL) were added aromatic acid(11 mmol), 4-dimethylaminopyridine(DMAP, 244 mg, 2 mmol) and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride(EDCI, 2.872 g, 15 mmol). The mixture was stirred at room temperature for 24 h and then concentrated *in vacuo*. The residue was extracted with DCM(50 mL×3), and the combined organic layer was washed with brine(30 mL×2), dried over anhydrous Na_2SO_4 and concentrated under vacuum. The resulting residue was purified on a silica gel column to give compound **10**.

*2.1.9 General Procedure for the Synthesis of N-{5-[5-(Aminomethyl)-4,5-dihydroisoxazol-3-yl]-6-methylpyridin-3-yl} Amide(**11**)*

To a stirred solution of compound **10**(8 mmol) in anhydrous DCM(6 mL) was added trifluoroacetic acid(6 mL) at 0 °C. The solution was stirred for 3 h and then concentrated *in vacuo*. The residue was extracted with DCM(40 mL×3), then 6 mol/L NaOH solution was added to basify pH=9—10 and a white precipitate was filtered to give compound **11**.

*2.1.10 General Procedure for the Synthesis of N-[5-(5-{[(2-Chloro-5-substitutedpyrimidin-4-yl)amino]-mehyl}-4,5-dihydroisoxazol-3-yl)-6-methylpyridin-3-yl] Amide(**12**)*

To a stirred solution of compound **11**(1.5 mmol) in EtOH (10 mL) were added TEA(2.25 mmol) and 2,4-dichloro-5-substitutedpyrimidine(1.65 mmol). The mixture was stirred at 70 °C overnight and then concentrated *in vacuo*. The residue was extracted with DCM, and the combined organic layer was washed with brine, then dried over anhydrous Na_2SO_4 and concentrated under vacuum. The resulting residue was purified on a silica gel column to give compound **12**.

*2.1.11 General Synthetic Procedures for Title Compounds **13**a—**13**v*

A solution of compound **12**(0.14 mmol), amine(0.42 mmol), and TsOH(0.112 mmol) in dioxane(5 mL) was stirred at reflux temperature overnight and then concentrated under vacuum. The reaction mixture was diluted with water and extracted with EtOAc(10 mL×3). The combined organic layer was subsequently washed with H_2O and brine, then dried over anhydrous Na_2SO_4 and concentrated under vacuum. The crude product was purified on a silica gel column to give compounds **13**a—**13**v.

N-{6-Methyl-5-[5-({[5-methyl-2-(4-methylpiperazin-1-yl)pyrimidin-4-yl]amino}methyl)-4,5-dihydroisoxazol-3-yl]-pyridin-3-yl}-3,5-bis(trifluoromethyl)benzamide(**13**a): a white solid, yield 39%, m. p. 97—100 °C. ^{1}H NMR(300 MHz, DMSO-d_6), *δ*: 10.89(s, 1H, NH of amide, exchangeable), 8.86(d, *J*=2.1 Hz, 1H, H6 pyridine), 8.62(s, 2H, Ar-H), 8.41(s, 1H, Ar-H), 8.21(d, *J*=2.1 Hz, 1H, H4 pyridine), 7.58(s, 1H, H6 pyrimidine), 6.75(t, *J*=5.7 Hz, 1H, NH, exchangeable), 5.01—4.92(m, 1H, CH, isoxazoline), 3.59—3.50(m, 8H, CH_2, piperazine), 2.65(s, 3H, CH_3, pyridine), 2.23—2.21(m, 4H, CH_2 and CH_2 of isoxazoline), 2.12(s, 3H, CH_3, *N*-CH_3), 1.86(s, 3H, CH_3, pyrimidine); ^{13}C NMR(75 MHz, DMSO-d_6), *δ*: 162.8, 161.0, 160.3, 155.9, 154.1, 151.8, 141.2, 136.4, 133.1, 130.8(q, $J_{F—C}$=32.9 Hz), 128.6, 128.0, 125.5, 124.9(q, $J_{F—C}$=271.4 Hz), 124.0, 102.5, 78.8, 54.4, 45.8, 43.4, 42.8, 25.1, 12.8; LC-MS, *m*/*z*: 637.2[M+H]$^+$.

N-[6-Methyl-5-(5-{[(5-methyl-2-morpholinopyrimidin-4-yl)amino]methyl}-4,5-dihydroisoxazol-3-yl)pyridin-3-yl]-3,5-bis(trifluoromethyl)benzamide(**13**b): a white solid, yield 85%, m. p. 116—119 °C. ^{1}H NMR(300 MHz, DMSO-d_6), *δ*: 10.88(s, 1H, NH of amide, exchangeable), 8.85(d, *J*=2.4 Hz, 1H, H6 pyridine), 8.62(s, 2H, Ar-H), 8.40(s, 1H, Ar-H), 8.21(d, *J*=2.4 Hz, 1H, H4 pyridine), 7.60(s, 1H, H6 pyrimidine), 6.80(t, *J*=5.7 Hz, 1H, NH, exchangeable), 5.02—4.93(m, 1H, CH, isoxazoline), 3.65—3.40(m, 12H, CH_2, CH_2 of isoxazoline and CH_2 of morpholine), 2.64(s, 3H, CH_3, pyridine), 1.86(s, 3H, CH_3, pyrimidine); ^{13}C NMR(75 MHz, $CDCl_3$), *δ*: 162.8, 161.0, 160.4, 155.9, 154.0, 151.8, 141.2, 136.3, 133.0, 130.8(q, $J_{F—C}$=

32.6 Hz), 128.5, 128.0, 125.5, 124.9(q, $J_{F—C}$=271.4 Hz), 124.0, 103.0, 78.7, 66.0, 44.1, 42.8, 25.0, 12.8; LC-MS, *m*/*z*: 624.2[M+H]$^+$.

N-{6-Methyl-5-[5-({[5-methyl-2-(4-methylpiperidin-1-yl)-pyrimidin-4-yl]amino}methyl)-4,5-dihydroisoxazol-3-yl]pyridin-3-yl}-3,5-bis(trifluoromethyl)benzamide(**13**c): a white solid, yield 63%, m. p. 85—87 °C. ^{1}H NMR(300 MHz, DMSO-d_6), δ: 10.91(s, 1H, NH of amide, exchangeable), 8.88(d, *J*=2.4 Hz, 1H, H6 pyridine), 8.63(s, 2H, Ar-H), 8.43(s, 1H, Ar-H), 8.21(d, *J*=2.4 Hz, 1H, H4 pyridine), 7.57(s, 1H, H6 pyrimidine), 6.72(t, *J*=6.0 Hz, 1H, NH, exchangeable), 5.02—4.93(m, 1H, CH, isoxazoline), 4.48(d, *J*=12.9 Hz, 2H, CH_2), 3.60—3.57(m, 2H, CH_2, piperidine), 3.60—3.57(m, 1H, CH_2, isoxazoline), 3.45—3.37 (m, 1H, CH_2, isoxazoline), 2.66(s, 3H, CH_3, pyridine), 2.62—2.58(m, 1H, CH_2, piperidine), 1.86(s, 3H, CH_3, pyrimidine), 1.54—1.43(m, 3H, CH_2 of piperidine and CH of piperidine), 1.04—0.84(m, 3H, CH_2, piperidine), 0.81(d, *J*=6.3 Hz, 3H, CH_3, piperidine); ^{13}C NMR(75 MHz, DMSO-d_6), δ: 162.7, 160.9, 160.2, 155.9, 154.2, 151.7, 141.1, 136.3, 133.0, 130.8(q, $J_{F—C}$=33.2 Hz), 128.5, 128.0, 125.5, 124.8(q, $J_{F—C}$=271.2 Hz), 124.0, 101.9, 78.7, 43.7, 42.7, 33.4, 30.7, 25.1, 21.8, 12.8; LC-MS, *m*/*z*: 636.3[M+H]$^+$.

N-{6-Methyl-5-[5-({[5-methyl-2-(piperidin-1-yl)pyrimidin-4-yl]amino}methyl)-4,5-dihydroisoxazol-3-yl]pyridin-3-yl}-3,5-bis(trifluoromethyl)benzamide(**13**d): a white solid, yield 57%, m. p. 109—111 °C. ^{1}H NMR(300 MHz, DMSO-d_6), δ: 8.85(d, *J*=2.4 Hz, 1H, H6 pyridine), 8.62(s, 2H, Ar-H), 8.41(s, 1H, Ar-H), 8.20(d, *J*=2.4 Hz, 1H, H4 pyridine), 7.55(s, 1H, H6 pyrimidine), 5.02—4.93(m, 1H, CH, isoxazoline), 3.61—3.54 (m, 4H, CH_2, piperidine), 3.53—3.47(m, 3H, CH_2 and CH_2 of isoxazoline), 3.36—3.39(m, 1H, CH_2, isoxazoline), 2.65(s, 3H, CH_3, pyridine), 1.84(s, 3H, CH_3, pyrimidine), 1.48(s, 2H, CH_2, piperidine), 1.39(s, 4H, CH_2, piperidine); ^{13}C NMR(75 MHz, DMSO-d_6), δ: 162.7, 160.9, 160.0, 155.9, 154.2, 151.7, 141.2, 136.3, 133.0, 130.8(q, $J_{F—C}$=32.2 Hz), 128.5, 128.0, 125.6, 124.8(q, $J_{F—C}$=271.2 Hz), 124.1, 101.8, 78.7, 44.3, 42.7, 25.1, 25.0, 24.5, 12.9; LC-MS, *m*/*z*: 622.3[M+H]$^+$.

N-{5-[5-({[2-(Cyclohexylamino)-5-methylpyrimidin-4-yl]-amino}methyl)-4,5-dihydroisoxazol-3-yl]-6-methylpyridin-3-yl}-3,5-bis(trifluoromethyl)benzamide(**13**e): an off-white solid, yield 60%, m. p. 63—65 °C. ^{1}H NMR(300 MHz, DMSO-d_6), δ: 10.92(s, 1H, NH of amide, exchangeable), 8.88(d, *J*=2.4 Hz, 1H, H6 pyridine), 8.64(s, 2H, Ar-H), 8.42(s, 1H, Ar-H), 8.23(d, *J*=2.4 Hz, 1H, H4 pyridine), 7.50(s, 1H, H6 pyrimidine), 6.75(s, 1H, NH, exchangeable), 6.15(s, 1H, NH, exchangeable), 5.05—4.95(m, 1H, CH, isoxazoline), 3.67—3.44(m, 3H, CH_2 and CH_2 of isoxazoline), 3.43—3.34(m, 1H, CH_2, isoxazoline), 2.67(s, 3H, CH_3, pyridine), 1.85(s, 3H, CH_3, pyrimidine), 1.83—1.76(m, 2H, CH_2, cyclohexane), 1.62(s, 2H, CH_2, cyclohexane), 1.54—1.50(m, 1H, CH, cyclohexane), 1.18—1.07(m, 6H, CH_2, cyclohexane); ^{13}C NMR(75 MHz, DMSO-d_6), δ: 162.5, 161.0, 159.5, 155.5, 152.7, 151.5, 141.2, 136.3, 132.7, 130.6(q, $J_{F—C}$=33.2 Hz), 128.2, 127.9, 124.8, 124.6(q, $J_{F—C}$=271.2 Hz), 123.8, 101.9, 78.6, 49.0, 42.8, 39.4, 32.4, 25.1, 24.4, 12.4; LC-MS, *m*/*z*: 636.2[M+H]$^+$.

N-{6-Methyl-5-[5-({[5-methyl-2-(phenylamino)pyrimidin-4-yl]amino}methyl)-4,5-dihydroisoxazol-3-yl]pyridin-3-yl}-3,5-bis(trifluoromethyl)benzamide(**13**f): a beige solid, yield 82%, m. p. 115—117 °C. ^{1}H NMR(300 MHz, DMSO-d_6), δ: 10.90(s, 1H, NH of amide, exchangeable), 8.89(d, *J*=1.8 Hz, 2H, H6 pyridine), 8.64(s, 2H, Ar-H), 8.43(s, 1H, Ar-H), 8.24(d, *J*=2.4 Hz, 1H, H4 pyridine), 7.75(s, 1H, H6 pyrimidine), 7.72(s, 2H, Ar-H), 7.18(t, *J*=7.5 Hz, 2H, Ar-H), 6.96(t, *J*=5.7 Hz, 1H, NH, exchangeable), 6.81(t, *J*=7.5 Hz, 1H, Ar-H), 5.16—5.01(m, 1H, CH, isoxazoline), 3.73—3.78(m, 1H, CH_2, exchangeable), 3.65—3.49(m, 2H, CH_2), 3.48—3.45(m, 1H, CH, isoxazoline), 2.68(s, 3H, CH_3, pyridine), 1.95(s, 3H, CH_3, pyrimidine); ^{13}C NMR(75 MHz, DMSO-d_6), δ: 162.4, 160.6, 157.9, 155.4, 153.4, 151.4, 141.0, 140.9, 135.9, 132.5, 130.4(q, $J_{F—C}$=33.2 Hz), 128.1, 128.0, 127.7, 124.9, 124.4(q, $J_{F—C}$=271.5 Hz), 123.6, 119.6, 117.7, 104.2, 78.1, 42.9, 24.5, 12.6; LC-MS, *m*/*z*: 630.2[M+H]$^+$.

N-{5-[5-({[2-(Benzylamino)-5-methylpyrimidin-4-yl]-amino}methyl)-4,5-dihydroisoxazol-3-yl]-6-methylpyridin-3-yl}-3,5-bis(trifluoromethyl)benzamide(**13**g): a white solid, yield 59%, m. p. 129—131 °C. ^{1}H NMR(300 MHz, DMSO-d_6), δ: 10.90(s, 1H, NH of amide, exchangeable), 8.88(d, *J*=2.1 Hz, 1H, H6 pyridine), 8.63(s, 2H, Ar-H), 8.42(s, 1H, Ar-H), 8.21(d, *J*=2.4 Hz, 1H, H4 pyridine), 7.50(s, 1H, H6 pyrimidine), 7.30—7.19(m, 4H, Ar-H), 7.12(m, *J*=6.6 Hz, 1H, Ar-H), 6.85(t, *J*=6.6 Hz, 1H, NH, exchangeable), 6.64(t, *J*=6.3 Hz, 1H, NH, exchangeable), 4.89(s, 1H, CH, isoxazoline), 4.37(d, *J*=6.0 Hz, 2H, CH_2, benzyl), 3.82—3.48(m, 1H, CH_2, isoxazoline), 3.49—3.38(m, 2H, CH_2), 3.24—3.23(m, 1H, CH_2, isoxazoline), 2.64(s, 3H, CH_3, pyridine), 1.84(s, 3H, CH_3, pyrimidine); ^{13}C NMR(75 MHz, DMSO-d_6), δ: 162.4, 160.8, 159.1, 155.4, 151.3, 140.9, 140.4, 135.9, 132.5, 130.4(q, $J_{F—C}$=33.3 Hz), 128.1, 127.6, 127.5, 126.5, 125.8, 125.0, 124.4(q, $J_{F—C}$=271.5 Hz), 124.1, 123.6, 102.4, 78.0, 43.8, 42.6, 24.4, 12.3; LC-MS, *m*/*z*: 644.2[M+H]$^+$.

N-{6-Methyl-5-[5-({[5-methyl-2-(propylamino)pyrimidin-4-yl]amino}methyl)-4,5-dihydroisoxazol-3-yl]pyridin-3-yl}-3,5-bis(trifluoromethyl)benzamide(**13**h): a white solid, yield 60%, m. p. 223—225 °C. ^{1}H NMR(300 MHz, DMSO-d_6), δ: 11.08(s, 1H, NH of amide, exchangeable), 8.88(d, *J*=2.1 Hz, 1H, H6 pyridine), 8.67(s, 2H, Ar-H), 8.41(s, 1H, Ar-H), 8.32(d, *J*=2.1 Hz, 1H, H4 pyridine), 7.61(s, 1H, H6 pyrimidine), 5.08—4.98(m, 1H, CH, isoxazoline), 3.76—3.68(m, 1H, CH_2, isoxazoline), 3.65—3.56(m, 2H, CH_2), 3.39(m, 1H, CH_2, isoxazoline), 3.26—3.20(m, 2H, CH_2), 2.66(s, 3H, CH_3, pyridine), 1.95(s, 3H, CH_3, pyrimidine), 1.62—1.43(m, 2H, CH_2), 0.84(t, *J*=7.2 Hz, 3H, CH_3); ^{13}C NMR(75 MHz, DMSO-d_6), δ: 162.8, 161.5, 155.9, 151.7, 141.3, 136.3, 133.1, 130.8(q, $J_{F—C}$=33.3 Hz), 128.6, 128.1, 125.4, 124.9(q, $J_{F—C}$=271.4 Hz), 123.9, 117.7, 102.9, 78.4, 43.1, 42.5, 29.0, 25.1, 22.2, 12.8, 11.3; LC-MS, *m*/*z*: 596.1[M+H]$^+$.

N-{5-[5-({[2-(Isopropylamino)-5-methylpyrimidin-4-yl]-amino}methyl)-4,5-dihydroisoxazol-3-yl]-6-methylpyridin-3-yl}-3,5-bis(trifluoromethyl)benzamide(**13**i): a white solid, yield 50%, m. p. 119—121 °C. ^{1}H NMR(300 MHz, DMSO-d_6), δ: 10.94(s, 1H, NH of amide, exchangeable), 8.87(d, *J*=2.1 Hz, 1H, H6 pyridine), 8.64(s, 2H, Ar-H), 8.43(s, 1H, Ar-H), 8.25(d, *J*=2.4 Hz, 1H, H4 pyridine), 7.53(s, 1H, H6 pyrimidine), 5.15—4.90(m, 1H, CH, isoxazoline), 3.87—3.90(m, 1H, CH_2,

isoxazoline), 3.63—3.49(m, 4H, CH_2, CH_2 of isoxazoline and CH), 2.66(s, 3H, CH_3, pyridine), 1.86(s, 3H, CH_3, pyrimidine), 1.07(dd, *J*=11.1, 6.3 Hz, 6H, CH_3); ^{13}C NMR(75 MHz, DMSO-d_6), *δ*: 162.8, 161.3, 155.9, 151.7, 141.2, 136.3, 133.0, 130.8(q, $J_{F—C}$=33.2 Hz), 128.6, 128.0, 125.5, 124.9(q, $J_{F—C}$=271.5 Hz), 124.0, 102.4, 78.5, 42.9, 42.0, 25.0, 22.4, 12.8; LC-MS, *m*/*z*: 596.3[M+H]$^+$.

N-{5-[5-({[2-(sec-Butylamino)-5-methylpyrimidin-4-yl]-amino}methyl)-4,5-dihydroisoxazol-3-yl]-6-methylpyridin-3-yl}-3,5-bis(trifluoromethyl)benzamide(**13**j): a white solid, yield 48%, m. p. 95—97 °C. ^{1}H NMR(300 MHz, DMSO-d_6), *δ*: 10.93(s, 1H, NH of amide, exchangeable), 8.88(d, *J*=2.4 Hz, 1H, H6 pyridine), 8.64(s, 2H, Ar-H), 8.43(s, 1H, Ar-H), 8.23(d, *J*=2.1 Hz, 1H, H4 pyridine), 7.51(s, 1H, H6 pyrimidine), 6.88(s, 1H, NH, exchangeable), 6.54(s, 1H, NH, exchangeable), 5.06—4.96(m , 1H, CH, isoxazoline), 3.68—3.46(m, 3H, CH_2 and CH_2 of isoxazoline), 3.44—3.38(m, 1H, CH_2, isoxazoline), 3.33—3.31(m, 1H, CH), 2.97(m, 2H, CH_2), 2.67(s, 3H, CH_3, pyridine), 1.86(s, 3H, CH_3, pyrimidine), 0.83—0.80(m, 6H, CH_3); ^{13}C NMR(75 MHz, DMSO-d_6), *δ*: 162.8, 161.2, 160.4, 155.9, 152.7, 151.8, 141.3, 136.4, 133.0, 130.8(q, $J_{F—C}$=33.1 Hz), 128.5, 128.1, 125.4, 124.9(q, $J_{F—C}$=271.2 Hz), 124.1, 102.1, 78.6, 48.5, 42.9, 27.9, 25.0, 20.2, 12.8; LC-MS, *m*/*z*: 610.3[M+H]$^+$.

N-{6-Methyl-5-[5-({[5-methyl-2-(methylamino)pyrimidin-4-yl]amino}methyl)-4,5-dihydroisoxazol-3-yl]pyridin-3-yl}-3,5-bis(trifluoromethyl)benzamide(**13**k): a white solid, yield 21%, m. p. 128—130 °C. ^{1}H NMR(300 MHz, DMSO-d_6), *δ*: 11.02(s, 1H, NH of amide, exchangeable), 8.89(d, *J*=2.1 Hz, 1H, H6 pyridine), 8.66(s, 2H, Ar-H), 8.42(s, 1H, Ar-H), 8.28(d, *J*=2.4 Hz, 1H, H4 pyridine), 7.56(s, 1H, H6 pyrimidine), 7.40(s, 1H, NH, exchangeable), 6.93(s, 1H, NH, exchangeable), 5.08—4.99(m, 1H, CH, isoxazoline), 3.71—3.62(m, 1H, CH_2, isoxazoline), 3.59—3.51(m, 2H, CH_2), 3.38—3.32(m, 1H, CH_2, isoxazoline), 2.75(d, *J*=4.7 Hz, 3H, CH_3), 2.66(s, 3H, CH_3, pyridine), 1.90(s, 3H, CH_3, pyrimidine); ^{13}C NMR(75 MHz, DMSO-d_6), *δ*: 162.9, 161.3, 160.5, 156.0, 152.1, 151.8, 141.4, 136.4, 133.1, 130.8(q, $J_{F—C}$=33.2 Hz), 128.6, 128.2, 125.3, 124.9(q, $J_{F—C}$=271.4 Hz), 124.1, 102.4, 78.6, 43.0, 27.8, 24.9, 12.8; LC-MS, *m*/*z*: 568.2[M+H]$^+$.

N-{6-Methyl-5-[5-({[2-(4-methylpiperidin-1-yl)pyrimidin-4-yl]amino}methyl)-4,5-dihydroisoxazol-3-yl]pyridin-3-yl}-3,5-bis(trifluoromethyl)benzamide(**13**l): a white solid, yield 70%, m. p. 115—117 °C. ^{1}H NMR(300 MHz, DMSO-d_6), *δ*: 10.92(s, 1H, NH of amide, exchangeable), 8.88(d, *J*=2.4 Hz, 1H, H6 pyridine), 8.65(s, 2H, Ar-H), 8.45(s, 1H, Ar-H), 8.24(d, *J*=2.4 Hz, 1H, H4 pyridine), 7.71(d, *J*=5.7 Hz, 1H, H6 pyrimidine), 7.30(s, 1H, NH, exchangeable), 5.80(d, *J*=5.7 Hz, 1H, H5 pyrimidine), 4.97—4.88(m, 1H, CH, isoxazoline), 4.56(d, *J*=12.3 Hz, 2H, CH_2), 3.63—3.54(m, 3H, CH_2 of piperidine and CH_2 of isoxazoline), 3.34—3.27(m, 1H, CH_2, isoxazoline), 2.76—2.71(m, 1H, CH_2, piperidine), 2.67(s, 3H, CH_3, pyridine), 1.59—1.50(m, 3H, CH_2 of piperidine and CH of piperidine), 1.02—0.90(m, 2H, CH_2, piperidine), 0.86(d, *J*=6.0 Hz, 3H, CH_3, piperidine), 0.82—0.75(m, 1H, CH_2, piperidine); ^{13}C NMR(75 MHz, DMSO-d_6), *δ*: 162.7, 162.6, 160.9, 155.9, 154.7, 151.8, 141.2, 136.4, 133.0, 130.8(q, $J_{F—C}$=33.1 Hz), 128.5, 128.0, 125.4, 124.9(q, $J_{F—C}$=271.4 Hz), 124.0, 95.3, 79.1, 43.0, 42.5, 39.4, 33.5, 30.7, 24.9, 21.7; LC-MS, *m*/*z*: 622.2[M+H]$^+$.

N-{5-[5-({[2-(Isopropylamino)pyrimidin-4-yl]amino}-methyl)-4,5-dihydroisoxazol-3-yl]-6-methylpyridin-3-yl}-3,5-bis(trifluoromethyl)benzamide(**13**m): a white solid, yield 60%, m. p. 112—114 °C. ^{1}H NMR(300 MHz, DMSO-d_6), *δ*: 10.92(s, 1H, NH of amide, exchangeable), 8.87(d, *J*=2.4 Hz, 1H, H6 pyridine), 8.64(s, 2H, Ar-H), 8.42(s, 1H, Ar-H), 8.24(d, *J*=2.4 Hz, 1H, H4 pyridine), 7.64(d, *J*=5.7 Hz, 1H, H6 pyrimidine), 7.35(s, 1H, NH, exchangeable), 6.40(s, 1H, NH, exchangeable), 5.79(d, *J*=5.7 Hz, 1H, H5 pyrimidine), 4.97—4.87(m, 1H, CH, isoxazoline), 4.00—3.89(m, 1H, CH_2, isoxazoline), 3.61—3.51 (m, 3H, CH_2 and CH), 3.31—3.23(m, 1H, CH_2, isoxazoline), 2.65(s, 3H, CH_3, pyridine), 1.08(dd, *J*=11.1, 6.3 Hz, 6H, CH_3); ^{13}C NMR(75 MHz, DMSO-d_6), *δ*: 162.8, 160.6, 156.0, 154.0, 151.8, 141.3, 136.4, 133.0, 130.8(q, $J_{F—C}$=33.1 Hz), 128.6, 128.1, 125.4, 124.9(q, $J_{F—C}$=271.4 Hz), 124.1, 117.7, 96.0, 79.0, 42.6, 41.8, 29.0, 24.9, 22.5; LC-MS, *m*/*z*: 582.2[M+H]$^+$.

N-{5-[5-({[2-(sec-Butylamino)pyrimidin-4-yl]amino}-methyl)-4,5-dihydroisoxazol-3-yl]-6-methylpyridin-3-yl}-3,5-bis(trifluoromethyl)benzamide(**13**n): a white solid, yield 50%, m. p. 95—97 °C. ^{1}H NMR(300 MHz, DMSO-d_6), *δ*: 10.92(s, 1H, NH of amide, exchangeable), 8.87(d, *J*=2.4 Hz, 1H, H6 pyridine), 8.63(s, 2H, Ar-H), 8.42(s, 1H, Ar-H), 8.23(d, *J*=2.4 Hz, 1H, H4 pyridine), 7.63(d, *J*=5.7 Hz, 1H, H6 pyrimidine), 5.78(d, *J*=5.7 Hz, 1H, H5 pyrimidine), 4.97—4.85(m, 1H, CH, isoxazoline), 3.60—3.50(m, 3H, CH_2 and CH_2 of isoxazoline), 3.31—3.22(m, 1H, CH_2, isoxazoline), 3.17—3.15(m, 1H, CH), 2.99(m, 2H, CH_2), 2.65(s, 3H, CH_3, pyridine), 0.83—0.80(m, 6H, CH_3); ^{13}C NMR(75 MHz, DMSO-d_6), *δ*: 162.8, 162.7, 161.5, 155.9, 154.2, 151.8, 141.3, 136.4, 133.0, 130.8(q, $J_{F—C}$=30.8 Hz), 128.5, 128.1, 125.4, 124.9(q, $J_{F—C}$=271.4 Hz), 124.0, 96.7, 79.0, 48.3, 42.5, 39.5, 27.8, 25.6, 20.2; LC-MS, *m*/*z*: 596.3[M+H]$^+$.

3-Fluoro-*N*-{5-[5-({[2-(isopropylamino)-5-methylpyrimidin-4-yl]amino}methyl)-4,5-dihydroisoxazol-3-yl]-6-methylpyridin-3-yl}-5-morpholinobenzamide(**13**o): a white solid, yield 65%, m. p. 112—114 °C. ^{1}H NMR(300 MHz, DMSO-d_6), *δ*: 10.51(s, 1H, NH of amide, exchangeable), 8.85(d, *J*=2.4 Hz, 1H, H6 pyridine), 8.28(d, *J*=2.4 Hz, 1H, H4 pyridine), 7.54(s, 1H, H6 pyrimidine), 7.35(s, 1H, Ar-H), 7.20—7.18(m, 1H, Ar-H), 7.05—7.03(m, 1H, Ar-H), 6.83(s, 1H, NH, exchangeable), 5.05—4.95(m, 1H, CH, isoxazoline), 4.01—3.85(m, 1H, CH_2, isoxazoline), 3.82—3.71(m, 4H, CH_2, morpholine), 3.70—3.59(m, 1H, CH_2, isoxazoline), 3.59—3.48(m, 2H, CH_2), 3.38—3.36(m, 1H, CH), 3.30—3.20(m, 4H, CH_2, morpholine), 2.64(s, 3H, CH_3, pyridine), 1.89(s, 3H, CH_3, pyrimidine), 1.20—1.14(m, 1H), 1.09(dd, *J*=10.2, 6.6 Hz, 6H, CH_3); ^{13}C NMR(75 MHz, DMSO-d_6), *δ*: 164.6, 164.4(d, $J_{F—C}$=240.1 Hz), 158.3, 155.7, 152.5(d, $J_{F—C}$=10.3 Hz), 150.9, 150.3, 141.1, 136.3(d, $J_{F—C}$=11.3 Hz), 133.2, 127.7, 123.6, 109.6, 104.3(d, $J_{F—C}$=25.7 Hz), 104.0(d, $J_{F—C}$=23.5 Hz), 102.4, 78.3, 65.6, 47.6, 42.9, 41.8, 24.3, 22.2, 21.7, 12.4; LC-MS , *m*/*z*: 563.3[M+H]$^+$.

N-{5-[5-({[2-(sec-Butylamino)-5-methylpyrimidin-4-yl]-amino}methyl)-4,5-dihydroisoxazol-3-yl]-6-methylpyridin-3-

yl}-3-fluoro-5-morpholinobenzamide(**13**p): a white solid, yield 53%, m. p. 250—252 °C. ^{1}H NMR(300 MHz, DMSO-d_6), δ: 10.46(s, 1H, NH of amide, exchangeable), 8.86(d, J=2.4 Hz, 1H, H6 pyridine), 8.23(d, J=2.4 Hz, 1H, H4 pyridine), 7.50(s, 1H, H6 pyrimidine), 7.34(s, 1H, Ar-H), 7.19—7.17(m, 1H, Ar-H), 7.04—7.02(m, 1H, Ar-H), 6.81(s, 1H, NH, exchangeable), 6.47(s, 1H, NH, exchangeable), 5.04—4.94(m, 1H, CH, isoxazoline), 3.79—3.73(m, 4H, CH_2, morpholine), 3.68—3.56 (m, 1H, CH_2, isoxazoline), 3.56—3.41(m, 2H, CH_2), 3.38—3.37(m, 1H, CH_2, isoxazoline), 3.33—3.31(m, 1H, CH), 3.27—3.20(m, 4H, CH_2, morpholine), 2.97(m, 2H, CH_2), 2.65(s, 3H, CH_3, pyridine), 1.85(s, 3H, CH_3, pyrimidine), 0.83—0.80(m, 6H, CH_3); ^{13}C NMR(75 MHz, DMSO-d_6), δ: 164.8, 164.6(d, $J_{F—C}$=241.4 Hz), 161.1, 160.7, 155.9, 153.2, 152.8(d, $J_{F—C}$=10.4 Hz), 151.2, 141.1, 136.5(d, $J_{F—C}$=8.9 Hz), 133.5, 127.9, 123.9, 109.8, 104.8(d, $J_{F—C}$=25.5 Hz), 104.3(d, $J_{F—C}$= 23.6 Hz), 78.4, 65.8, 48.5, 47.7, 42.8, 27.9, 25.1, 20.2, 12.9; LC-MS , m/z: 577.3$[M+H]^+$.

3,5-Difluoro-*N*-{5-[5-({[2-(isopropylamino)-5-methylpyrimidin-4-yl]amino}methyl)-4,5-dihydroisoxazol-3-yl]-6-methylpyridin-3-yl}benzamide(**13**q): a white solid, yield 71%, m. p. 225—227 °C. ^{1}H NMR(300 MHz, DMSO-d_6), δ: 10.71(s, 1H, NH of amide, exchangeable), 8.86(d, J=2.4 Hz, 1H, H6 pyridine), 8.30(d, J=2.4 Hz, 1H, H4 pyridine), 7.81—7.70(m, 2H, Ar-H), 7.62—7.51(m, 2H, Ar-H and H6 pyrimidine), 7.09(s, 1H, NH, exchangeable), 5.06—4.96(m, 1H, CH, isoxazoline), 4.00—3.88(m, 1H, CH_2, isoxazoline), 3.71—3.62(m, 1H, CH), 3.59—3.51(m, 2H, CH_2), 3.38—3.37(m, 1H, CH_2, isoxazoline), 2.65(s, 3H, CH_3, pyridine), 1.91(s, 3H, CH_3, pyrimidine), 1.11(dd, J=10.2, 6.5 Hz, 6H, CH_3); ^{13}C NMR(75 MHz, DMSO-d_6), δ: 164.0, 163.2, 161.8, 160.4(d, $J_{F—C}$=245.9 Hz), 156.0, 151.5, 141.1, 133.3, 127.9, 123.8, 111.4(d, $J_{F—C}$=26.3 Hz), 111.3(d, $J_{F—C}$=9.0 Hz), 107.7, 107.4(d, $J_{F—C}$=26.4 Hz), 103.5, 78.1, 43.3, 42.4, 25.1, 22.2, 21.7, 12.8; LC-MS, m/z: 496.2$[M+H]^+$.

N-{5-[5-({[2-(sec-Butylamino)-5-methylpyrimidin-4-yl]-amino}methyl)-4,5-dihydroisoxazol-3-yl]-6-methylpyridin-3-yl}-3,5-difluorobenzamide(**13**r): a white solid, yield 62%, m. p. 113—115 °C. ^{1}H NMR(300 MHz, DMSO-d_6), δ: 10.62(s, 1H, NH of amide, exchangeable), 8.86(d, J=2.4 Hz, 1H, H6 pyridine), 8.23(d, J=2.4 Hz, 1H, H4 pyridine), 7.77—7.66(m, 2H, Ar-H), 7.57(m, 1H, Ar-H), 7.50(s, 1H, H6 pyrimidine), 5.05—4.93(m, 1H, CH, isoxazoline), 3.68—3.56(m, 1H, CH_2, isoxazoline), 3.55—3.42(m, 2H, CH_2), 3.39—3.37(m, 1H, CH), 3.33—3.31(m, 1H, CH_2, isoxazoline), 2.97(m, 2H, CH_2), 2.65(s, 3H, CH_3, pyridine), 1.85(s, 3H, CH_3, pyrimidine), 0.83—0.81(m, 6H, CH_3); ^{13}C NMR(75 MHz, DMSO-d_6), δ: 164.0, 163.1, 161.1, 160.5(d, $J_{F—C}$=244.5 Hz), 155.9, 153.4, 151.6, 141.0, 133.2, 131.5, 128.7, 127.9, 124.0, 111.4(d, $J_{F—C}$=26.3 Hz), 111.2(d, $J_{F—C}$=8.9 Hz), 107.4(d, $J_{F—C}$=26.0 Hz), 78.5, 65.0, 48.5, 42.8, 27.6, 25.2, 20.3, 12.9; LC-MS, m/z: 510.2$[M+H]^+$.

N-{5-[5-({[2-(Isopropylamino)-5-methylpyrimidin-4-yl]-amino}methyl)-4,5-dihydroisoxazol-3-yl]-6-methylpyridin-3-yl}-3-(methylsulfonamido)-5-(trifluoromethyl)benzamide(**13**s): a white solid, yield 34%, m. p. 153—155 °C. ^{1}H NMR(300 MHz, DMSO-d_6), δ: 10.74(s, 1H, NH of amide, exchangeable), 8.85(d, J=2.1 Hz, 1H, H6 pyridine), 8.23(d, J=2.4 Hz, 1H, H4 pyridine), 8.07—8.05(m, 2H, Ar-H), 7.70(s, 1H, Ar-H), 7.51(s, 1H, H6 pyrimidine), 6.87(s, 1H, NH, exchangeable), 6.21(s, 1H, NH, exchangeable), 5.06—4.93(m, 1H, CH, isoxazoline), 3.95—3.84(m, 1H, CH_2, isoxazoline), 3.67—3.59(m, 1H, CH), 3.54—3.45(m, 2H, CH_2), 3.38—3.37(m, 1H, CH_2, isoxazoline), 3.13(s, 3H, SO_2CH_3), 2.65(s, 3H, CH_3, pyridine), 1.86(s, 3H, CH_3, pyrimidine), 1.07(dd, J=10.2, 6.6 Hz, 6H, CH_3); ^{13}C NMR(75 MHz, DMSO-d_6), δ: 164.1, 161.1, 159.9, 156.4, 155.9, 153.3, 151.5, 142.8, 142.4, 141.3, 140.5, 135.6, 130.4(q, $J_{F—C}$=32.0 Hz), 128.1(q, $J_{F—C}$=32.6 Hz), 123.8, 122.2(q, $J_{F—C}$=275.1 Hz), 120. 7, 102.0; LC-MS, m/z: 621.2$[M+H]^+$.

N-{5-[5-({[2-(sec-Butylamino)-5-methylpyrimidin-4-yl]-amino}methyl)-4,5-dihydroisoxazol-3-yl]-6-methylpyridin-3-yl}-3-(methylsulfonamido)-5-(trifluoromethyl)benzamide(**13**t): a white solid, yield 30%, m. p. 136—138 °C. ^{1}H NMR(300 MHz, DMSO-d_6), δ: 10.72(s, 1H, NH of amide, exchangeable), 8.86(d, J=2.4 Hz, 1H, H6 pyridine), 8.22(d, J=2.4 Hz, 1H, H4 pyridine), 8.04(m, 2H, Ar-H), 7.69(s, 1H, Ar-H), 7.49(s, 1H, H6 pyrimidine), 6.71(s, 1H, NH, exchangeable), 6.36(s, 1H, NH, exchangeable), 5.04—4.95(m, 1H, CH, isoxazoline), 3.68—3.57(m, 1H, CH_2, isoxazoline), 3.56—3.43(m, 2H, CH_2), 3.39—3.38(m, 1H, CH), 3.34—3.32(m, 1H, CH_2, isoxazoline) 3.12(s, 3H, SO_2CH_3), 2.65(s, 3H, CH_3, pyridine), 1.85(s, 3H, CH_3, pyrimidine), 0.81—0.79(m, 6H, CH_3); ^{13}C NMR(75 MHz, DMSO-d_6), δ: 164.0, 161.1, 160.7, 155.9, 153.3, 151.5, 141.2, 140.4, 136.4, 133.2, 130.8(q, $J_{F—C}$=31.2 Hz), 129.9(q, $J_{F—C}$=31.7 Hz), 128.1, 125.4(q, $J_{F—C}$=270.8 Hz), 124.0, 122.2, 118.3, 101.6, 78.6, 68.9, 48.5, 42.8, 27.9, 25.0, 20.2, 12.8; LC-MS, m/z: 635.2$[M+H]^+$.

N-{5-[5-({[2-(Isopropylamino)-5-methylpyrimidin-4-yl]-amino}methyl)-4,5-dihydroisoxazol-3-yl]-6-methylpyridin-3-yl}-3-(4-methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)benzamide (**13**u): a white solid, yield 53%, m. p. 169—170 °C. ^{1}H NMR (300 MHz, DMSO-d_6), δ: 10.99(s, 1H, NH of amide, exchangeable), 8.91(d, J=2.4 Hz, 1H, H6 pyridine), 8.58(s, 1H, Ar-H), 8.45(s, 1H, Ar-H), 8.35(d, J=2.4 Hz, 1H, H4 pyridine), 8.27(s, 1H, Ar-H), 8.21(s, 1H, imidazole-H), 7.87(s, 1H, NH, exchangeable), 7.76(s, 1H, imidazole-H), 7.57(s, 1H, H6 pyrimidine), 7.31(s, 1H, NH, exchangeable), 5.07—4.97(m, 1H, CH, isoxazoline), 4.10—3.85(m, 1H, CH_2, isoxazoline), 3.74—3.50 (m, 3H, CH_2 and CH), 3.39—3.38(m, 1H, CH_2, isoxazoline), 2.66(s, 3H, CH_3, pyridine), 2.19(s, 3H, CH_3, imidazole), 1.92(s, 3H, CH_3, pyrimidine), 1.12(dd, J=10.2, 6.6 Hz, 6H, CH_3); ^{13}C NMR(75 MHz, DMSO-d_6), δ: 163.4, 161.9, 156.0, 151.5, 141.2, 138.9, 137.7, 136.6, 135.3, 133.3, 131.1(q, $J_{F—C}$= 32.7 Hz), 128.0, 125.3(q, $J_{F—C}$=271.5 Hz), 123.7, 122.6, 122.2, 119.0, 114.3, 104.0, 78.0, 43.4, 42.6, 39.6, 25.1, 22.1, 13.5, 12.8; LC-MS , m/z: 608.2$[M+H]^+$.

N-{5-[5-({[2-(sec-Butylamino)-5-methylpyrimidin-4-yl]-amino}methyl)-4,5-dihydroisoxazol-3-yl]-6-methylpyridin-3-yl}-3-(4-methyl-1*H*-imidazol-1-yl)-5-(trifluoromethyl)benzamide (**13**v): a white solid, yield 60%, m. p. 165—167 °C. ^{1}H NMR (300 MHz, DMSO-d_6), δ: 11.05(s, 1H, NH of amide, exchangeable), 8.93(s, 1H, H6 pyridine), 8.60(s, 1H, Ar-H), 8.47(s, 1H, Ar-H), 8.36(s, 1H, H4 pyridine), 8.27(s, 1H, Ar-H), 8.21(s, 1H, imidazole-H), 7.78(s, 1H, imidazole-H), 7.60(s, 1H, H6

pyrimidine), 5.13—4.81(m, 1H, CH, isoxazoline), 3.75—3.66 (m, 1H, CH_2, isoxazoline), 3.64—3.55(m, 2H, CH_2), 3.40—3.38(m, 1H, CH), 3.34—3.34(m, 1H, CH_2, isoxazoline), 3.09—3.07(m, 2H, CH_2), 2.66(s, 3H, CH_3, pyridine), 2.19(s, 3H, CH_3, imidazole), 1.93(s, 3H, CH_3, pyrimidine), 0.83(d, J=6.3 Hz, 6H, CH_3); ^{13}C NMR(75 MHz, DMSO-d_6), δ: 163.5, 161.1, 160.8, 156.4, 153.3, 142.9, 142.5, 141.2, 139.0, 137.8, 136.8, 135.7, 135.3, 133.2, 131.1(q, $J_{F—C}$=32.6 Hz), 128.0, 125.2(q, $J_{F—C}$=271.6 Hz), 123.8, 122.7, 120.7, 114.3, 102.0, 78.0, 48.5, 42.9, 39.7, 29.0, 28.2, 24.2, 20.2, 13.2; LC-MS, *m*/*z*: 622.2$[M+H]^+$.

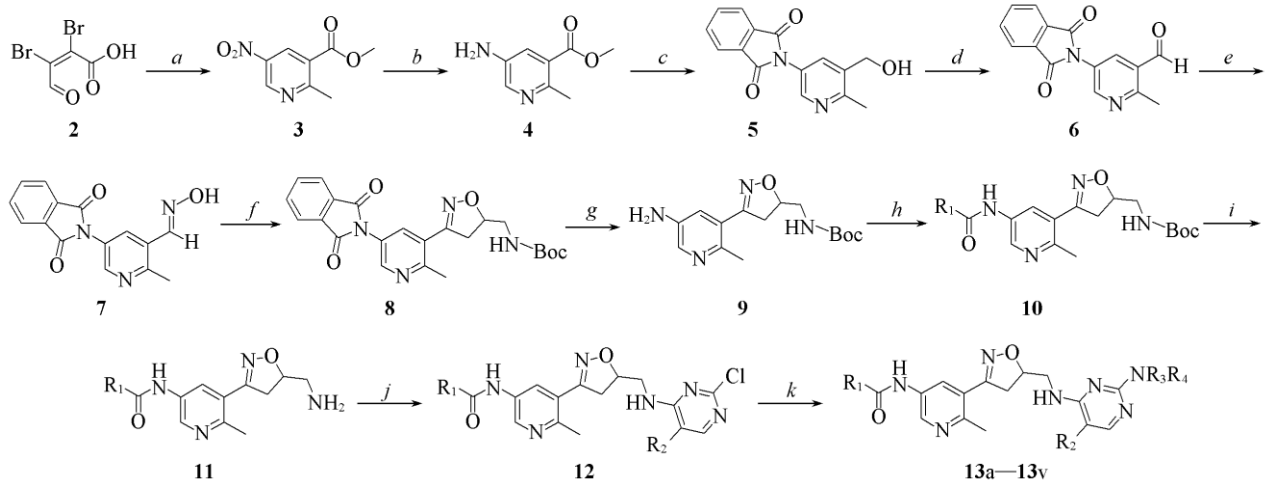
2.2 Biological Evaluation

The anti-proliferative activities of compounds **13**a—**13**v were assessed against MCF-7(human breast cancer cell lines) by MTT assay. The MCF-7 cells were plated in 96-well flat-bottomed microtiter plates(cells were suspended in 100 μL culture medium per well) and incubated at 37 °C for 18 h under 100% relative humidity atmosphere containing 5% CO_2. A 100 μL aliquot of culture medium containing the tested compound was added to the wells. The plates were incubated for additional 52 h. After removal of culture medium, the culture medium containing 3-(4,5-dimethylthiazol-2-yl-5-(3-carboxymethoxyphenyl)-2-(4-sulfphenyl)-2*H*-tetrazolium, inner salt(MTS, 2 mg/mL)/phenazine methosulfate(PMS, 0.92 mg/mL)(volume tratio 20:1) was added and incubated at 37 °C for 4 h. The absorbance was measured on a Synergy HT multifunctional microplate reader at 490 nm. The percentage of inhibition at each compound concentration was calculated according to formula: percentage of inhibition(%)=(A_{sample}−$A_{negative}$)/(A_{blank}−$A_{negative}$)×100%. Compounds were studied for dose-response relationship at 100, 50.0, 16.667, 5.556, 1.852, 0.617, 0.206, 0.069, 0.023 and 0.007 μmol/L. Their half maximal inhibitory concentrations(IC_{50}s) were calculated using GraphPad Prism 5. Seven compounds(**13**c, **13**f—**13**j and **13**v) which exploited potent anti-proliferative activities against MCF-7 were selected against another two types of human cancer cell lines: liver cancer cell line HepG2 and cervical cancer cell line HeLa, and the test method was also MTT assay as described above against MCF-7.

3 Results and Discussion

3.1 Chemistry

The long linear synthetic pathway employed to obtain the target pyridinyl-4,5-2*H*-isoxazole derivatives **13**a—**13**v is illustrated in Scheme 1. Intermediate **3** was obtained from the starting material mucobromic acid **2** through nitration and condensation with methyl 3-aminocrotonate[23]. Intermediate **5** was prepared by reduction reaction and Pht protection after catalytic hydrogenation of compound **3**. For the synthesis of intermediate **6**, oxidation of primary alcohol was accomplished under Parikh-Doering condition, then it reacted with hydroxylamine to give compound **7**. The 1,3-dipolar cycloaddition reaction of compound **7** with *t*-butyl allylcarbamate in the presence of triethylamine gave the key intermediate **8** after halogenation with *N*-chlorosuccinimide. After that, Pht protection group was removed and various aromatic acids were introduced to give compound **10**. Subsequently, Boc protection group was removed and compound **11** reacted with 2,4-dichloro-pyrimidine analogues to give 4-substituted pyrimidine derivatives **12**[24]. Then, various amines reacted with intermediates **12** to prepare final products **13**a—**13**v in the presence of *p*-toluenesulfonic acid in dioxane at reflux.



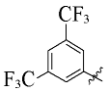
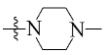
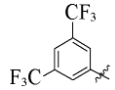
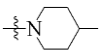
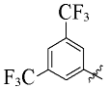
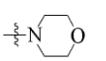
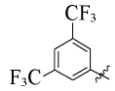
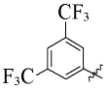
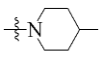
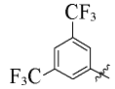
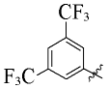
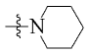
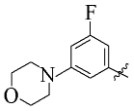
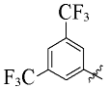
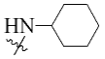
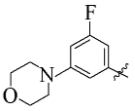
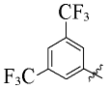
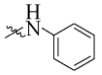
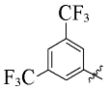
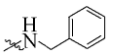
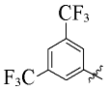
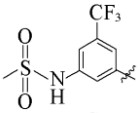
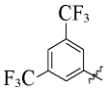
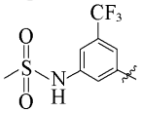
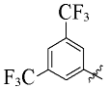
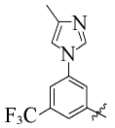
Reagents and conditions: *a*. (1) $NaNO_2$, EtOH, H_2O, 0—60 °C, 0.5 h; (2) methyl 3-aminocrotonate, acetic acid, EtOH, 50 °C, overnight, 23%. *b*. H_2, Pd/C, MeOH, r. t., overnight, 85%. *c*. (1) LAH, anhydrous THF, 0 °C to r. t., 2 h; (2) *o*-phthalic anhydride, TsOH, dioxane, r. t. to 100 °C, 1.5 d, 81%. *d*. Py·SO_3, anhydrous DMSO, anhydrous TEA, anhydrous DCM, r. t., 3 h, 90%. *e*. NH_2OH HCl, EtOH, 60 °C, 3 h, 70%. *f*. (1) NCS, anhydrous DMF, 0—50 °C, 3 h; (2) *t*-butyl allylcarbamate, anhydrous TEA, anhydrous DCM, 0 °C to r .t., overnight, 52%. *g*. methylamine EtOH, 60 °C, 3 h, 99%. *h*. aromatic acid, DMAP, EDCI, DCM, r. t., overnight, 70%—90%. *i*. TFA, anhydrous DCM, 0 °C to r. t., 2 h, 100%. *j*. 2,4-dichloropyrimidine or 2,4-dichloro-5-methylpyrimidine, TEA, EtOH, 70 °C, overnight, 77%. *k*. amine, TsOH, dioxane, reflux, 0.5—3 d, 29%—85%.

Scheme 1 Synthetic routes of target compounds 13a—13v

3.2 Biological Evaluation

All the newly synthesized 22 compounds were investigated for their *in vitro* anti-proliferative activities against human breast cancer cell line(MCF-7) by MTT assay and the results are tabulated in Table 1.

Table 1 Anti-proliferative activities of compounds 13a—13v against MCF-7 cell line

Compound	R_1	R_2	NR_3R_4	IC_{50}/(μmol·L^{-1})	Compound	R_1	R_2	NR_3R_4	IC_{50}/(μmol·L^{-1})
13a		Me		>10	**13**l		H		6.9
13b		Me		>10	**13**m		H		>10
13c		Me		1.9	**13**n		H		>10
13d		Me		>10	**13**o		Me		NA*
13e		Me		>10	**13**p		Me		>10
13f		Me		2.5	**13**q		Me		NA*
13g		Me		2.3	**13**r		Me		>10
13h		Me		2.9	**13**s		Me		NA*
13i		Me		2.7	**13**t		Me		NA*
13j		Me		1.5	**13**u		Me		9.0
13k		Me		>10	**13**v		Me		3.1
					INK128	—	—	—	0.36

* Inactive against this cell line.

From the results, it is evident that most of the tested compounds, including **13**o, **13**q, **13**s and **13**t, displayed potent inhibitory activity toward the tested human breast cancer cell line. Compounds **13**c and **13**j showed the highest antitumor activities with IC_{50} values of 1.9 and 1.5 μmol/L, respectively. Most of the tested compounds were found to be more potent than compound **1**.

Subsequently, the SAR could be inferred from Table 1. While keeping R_1 and R_2 constant[R_1=3,5-bis(trifluoromethyl)-benzyl and R_2=methyl], various pharmaceutical molecular fragments[25—27] on the 2-position of pyrimidine ring(NR_3R_4) were examined. With cyclic aliphatic substituents, most derivatives showed poor activities besides **13**c(IC_{50}=1.9 μmol/L). In comparison, derivatives with both chain aliphatic substituents and aromatic substituents(**13**f—**13**j) showed substantial anti-proliferative activities, with IC_{50}s in the low to mid single digit micromolar range. While keeping R_2=H, three substituent groups on the 2-position of pyrimidine ring(NR_3R_4) were examined(**13**l—**13**n). All of them exhibited worse activities than the compounds with R_2=methyl(**13**c, **13**i and **13**j). This result suggested that the methyl on pyrimidine ring might play an important role in the anti-cancer activities. Otherwise, keeping R_2 and NR_3R_4 constant(R_2=methyl and NR_3R_4=isopropyl or isobutyl), both hydrophilic and hydrophobic substituent groups on the amide(R_1) were examined. Only the derivatives with hydrophobic groups on phenyl displayed potent biological

activities (**13**u, IC_{50}=9.0 μmol/L; **13**v, IC_{50}=3.1 μmol/L)(Fig.2).

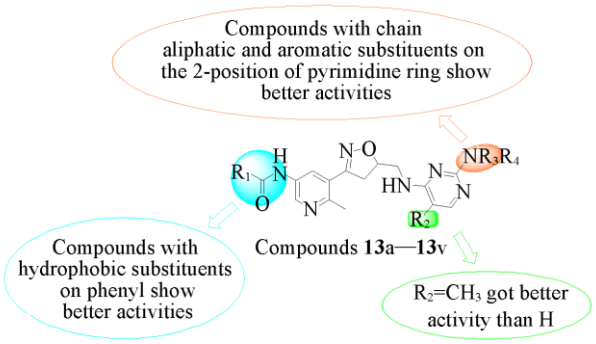


Fig.2 Structure-activity relationship of compounds 13a—13v

To further study the anticancer activities, compounds **13**c, **13**f—**13**j, and **13**v were selected for evaluation of their inhibitory activities against another two types of human cancer cell lines: human liver cancer HepG2 cell line and cervical cancer HeLa cell line. As shown in Table 2, most of them, besides **13**f, exhibited good anti-proliferative activities against the other two cancer cell lines. It is amazing that compounds **13**g and **13**v exhibited more stable activities against these three human cancer cell lines, and can be used as molecular probes for screening various targets. These broad-spectrum anti-proliferative activities are encouraging for the further development of new chemotherapeutic agents.

Table 2 Anti-proliferative activities of compounds 13c, 13f—13j and 13v against MCF-7, HepG2 and HeLa cell lines

Compound	IC_{50}/(μmol·L^{-1})		
	MCF-7	HepG2	HeLa
13c	1.9	11.2	7.3
13f	2.5	NA*	NA*
13g	2.3	5.4	8.6
13h	2.9	9.2	15.8
13i	2.7	14.0	8.2
13j	1.5	16.0	9.6
13v	3.1	4.4	8.7

* Inactive against this cell line.

4 Conclusions

Twenty-two pyridinyl-4,5-2*H*-isoxazole derivatives were prepared and their anti-proliferative activities against MCF-7 cell line were evaluated by MTT assay. Among them, compounds **13**c and **13**j showed the highest antitumor activities. The SAR analysis indicated that both the chain aliphatic substituents and aromatic substituents on the 2-position of pyrimidine ring were sufferable. Secondly, the methyl on 5-position of pyrimidine was necessary. Last, the amide derivatives with hydrophobic substituents on phenyl displayed potent biological activities. Seven of them were selected against HepG2 and Hela cell lines. The results showed that this kind of compounds possessed a broad spectrum of anti-proliferative activities. Our results provide useful information for the design of novel compounds with better potency as antitumor agents. Further study on the antitumor activities *in vivo* and exact biological mechanism is underway.

References

[1] Devita V. T., Rosenberg S. A., *N. Engl. J. Med.*, **2012**, *366*(23), 2207
[2] Daly J. M., Weintraub F. N., Shou J., Rosato E. F., Lucia M., *Ann. Surg.*, **1995**, *221*(4), 327
[3] Kaliberov S. A., Buchsbaum D. J., *Cancer Res.*, **2012**, *115*, 221
[4] Gerber D. E., Schiller J. H., *J. Clin. Oncol.*, **2013**, *31*(8), 1009
[5] Zhang Y. H., Ye L. H., Peng H. Y., Zhang X., *J. Nanjing Univ. Tradit. Chin. Med.*, **2011**, *1*, 4
[6] Flaherty K. T., *Clin. Cancer Res.*, **2006**, *12*(7), 2366
[7] Kim J., Lee J. E., Lee S. H., Yu J. H., Lee J. H., Park T. G., Hyeon T., *Adv. Mater.*, **2008**, *20*(3), 478
[8] Demartino J. K., Boger D. L., *Drug Future*, **2008**, *33*(11), 969
[9] Ferlay J., Autier P., Boniol M., Heanue M., Colombet M., Boyle P., *Ann. Oncol.*, **2007**, *18*(3), 581
[10] Nussbaumer S., Bonnabry P., Jean-Luc V., Fleury-Souverain S., *Talanta*, **2011**, *85*(5), 2265
[11] Jemal A., Siegel R., Ward E., Murray T., Xu J., Smigal C., Thun M. J., *Cancer J. Clin.*, **2006**, *56*(2), 106
[12] Kaushik G., Kaushik T., Khanduja S., Pathak C. M., Khanduja K. L., *Cancer Lett.*, **2008**, *270*(1), 120
[13] Bhuva H. A., Kini S. J., *J. Mol. Graph. Model.*, **2010**, *29*(1), 32
[14] Albreht T., Mckee M., Alexe D. M., Coleman M. P., Martin-Moreno J. M., *Eur. J. Cancer*, **2008**, *44*(10), 1451
[15] Madhusudan S., Trivadi S., Ganesan T. S., *Clinical. Biochem.*, **2004**, *37*, 618
[16] Sawyers C., *Nature*, **2004**, *432*(7015), 294
[17] Eckhardt S., *Curr. Med. Chem. Anti-Cancer Agents*, **2002**, *2*(3), 419
[18] Li Q. B., Xu W. F., *Curr. Med. Chem. Anti-Cancer Agents*, **2005**, *5*(1), 53
[19] Bagalkot V., Zhang L. F., Levy-Nissenbaum E., Jon S. Y., Kantoff P. W., Langer R., Farokhzad O. C., *Nano Lett.*, **2007**, *7*(10), 3065
[20] Willmann J. K., Bruggen N. V., Dinkelborg L. M., Gambhir S. S., *Nat. Rev. Drug Discov.*, **2008**, *7*(7), 591
[21] Dong B., Zhu Y. M., *Chin. J. Cancer*, **2010**, *29*(3), 340
[22] Yang H. L., Xu G. X., Bao M. Y., Zhang D. P., Li Z. W., Pei Y. Z., *Chem. J. Chinese Universities*, **2014**, *35*(12), 2584
[23] Fanta P. E., *J. Am. Chem. Soc.*, **1953**, *75*, 737
[24] Altmann E., Aichholz R., Betschart C., Buhl T., Green J., Irie O., Teno N., Lattmann R., Tintelnot-Blomley M., Missbach M., *J. Med. Chem.*, **2007**, *50*(4), 591
[25] Rogez-Florent T., Meignan S., Foulon C., Six P., Gros A., Bal-Mahieu C., Supuran C. T., Scozzafava A., Frédérick R., Masereel B., Depreux P., Lansiaux A., Goossens J. F., Gluszok S., Goossens L., *Bioorg. Med. Chem.*, **2013**, *21*(6), 1451
[26] Hartshorn M. J., Murray C. W., Cleasby A., Frederickson M., Tickle I. J., Jhoti H., *J. Med. Chem.*, **2005**, *48*(2), 403
[27] Badrinarayan P., Sastry G. N., *J. Chem. Inf. Model.*, **2011**, *51*(1), 115