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Selective and Orally Bioavailable CHK1 Inhibitors of Some Synthesized Substituted Thieno[2,3-b]pyridine Candidates

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ABSTRACT
A series of the newly substituted thienopyrimidine derivatives 2-13 were synthesized by using 3-amino-6-(4-chlorophenyl)-4-(4-isopropylphenyl) thieno[2,3-b]pyridine-2-carbohydrazide 1 as starting material. Reaction of compound 1 with p-nitrobenzaldehyde in refluxing ethanol afforded the corresponding Shiff base 2, which was cyclized with triethyl orthoformate to give thienopyrimidine derivative 3. Compound 1 was treated with formic acid or acetic anhydride to afford the corresponding thienopyrimidines 4 and 5, respectively. Treatment of 1 with acetylacetone afforded pyrazolothienopyrimidine 6. Treatment of 1 with triethyl orthoformate, carbon disulfide, dimethylformamide and ethyl acetoacetate or diethyl malonate gave compounds 7-11, respectively. Cyclization of 1 with m-nitrophthalic anhydride or formaldehyde afforded thienopyrimidines 12 and 13, respectively. The newly synthesized compounds were found to be potent selective and orally bioavailable CHK1 inhibitors. This elucidated and confirmed via determination of checkpoint abrogation, antiproliferative activity and potentiation of genotoxic drug efficacy in cancer cell lines and also determination of compound concentrations *in vivo* at selected time points following i.v. and oral dosing.

Key words: Synthesis, thienopyridine carbohydrazide, thienopyrimidines, potent selective and orally bioavailable CHK1 inhibitors

INTRODUCTION

Thienopyrimidines are analogs to biogenic purines and were considered as potential nucleic acid antimetabolites. Earlier, various aspects of the chemistry and biology of isomeric thienopyrimidines have been reviewed (Litvinov, 2004). Many of these derivatives were found to possess a variety of pronounced activities such as antimalarial (Rosowsky *et al*., 1973), antimicrobial (Dave and Shah, 2002; Chambhare *et al*., 2003), anti-inflammatory (Santagati *et al*., 1995) and antiallergic (Gillespie *et al*., 1985). In previous studies, we have found some substituted pyridine and their derivatives showed anticonvulsant, antiparkinsonian, antimicrobial and analgesic (Abd El-Latif *et al*., 2007; Amr *et al*., 2003) and antitumor activities (Amr *et al*., 2006; Abo-Ghalia and Amr, 2004). Also, the biological and analgesic activities of some sulfur heterocyclic compounds have been reviewed (Fahmy and El-Eraki, 2001). Additionally, thioxopyrimidine and thienopyrimidine derivatives have been promising pharmacological (De Clercq, 1986) and anticancer activities (Brana *et al*., 1993). Recently, some of thienopyrimidinone and oxazinone derivatives have been prepared and they are screening as anti-inflammatory, antimicrobial and anti-HIV agents (Al-Omar and Amr, 2010; Mohamed *et al*., 2012). Also, many pyridine fused to different heterocyclic ring systems were found to have potent kinase inhibitor activities were discovered and we will discus in the results and discussion sector.

Many pyridine fused ring systems synthesized and screened for their kinase inhibitor activities and were to have this property on a wide diverse and different kinases. Some of which were founded to have potent selective inhibitor activities on one kinase only and in rare cases some pyridine derivative were founded to be highly selective against certain one isomer of the kinase itself rather than other isomers. This considered as a lead to screen the newly synthesized thienopyrimidine candidates first for their kinases inhibitor activities and then for selectivity.

MATERIALS AND METHODS

Chemistry: Melting points were determined in open glass capillary tubes with an Electro Thermal Digital melting point apparatus, (model: IA9100) and are uncorrected. Elemental microanalysis for carbon, hydrogen and nitrogen (Microanalytical Unit, NRC) was found within the acceptable limits of the calculated values. Infrared spectra (KBr) were recorded on a Nexus 670 FTIR Nicolet, Fourier Transform infrared spectrometer. Proton Nuclear Magnetic Resonance (^{1}H NMR) spectra were run in DMSO-d_6 on Jeol 500 MHz instruments. Analytical Thin Layer Chromatography (TLC) was performed on silica gel aluminum sheets, 60 F_{254} (E. Merck).

3-Amino-6-(4-chlorophenyl)-4-(4-isopropylphenyl) thieno[2,3-b]pyridine-2-carbohydrazide (1): A mixture of thienopyridine ethyl ester (0.01 mol) and hydrazine hydrate 98% (1 mL, 0.04 mol) was refluxed in absolute ethanol for 3 h, then the reaction mixture was cooled. The formed precipitate was filtered off, dried and crystallized from acetic acid to give yellow crystals of 1; Yield 65%, mp 182-184°C. IR (KBr, cm^{-1}): 3401, 3280, 3189 ($2NH_2$ and NH) and 1649 C = O, amide). ^{1}H NMR (DMSO-d_6, δ ppm): δ = 1.26 (d, 6H, J = 6.50 Hz, $(CH_3)_2CH$), 3.0 (m, 1 H, $(CH_3)_2CH$), 4.10 (s, 2H, NH_2), 4.41 (s, 2H, NH_2), 7.48 (d, 2H, J = 7.50 Hz, Ar-H), 7.57 (d, 2H, J = 8.0 Hz, Ar-H), 7.69 (d, 2H, J = 7.50 Hz, Ar-H), 7.94 (s, 1H, pyridine-H-5), 8.31 (d, 2H, J = 8.0 Hz, Ar-H), 9.43 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, δ ppm): δ = 24.1 ($(CH_3)_2CH$), 33.8 ($(CH_3)_2CH$), 103.1, 116.2, 116.8, 127.3, 129.2, 129.3, 129.7, 130.0, 133.5, 135.9, 136.1, 151.3, 154.7, 157.2, 162.7 and 166.9 (Ar-C and C = O). $C_{23}H_{21}ClN_4OS$ (436.96): calcd. C, 63.22; H, 4.84; Cl, 8.11; N, 12.82; S, 7.34; found C, 63.10; H, 4.76; Cl, 8.04; N, 12.75; S, 7.30.

3-Amino-6-(4-chlorophenyl)-4-(4-isopropylphenyl)-N'-(4-nitrobenzylidene)thieno[2,3-b]-pyridine-2-carbohydrazide (2): A mixture of 1 (0.01 mole) and 4-nitrobenzaldehyde (0.01 mole) in ethanol (10 mL) was refluxed for 8 h. The solvent was evaporated under reduced pressure and the residue washed with petroleum ether 60-80°C. The obtained solid was filtered off, dried and recrystallized from ethanol to give pale yellow crystals of 2. Yield 55%, mp 250-252°C. IR (KBr, cm^{-1}): 3315, 3150 (NH_2 and NH), 1651 (C = O, amide) and 1602 C = N). ^{1}H NMR (DMSO-d_6, δ ppm): δ = 1.25 (d, 6H, J = 6.50 Hz, $(CH_3)_2CH$), 2.99 (m, 1H, $(CH_3)_2CH$), 4.26 (s, 2H, NH_2), 4.74 (s, 1H, N=CH), 6.89 (s, 1H, pyridine-H-5), 7.45 (d, 2H, J = 8.40 Hz, Ar-H), 7.60 (d, 2H, J = 8.0 Hz, Ar-H), 7.68 (d, 2H, J = 8.40 Hz, Ar-H), 7.93 (d, 2H, J = 8.0 Hz, Ar-H), 8.22 (d, 2H, J = 8.0 Hz, Ar-H), 8.41 (d, 2H, J = 8.0 Hz, Ar-H), (s, 1H, NH). $C_{30}H_{24}ClN_5O_3S$ (570.06): calcd. C, 63.21; H, 4.24; Cl, 6.22; N, 12.29; S, 5.62; found C, 63.15; H, 4.20; Cl, 6.16; N, 12.20; S, 5.55.

7-(4-Chlorophenyl)-9-(4-isopropylphenyl)-3-(4-nitrobenzylideneamino)-4-oxo-3,4-dihydro-pyrido[3',2':4,5]thieno[3,2-d]pyrimidine (3): To a mixture of hydrazide 2 (0.01 mol) in ethanol (20 mL), triethyl orthoformate (0.01 mol) and few drops of acetic acid were added; the reaction mixture was refluxed for 3 h. The formed precipitate was filtered off, dried and recrystallized from acetic acid to give pale yellow crystals of 3. Yield 68%, mp 274-276C. IR (KBr, cm^{-1}): 1676 C = O, amide) and 1605 (C =N). ^{1}H NMR (DMSO-d_6, δ ppm): δ = 1.25 (d, 6H, J = 7.0 Hz, $(CH_3)_2CH$), 2.98 (m, 1H, $(CH_3)_2CH$), 4.73 (s, 1H, N = CH), 7.31-8.25 (m, 14H, Ar-H). ^{13}C NMR (DMSO-d_6, δ ppm): δ = 24.1 ($(CH_3)_2CH$), 33.8 ($(CH_3)_2CH$), 61.7 (CH = N), 98.0, 105.3, 108.4, 115.7, 116.4, 119.1, 124.5, 127.3, 128.2, 129.0, 129.1, 129.2, 129.7, 132.9, 135.3, 140.1, 142.3, 146.8, 150.8, 153.1 and 168.5 (Ar-C, N = CH and C = O). $C_{31}H_{22}ClN_5O_3S$ (580.06): calcd. C, 64.19; H, 3.82; Cl, 6.11; N, 12.07; S, 5.53. found. C, 64.19; H, 3.82; Cl, 6.11; N, 12.07; S, 5.53.

7-(4-Chlorophenyl)-9-(4-isopropylphenyl)-3-formylamino-4-oxo-3,4-dihydropyrido[3',2':4,5]-thieno[3,2-d]pyrimidine (4): A solution of hydrazide 1 (0.01 mol) in formic acid (20 mL) was heated under reflux for 5 h. The reaction mixture was cooled and poured into ice water. The formed precipitate was filtered off, dried and recrystallized from ethanol to give yellow crystals of 4. Yield 80%, mp 224-226°C. IR (KBr, cm^{-1}): 3310 (NH), 1670 (C = O, amide). ^{1}H NMR (DMSO-d_6, ppm): δ = 1.25 (d, 6H, J = 6.0 Hz, $(CH_3)_2CH$), 2.99 (m, 1H, $(CH_3)_2CH$), 6.88 (s, 1H, pyridine-H-8), 7.43 (d, 2H, J = 7.50 Hz, Ar-H), 7.58 (d, 2H, J = 7.0 Hz, Ar-H), 7.67 (d, 2H, J = 7.50 Hz, Ar-H), 7.93, (d, 2H, J = 7.0 Hz, Ar-H), 8.29 (s, 1H, pyrimidine-H-2), 10.41 (s, 1H, CHO), 12.83 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, δ ppm): δ = 24.1 ($(CH_3)_2CH$), 33.3 ($(CH_3)_2CH$), 103.0, 116.7, 117.0, 127.2, 127.3, 128.8, 129.2, 129.3, 130.0, 133.4, 133.8, 135.9, 136.4, 151.5, 157.2, 159.9, 162.6 and 170.3 (Ar-C, C = O and HC=O). $C_{25}H_{19}ClN_4O_2S$ (474.96): calcd. C, 63.22; H, 4.03; Cl, 7.46; N, 11.80; S, 6.75; found. C, 63.12; H, 4.00; Cl, 7.40; N, 11.72; S, 6.70.

3-Acetylamino-7-(4-chlorophenyl)-9-(4-isopropylphenyl)-2-methyl-4-oxo-3,4-dihydropyrido [3',2':4,5]-thieno[3,2-d]pyrimidine (5): A mixture of hydrazide 1 (0.01 mol) and acetic anhydride (15 mL) was heated under reflux for 3 h and after cooling, the reaction mixture was poured into ice

water, the formed precipitate was filtered off, dried and recrystalized from ethanol to give pale yellow crystals of 5. Yield 74%, mp 274-276°C. IR (KBr, cm^{-1}): 3220 (NH), 1685 (C = O, amide). ^{1}H NMR (DMSO-d_6, ppm): δ = 1.25 (d, 6H, J = 6.0 Hz, $(CH_3)_2CH$), 2.98 (m, 1H, $(CH_3)_2CH$), 1.92 (s, 3H, $NCOCH_3$), 2.34 (s, 3H, CH_3-pyrimidine ring), 6.89 (s, 1H, pyridine-H-8), 7.43 (d, 2H, J = 7.50 Hz, Ar-H), 7.58 (d, 2H, J = 7.0 Hz, Ar-H), 7.67 (d, 2H, J = 7.50 Hz, Ar-H), 7.94 (d, 2H, J = 7.0 Hz, Ar-H), 12.78 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, δ ppm): δ = 22.1 ($COCH_3$), 23.0 (CH_3-pyrimidine ring), 24.1 ($(CH_3)_2CH$), 33.8 ($(CH_3)_2CH$), 98.4, 107.1, 117.0, 127.2, 128.8, 129.3, 129.8, 130.0, 131.8, 133.9, 136.4, 142.0, 150.8, 151.5, 159.9, 162.6, 166.0 and 171.3 (Ar-C and 2C = O). $C_{21}H_{19}ClN_4O_2S$ (426.92): calcd. C, 59.08; H, 4.49; Cl, 8.30; N, 13.12; S, 7.51; found. C, 59.00; H, 4.40; Cl, 8.22; N, 13.05; S, 7.45.

(3-Amino-6-(4-chlorophenyl)-4-(4-isopropylphenyl)-thieno[2,3-b]pyridin-2-yl)(3,5-dimethyl-1H-pyrazol-1-yl) methanone (6): A mixture of hydrazide 1 (0.01 mol) and acetylacetone (0.02 mol) in ethanol (30 mL) was heated under reflux for 6 h. The reaction mixture was cooled and poured into ice water. The formed precipitate was filtered off, dried and recrystallized from ethanol to give yellow crystals of 6; Yield 55%, mp 282-284°C. IR (KBr, cm^{-1}): 3411, (NH_2), 1679 (C=O, amide). ^{1}H NMR (DMSO-d_6, ppm): δ = 1.24 (d, 6H, J = 6.0 Hz, $(CH_3)_2CH$), 2.32 (s, 3H, CH_3), 2.38 (s, 3H, CH_3), 2.98 (m, 1H, $(CH_3)_2CH$), 3.40 (s, 2H, NH_2), 6.77 (s, 1H, pyrazole-H), 6.88 (s, 1H, pyridine-H-5), 7.42 (d, 2H, J = 8.0 Hz, Ar-H), 7.57 (d, 2H, J = 8.50 Hz, Ar-H), 7.66 (d, 2H, J = 8.0 Hz, Ar-H), 7.93 (d, 2H, J = 8.50 Hz, Ar-H). $C_{22}H_{21}ClN_4OS$ (424.95): calcd. C, 62.18; H, 4.98; Cl, 8.34; N, 13.18; S, 7.55; found. C, 62.10; H, 4.90; Cl, 8.30; N, 13.10; S, 7.50.

7-(4-Chlorophenyl)-9-(4-isopropylphenyl)-4-oxo-3,4-dihydropyrido[3',2':4,5]-thieno[3,2-d]pyrimidine (7): A mixture of hydrazide 1 (0.01 mole in methanol (30 mL), triethyl orthoformate (5 mL) and few drops of acetic acid was refluxed for 5 h. After cooling, the precipitate was filtered off, washed with water and recrystallized from methanol to give pale yellow crystals of 7. Yield 55%, mp 260-262°C. IR (KBr, cm^{-1}): 3356 (NH) and 1665 (C = O). ^{1}H NMR (DMSO-d_6, δ ppm): δ = 1.25 (d, 6H, J = 6.50 Hz, $(CH_3)_2CH$), 2.99 (m, 1H, $(CH_3)_2CH$), 6.89 (s, 1H, pyridine-H-8), 7.43 (d, 2H, J = 8.0 Hz, Ar-H), 7.58 (d, 2H, J = 8.0 Hz, Ar-H), 7.67 (d, 2H, J = 7.50 Hz, Ar-H) 7.94 (d, 2H, J = 7.50 Hz, Ar-H), 8.30 (s, 1H, pyrimidine-H-2), 12.86 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, δ ppm): δ = 24.1 ($(CH_3)_2CH$), 33.8 ($(CH_3)_2CH$), 107.0, 116.2, 117.0, 127.2, 127.3, 128.8, 129.2, 129.3, 130.0, 131.7, 133.8, 136.4, 140.1, 151.6, 159.9 and 162.6, 169.0 (Ar-C and C = O). $C_{24}H_{18}ClN_3OS$ (431.94): calcd. C, 66.74; H, 4.20; Cl, 8.21; N, 9.73; S, 7.42; found. C, 66.65; H, 4.12; Cl, 8.15; N, 9.66; S, 7.34.

5-(3-Amino-6-(4-chlorophenyl)-4-(4-isopropylphenyl) thieno[2,3-b]pyridin-2-yl)-1,3,4-thia-diazole-2(3H)-thione (8): A mixture hydrazide 1 (0.01 mol), carbon disulfide (0.6 mL) and potassium hydroxide (0.28 g) in ethanol (25 mL) was stirred for 3 h and then acidified by HCl. The formed precipitate was crystalized from methanol to give pale yellow crystals of 8, Yield 60%, mp 130-132°C. ^{1}H NMR (DMSO-d6, ppm): δ = 1.25 (d, 6H, J = 6.50 Hz, $(CH_3)_2CH$), 2.98 (m, 1H, $(CH_3)_2CH$), 5.83 (s, 2H, NH_2), 6.88 (s, 1H, pyridine-H-5), 7.44 (d, 2H, J = 8.0 Hz, Ar-H), 7.57 (d, 2H, J = 8.0 Hz, Ar-H), 7.68 (d, 2H, J = 8.0 Hz, Ar-H), 7.93 (d, 2H, J = 8.0 Hz, Ar-H) 12.96 (s, 1H, SH),. ^{13}C NMR (DMSO-d_6, δ ppm): δ = 24.1 ($(CH_3)_2CH$), 33.8 ($(CH_3)_2CH$), 95.5, 102.9, 116.8, 118.7, 126.9, 127.2, 128.8, 129.1, 129.3, 130.0, 133.8, 134.1, 135.3, 136.4, 151.5, 161.3 and 164.7 (Ar-C). $C_{24}H_{19}ClN_4S_3$ (495.08): calcd. C, 58.22; H, 3.87; Cl, 7.16; N, 11.32; S, 19.43; found. C, 58.08; H, 3.80; Cl, 7.10; N, 11.24; S, 19.32.

3-Amino-7-(4-chlorophenyl)-9-(4-isopropylphenyl)-4-oxo-3,4-dihydropyrido[3',2':4,5]-thieno[3,2-d]pyrimidine (9): A mixture of hydrazide 1 (0.01 mol) and DMF (5 mL) was refluxed for 12 h, The reaction mixture was cooled and poured into ice water. The formed precipitate was filtered off and recrystalized from ethanol to give pale yellow crystals of 9. Yield 60%, mp 140-142°C. IR (KBr, cm^{-1}): 3390 (NH_2), 1667 (C = O, amide). ^{1}H NMR (DMSO-d_6, δ ppm): δ = 1.27 (d, 6H, J = 7.0 Hz, $(CH_3)_2CH$), 3.02 (m, 1H, $(CH_3)_2CH$), 5.86 (s, 2H, NH_2), 6.93 (s, 1H, pyridine-H-8), 7.44 (d, 2H, J = 8.0 Hz, Ar-H), 7.60 (d, 2H, J = 8.50 Hz, Ar-H), 7.69 (d, 2H, J = 8.0 Hz, Ar-H), 7.75 (s, 1H, pyrimidine-H-2),7.94 (d, 2H, J = 8.50 Hz, Ar-H). ^{13}C NMR (DMSO-d_6, δ ppm): δ = 24.1 ($(CH_3)_2CH$), 33.8 ($(CH_3)_2CH$), 118.8, 121.0, 127.2, 127.3, 128.8, 129.1, 129.5, 129.7, 130.0, 134.1, 135.3, 136.5, 148.7, 150.1, 155.5, 161.3 and 164.7 (Ar-C and C=O). $C_{24}H_{19}ClN_4OS$ (446.95): calcd. C, 64.49; H, 4.28; Cl, 7.93; N, 12.54; S, 7.17; found. C, 64.40; H, 4.20; Cl, 7.90; N, 12.50; S, 7.10.

1-(3-Amino-6-(4-chlorophenyl)-4-(4-isopropylphenyl)thieno [2,3-b]pyridine-2-carbonyl)-5-methyl-1H-pyrazol-3(2H)-one (10): A mixture of hydrazide 1 (0.01 mol) and ethyl acetoacetate (0.02 mol) in ethanol (30 mL) was heated under reflux for 6 h. The reaction m ixture was cooled and poured into ice water. The formed precipitate was filtered off, dried and recrystallized from ethanol to give yellow crystals of 10; Yield 52%, mp 118-120°C. IR (KBr, cm^{-1}): 3412, 3318 (NH_2 and NH), 1656 (C = O, amide). ^{1}H NMR (DMSO-d_6, ppm): δ = 1.25 (d, 6H, J = 6.50 Hz, $(CH_3)_2CH$), 1.91 (s, 3H, CH_3), 2.99 (m, 1H, $(CH_3)_2CH$), 5.87 (s, 2H, NH_2), 6.89 (s, 1H, pyridine-H-5), 7.25 (s, 1H, pyrazole-H), 7.44 (d, 2H, J = 8.0 Hz, Ar-H), 7.56 (d, 2H, J = 7.50 Hz, Ar-H), 7.68 (d, 2H, J = 8.0 Hz, Ar-H), 7.94 (d, 2H, J = 7.50 Hz, Ar-H), 12.69 (s, H, NH). ^{13}C NMR (DMSO-d_6, δ ppm): δ = 14.8 (CH_3), 24.1 ($(CH_3)_2CH$), 33.8 ($(CH_3)_2CH$), 98.6, 100.9, 118.8, 127.2, 127.3, 128.8, 129.1, 129.3, 129.5, 130.1, 133.9, 134.1, 135.3, 136.4, 136.5, 148.7, 150.1, 166.8

and 168.0 (Ar-C and 2C = O). $C_{27}H_{23}ClN_4O_2S$ (503.02): calcd. C, 64.47; H, 4.61; Cl, 7.05; N, 11.14; S, 6.37; found. C, 64.40; H, 4.54; Cl, 7.00; N, 11.10; S, 6.30.

1-(3-Amino-6-(4-chlorophenyl)-4-(4-isopropylphenyl) thieno[2,3-b]pyridine-2-carbonyl)-pyrazolidine-3,5-dione (11): A mixture of hydrazide 1 (0.01 mol) and diethyl malonate (0.02 mol) in ethanol (30 mL) was heated under reflux for 6 h. The reaction mixture was cooled and poured into ice water. The formed precipitate was filtered off, dried and recrystallized from ethanol to give yellow crystals; Yield 50%, mp 130-132ºC. IR (KBr, cm^{-1}): 3420, 3315 (NH_2 and NH), 1680, 1649 (2C=O, amide). ^{1}H NMR (DMSO-d_6, ppm): δ = 1.24 (d, 6H, J = 6.50 Hz, $(CH_3)_2CH$), 2.99 (m, 1H, $(CH_3)_2CH$), 3.75 (s, 2H, pyrazolidine-H), 5.85 (s, 2H, NH_2), 6.88 (s, 1H, pyridine-H-5), 7.43 (d, 2H, J = 8.0 Hz, Ar-H), 7.58 (d, 2H, J = 8.20 Hz, Ar-H), 7.67 (d, 2H, J = 8.0 Hz, Ar-H), 7.94 (d, 2H, J = 8.20 Hz, Ar-H), 12.68 (s, H, NH). ^{13}C NMR (DMSO-d_6, δ ppm): δ = 24.1 ($(CH_3)_2CH$), 33.8 ($(CH_3)_2CH$), 60.6 (CH_2- pyrazolidine), 117.0, 118.7, 127.2, 127.3, 128.8, 129.1, 129.3, 129.4, 130.0, 133.8, 135.3, 136.4, 147.8, 148.7, 150.1, 155.4, 161.3 and 164.7 (Ar-C and 3C = O). $C_{26}H_{21}ClN_4O_3S$ (504.99): calcd. C, 61.84; H, 4.19; Cl, 7.02; N, 11.09; S, 6.35; found. C, 61.76; H, 4.10; Cl, 7.00; N, 11.00; S, 6.30.

10-(4-Chlorophenyl)-8-(4-isopropylphenyl)-2,13-dioxo-5-nitro-1,2,13-trihydropyrido[3",2"-4',5']thieno[3',2'-4,5]pyrimido[1,2-b]phthalazine (12): To a mixture of hydrazide 1 (0.01 mol) in DMF (10 mL), 3-nitrophthalic anhydride (0.01 mol) was added. The reaction mixture was refluxed for 6 h. The reaction mixture was poured into ice water (20 mL). The formed precipitate was filtered off, washed with ethanol, dried and recrystallized from n-butanol to give pale yellow crystals of 12. Yield 55%, mp 168-170ºC. IR (KBr, cm^{-1}): 3346 (NH), 1661 (C = O, amide). ^{1}H NMR (DMSO-d_6, δ ppm): δ = 1.25 (d, 6H, J = 6.0 Hz, $(CH_3)_2CH$), 2.99 (m, 1H, $(CH_3)_2CH$), 6.88 (s, 1H, pyridine-H-8), 7.43-8.24 (m, 11H, Ar-H), 12.80 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, δ ppm): δ = 24.1 ($(CH_3)_2CH$), 33.8 ($(CH_3)_2CH$), 107.1, 117.0, 118.7, 126.6, 127.2, 127.3, 128.8, 129.1, 129.3, 129.4, 130.0, 133.8, 134.1, 135.3, 136.4, 136.5, 147.8, 148.7, 150.1, 151.5, 155.4, 159.9, 161.4 and 162.6 (Ar-C and 2C = O). $C_{31}H_{20}ClN_5O_4S$ (594.04): calcd. C, 62.68; H, 3.39; Cl, 5.97; N, 11.79; S, 5.40; found. C, 62.60; H, 3.30; Cl, 5.90; N, 11.70; S, 5.32.

3-Amino-7-(4-chlorophenyl)-9-(4-isopropylphenyl)-4-oxo-1,2,3,4-tetrahydropyrido[3',2':4,5]-thieno[3,2-d]pyrimidine (13): A mixture of hydrazide 1 (0.01 mol) and formaldehyde (5 mL) in methanol (20 mL) was heated under reflux for 2 h. The solvent was evaporated under reduced pressure; the residue was treated with water. The formed precipitate was filtered off, dried and recrystallized from ethanol to give pale yellow crystals of 13. Yield 85%, mp 212-214ºC. The IR (KBr, cm^{-1}): 3430 (NH_2, NH), 1660 (C = O, amide) and 1602 (C=N). ^{1}H NMR (DMSO-d_6, δ ppm): δ = 1.25 (d, 6H, J = 7.0 Hz, $(CH_3)_2CH$), 2.74 (s, 2H, pyrimidine-H-2), 3.00 (m, 1H, $(CH_3)_2CH$), 5.84 (s, 2H, NH_2), 6.87 (s, 1H, pyridine-H-8), 7.44 (d, 2H, J = 8.0 Hz, Ar-H), 7.59 (d, 2H, J = 7.50 Hz, Ar-H), 7.67 (d, 2H, J = 8.0 Hz, Ar-H), 7.93 (d, 2H, J = 7.50 Hz, Ar-H), 12.85 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, δ ppm): δ = 24.1 ($(CH_3)_2CH$), 33.8 ($(CH_3)_2CH$), 62.8 (CH_2), 95.6, 118.7, 127.2, 128.8, 129.1, 129.3, 129.4, 130.0, 134.1, 136.4, 147.8, 150.1, 151.5, 155.4, 161.3 and 164.7 (Ar-C and C = O). $C_{24}H_{21}ClN_4OS$ (448.97): calcd. C, 64.20; H, 4.71; Cl, 7.90; N, 12.48; S, 7.14; found. C, 64.10; H, 4.66; Cl, 7.82; N, 12.40; S, 7.10.

Pharmacological activity

Kinase screening at 30 µM: Microfluidic mobility shift assays to determine kinase inhibition were carried out using ProfilerPro assay kits (Caliper Life Sciences); kit 1 was used for the 24-member kinase screen and kits 1-4 were used for the 96-member kinase screen. Each kit contained an enzyme plate, a substrate plate, reconstitution buffer, 1M DTT, protease inhibitor cocktail and termination buffer. Test compounds were added as a solution in DMSO, at the appropriate concentration. The peptide phosphorylation assay was carried out as per the manufacturer's instructions. The electrophoretic separation and analysis of the phosphorylated and non-phosphorylated peptides was carried out on a 4-sipper chip using a LabChip EZReader II (Caliper LifeSciences). Results were expressed as percentage inhibition of peptide phosphorylation relative to control wells for 0% (no test compound) and 100% (no ATP) inhibition. A plate layout was used to accommodate up to 12 test compounds in each assay run. The pan-kinase inhibitor H-89 (30 µM) were incorporated as a test compound in each separate assay plate run.

Determination of CHK1 and CHK2 inhibition: CHK1 kinase activities determined with the microfluidic assay that monitored the separation of a phosphorylated product from its substrate. The assay were run on an EZ Reader II (Caliper Life Sciences Ltd, Runcorn, UK) using separation buffer (#760367 Caliper LS) containing CR-8 (500nM, #760278, Caliper LS). An ECHOR 550 (Labcyte Inc™) acoustic dispenser used to generate duplicate 8 pt dilution curves directly into 384 polypropylene assay plates (Greiner Bio-One, Gloucestershire, UK). For every compound a 50 µM stock concentration in 100% DMSO was used. The total amount of DMSO dispensed per well was 250 nL to give a final assay concentration of 2.5% DMSO and compound concentrations in the range 0.5-1000 nM. To this assay plate, 6 PL CHK1 (2 nM final concentration, in-house protein preparation), Two PL peptide 10 (5-FAM-KKKVSRSGLYRSPSMPENLNRPR-COOH, 1.5 pM final concentration, #760354 Caliper LS) and 2 pL ATP (90 pM final concentration) all diluted in kinase buffer (HEPES 50 mM, NaN3 0.02%, BSA 0.01%, sodium orthovanadate 0.1 mM, DTT 1 mM, $MgCl_2$ 2 mM, Tween20 0.1%) were added. The plate was sealed and centrifuged

(1 min, 1000 rpm) before incubation for 1 h at room temperature. The reaction was stopped by the addition of separation buffer (90 PL). The plate read on an EZ Reade II, using a 12-sipper chip (#760404, Caliper LS) with instrument settings of -1.5 psi and 1750 ΔV. The percentage conversion of product from substrate was generated automatically and the percentage inhibition was calculated relative to blank wells (containing no enzyme and 2.5% DMSO) and total wells (containing all reagents and 2.5% DMSO). IC_{50} values calculated in GraphPad Prism 5 via a non linear regression fit of the log (inhibitor) vs response with variable slope equation.

CHK2 kinase activity measured in a DELFIAR assay that monitored phosphorylation of a CDC25C peptide via a specific phospho antibody. The enzyme reaction was carried out in 96-well polypropylene plates (Greiner). The reaction mix (total volume 25 μL) contained enzyme and peptide mix (15 μL) (containing CHK2, prepared in-house, 1 nM; Biotin-KKKVSRSGLYRSPSMPENLNRPR, 1 μM), ATP (30 μM, 5 μL) and either DMSO (2.5%) or test compound (5 μL) diluted to give a range of concentrations (0-100 μM in 2.5% DMSO, final concentrations) in assay buffer (40 mM HEPES (pH7.4), 40 mM KCl, 2 mM $MgCl_2$, 10 mM DTT and 0.02% Tween 20). The reaction mixture was incubated for 30 min at room temperature and stopped by the addition of buffer (125 μL) containing 40 mM EDTA, 0.05% Tween 20 and 0.1% BSA in TBS (10x concentrate, Sigma). An aliquot (100 μL) of the reaction mix was transferred to a black neutravidin-coated 96-well plate (Perbio) and incubated for 1 h on a shaker (Titertek, Flow Laboratories) at room temperature. The plates were washed four times with wash buffer (25 mM Tris (pH 8), 150 mM NaCl and 0.1% polysorbate 20) (WellWash4, Thermo Life Sciences) and incubated for 1 h as before with antibody mix (100 μL) consisting of anti-phospho CDC25C (diluted 1/4000 equivalent to 0.35-1.25 nM, #9528, Cell Signaling Technology) and europium-labeled anti rabbit IgG, (0.3 μg mL^{-1}, AD0105, PerkinElmer Life Sciences) diluted in DELFIA assay buffer (PerkinElmer Life Sciences). The plates were washed a further four times with wash buffer before the addition of enhancement solution (100 μL/well, PerkinElmer Life Sciences). The plate was read on a EnVision™ 2103 multilabel counter (PerkinElmer Life Sciences) using a time-resolved measurement mode reading fluorescence at 615 nM. IC_{50} values were calculated in GraphPad Prism5 using a non linear regression fit of the log (inhibitor) vs response with variable slope equation.

Determination of checkpoint abrogation, antiproliferative activity and potentiation of genotoxic drug efficacy in cancer cell lines: Checkpoint abrogation by CHK1 kinase inhibitors in combination with genotoxic agents was assessed using a europium based ELISA assay designed to quantify the number of cells trapped in mitosis after treatment with a genotoxic agent (to induce G2 arrest) followed by a test compound in combination with nocodazole to abrogate this arrest. The HT29 cells were seeded at 104 cells per well into 96 well plates in a volume of 160 μL and left to attach for 36 h. Etoposide (10 mM stock in DMSO) was diluted in medium to 250 μM and then 40 μL was added to appropriate wells to give a final concentration of 50 μM and incubated for 1 h. This treatment had previously been optimised to induce a G2 arrest in 80% of cells 16 h following treatment. After genotoxic drug exposure, the medium was removed and replaced with fresh medium (160 μL). Cells were either untreated (untreated control or etoposide pre-treatment alone), exposed to nocodazole following etoposide pretreatment or nocodazole alone (100 ng mL^{-1} final concentration), or exposed to increasing concentrations of test compound (200 μM to 0.01 nM final concentration) in combination with nocodazole (100 ng mL^{-1} final concentration). Test compounds were added in 40 μL using quadruplicate wells for each dose. After 21 h exposure, the medium was removed and cells were fixed in 4% formaldehyde in PBS (pH 7.4, pre-cooled to 4°C) for 30 min at 4°C, followed by 100% MeOH (pre-cooled to -20°C) for 10 min at ambient temperature. Wells were washed with PBS and blocked with 5% dried milk (Marvel) in Tris-buffered saline (TBS, pH 7.4) at 37°C for 30 min. Each well was washed three times with water containing 0.1% Tween20. Primary antibody (MPM-2, Upstate cat# 05-368, 1 μg mL^{-1} in 5% milk in TBS) was added to each well and incubated overnight with shaking at 4°C. Primary antibody was removed and wells were washed with water containing 0.1% Tween20. The secondary antibody (europium labeled anti-mouse, Perkin-Elmer cat# AD0124, 333 ng mL^{-1} in assay buffer, Perkin-Elmer cat# 1244-111) was added to each well and incubated at 37°C for 1 h. Each well was washed with water containing 0.1% Tween20 and treated with enhancement solution (Perkin-Elmer cat# 1244-105). Europium emissions were counted on a Wallac, Victor 2 counter (Perkin-Elmer, Bucks UK). Appropriate controls were included and results were expressed as the concentration of test compound required to allow 50% of cells to enter mitosis (Checkpoint abrogation IC_{50}).

Compound cytotoxicity and the ability of CHK1 inhibitors to enhance SN38 (the active metabolite of the topoisomerase I inhibitor irinotecan) and gemcitabine (an antimetabolite) cytotoxicity was assessed using a 96 h sulforhodamine B assay (SRB, Sigma cat# S9012). The HT29 or SW620 cells were seeded at 1.6-3.2×103 cells per well in 96-well plates in a volume of 160 μL medium and allowed to attach for 36 h prior to treatment. For cytotoxicity assays, CHK1 inhibitors (10 mM stock in DMSO) were serially diluted in medium from a starting concentration of 250 pM and then 40 PL was added to appropriate wells in quadruplicate to give a final concentration range of 50-0.1 pM (10 concentrations). Genotoxic agents (SN38, LKT laboratories cat#C0154 and gemcitabine, Lilly "Gemzar"; 10 mM stock in DMSO) were serially diluted in medium from a starting concentration of 2 pM and 40 PL was added to each well in quadruplicate to give final concentrations from 200-0.39 nM (10 concentrations). Cells were incubated for 96 h (four doublings) at 37°C in a

humidified 5% CO_2 environment and then fixed and stained with SRB. Appropriate controls were included and results were expressed as the concentration of test compound required to inhibit cell growth by 50% relative to untreated controls (SRB IC_{50}). Potentiation assays involved adding a fixed SRB IC_{50} concentration of either gemcitabine or SN38 in a volume of 20 μL of medium (10x final concentration), to each well in quadruplicate and mixing for 1 min. CHK1 inhibitor (10 mM stock) was serially diluted from a starting concentration of 50 pM in medium and 20 pL was added per well in quadruplicate to give a final concentration range of 5-0.039 pM (8 concentrations). After mixing for 1 min the cells were incubated at 37°C in a humidified atmosphere for 96 h (four doublings) prior to fixing and SRB staining. Untreated and genotoxic alone treated controls were included and results were expressed as the concentration of CHK1 inhibitor required to inhibit cell growth by 50% (potentiation IC_{50}). The Potentiation Index (PI) was used as a measure of the ability of the CHK1 inhibitor to enhance SN38 or gemcitabine cytotoxicity and was the ratio of the SRB IC_{50} versus potentiation IC_{50} (i.e. PI = SRB IC_{50}/Potentiation IC_{50}).

Determination of compound concentrations *in vivo* at selected time points following i.v. and oral dosing: Female BALB/c mice (6 weeks old) (Charles River UK Ltd, Margate, UK) were kept in a controlled environment with food and sterilized water available *ad libitum*. Animals divided to 13 groups each of 8 animals and each group received separately single derivatives from 1 to 13 . Animals weighed 20±2 g at the time of experiment. All procedures were conducted in accordance with the local and national guidelines for animal experimentation. Dosing solutions were prepared by dissolving the compounds in 10% DMSO and 5% Tween 20 in 85% saline. The compounds were administered i.v. and p.o., individually. Animals were warmed before receiving a single i.v. bolus injection into a lateral tail vein. Oral administration was by oral gavage. Blood (0.5 mL) was collected at selected time points (1 and 6 h after dosing) by cardiac puncture under anesthesia into heparinized syringes, transferred to micro centrifuge tubes and centrifuged at 4500×g for 2 min to obtain plasma. Quantitative analysis was performed by high performance liquid chromatography tandem mass spectrometry on a triple quadrupole instrument (Agilent 6410) using multiple reaction monitoring of selected transitions with olomoucine used as internal standard. Quantitation was performed against a standard curve ranging from concentrations of 2-1000 nM in the matrix measured. Quality controls were included at the level of 25, 250 and 750 nM. If required, samples were diluted in the matrix of interest. The doses selected were elected depending on the response of tested agents depending on similar experiments (Posy *et al*., 2011; Bamborough *et al*., 2011; Smyth *et al*., 2011; Matthews *et al*., 2010).

Statistical analysis: Statistical comparison of the difference between control group and treated groups was done by one-way ANOVA and Duncan's multiple comparison test $*p<0.05$.

RESULTS AND DISCUSSION

Chemistry: A series of the newly substituted thienopyrimidine derivatives 2-13 were synthesized by using 3-amino-6-(4-chlorophenyl)-4-(4-isopropylphenyl)thieno[2,3-b]pyridine-2-carbohydrazide 1 as starting material. Reaction of compound 1 with p-nitrobenzaldehyde in refluxing ethanol afforded the corresponding Shiff base 2, which was cyclized with triethyl orthoformate to afford thienopyrimidine derivative 3. Compound 1 was treated with formic acid or acetic anhydride to afford the corresponding thienopyrimidines 4 and 5, respectively. Treatment of 1 with acetylacetone afforded pyrazolyl derivative of thienopyridine 6 (Fig. 1).

Treatment of 1 with triethyl orthoformate in presence of acetic acid in refluxing methanol or carbon disulfide in the presence of potassium hydroxide afforded the corresponding thienopyrimidine derivative 7 and thiadiazolyl thienopyridine derivative 8, respectively. Compound 1 was reacted with dimethylformamide, ethyl acetoacetate or diethyl malonate to give the corresponding compounds 9-11, respectively (Fig. 2).

Finally, cyclization of 1 with m-nitrophthalic anhydride or formaldehyde afforded the corresponding 10-(4-chlorophenyl)-8-(4-isopropylphenyl)-2,13-dioxo-6-nitro-1,2,13-trihydro-pyrido[3",2"-4',5']-thieno[3',2'-4,5]pyrimido[1,2-b]phthalazine (12) and 3-amino-7-(4-chloro-phenyl)-9-(4-isopropyl-phenyl)-4-oxo-3,4-dihydropyrido[3',2':4,5]thieno[3,2-d]pyrimidine (13), respectively (Fig. 3).

Pharmacological activity: Some substituted 3,6-diamino-1H-pyrazolo[3,4-b]pyridine derivatives exhibited inhibition on a selection of disease-relevant protein kinases (Chioua *et al*., 2009). A series of 3,5-disubstituted pyrazolo[3,4-b]pyridine Cyclin-Dependent Kinase (CDK) inhibitors was synthesized. These compounds showed potent and selective CDK inhibitory activities and inhibited *in vitro* cellular proliferation in cultured human tumor cells (Lin *et al*., 2007). Synthesis and structure-activity relationships of new 1H-pyrrolo [2,3-b]pyridine derivatives identified as inhibitors of Cdc7 kinase (Ermoli *et al*., 2009). Docking experiments showed that the novel pyrazolo[3,4-b]pyridines share the similar interaction mode with Aurora-A kinase as PHA739358 (Shi *et al*., 2010). A novel series of pyrazolo[1,5-a]pyridines as PI3 kinase inhibitors and demonstrated their selectivity for the p110α isoform over the other Class Ia PI3 kinases (Kendall *et al*., 2012) and also, pyrazolo[1,5-a]pyrimidine showed potent, selective CHK1 inhibitors (Dwyer *et al*., 2011). A novel series of pyrazolo[3,4-b]pyridines has been identified that are potent inhibitors of glycogen synthase kinase-3 (GSK-3) (Witherington *et al*., 2003). Four series of dihydropyrazolo[3,4-b]pyridines and benzo[4,5]imidazo[1,2-a]pyrimidines were designed and synthesized as dual KSP and Aurora-A kinase (Fu *et al*., 2010).

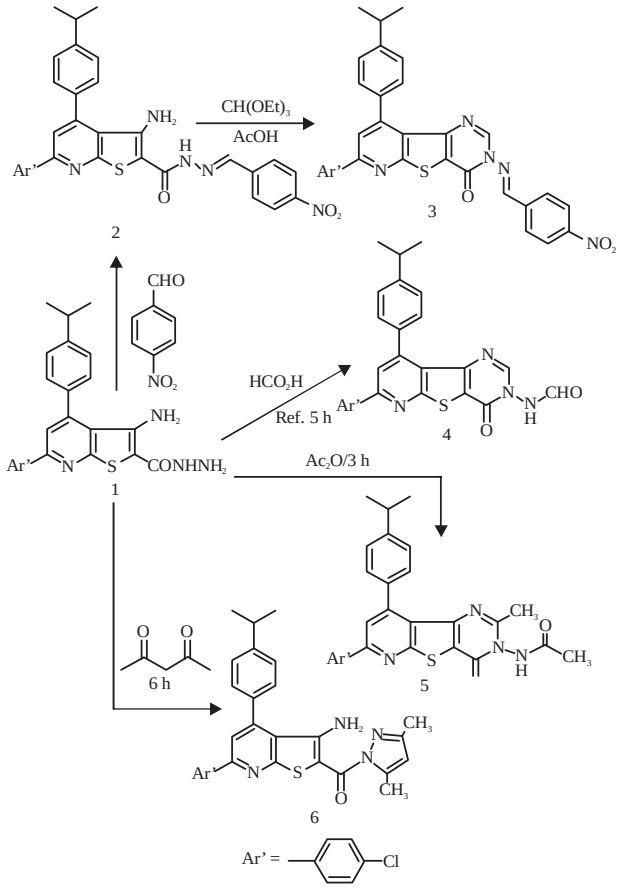

Fig. 1: Synthetic routs for synthesis of compounds 2-6

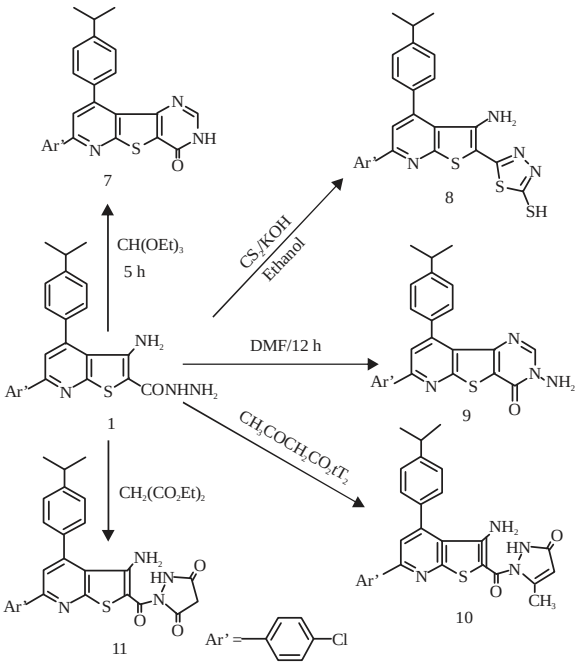

Fig. 2: Synthetic routs for synthesis of compounds 7-10

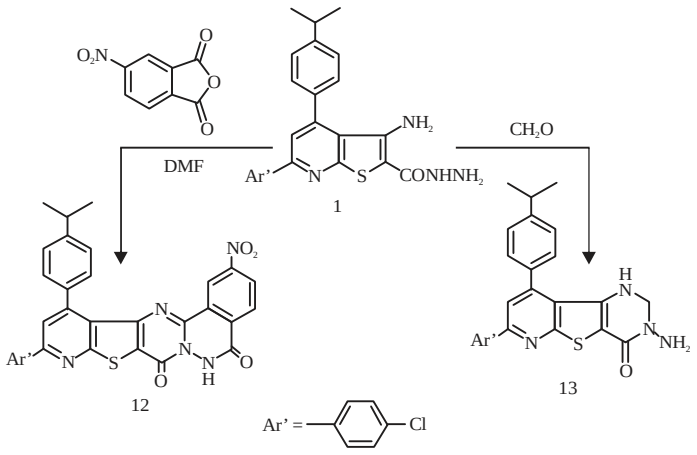

Fig. 3: Synthetic routs for compounds 12 and 13

Due to some aspects of structural similarity between our newly synthesized compounds and the orally active 3-alkoxyamino-5-(pyridin-2-ylamino)pyrazine-2-carbonitrile CHK1 inhibitors in containing nearly the same bio-isosteres, the authors screened some representative for their CHK1 inhibitors alongside their oral bioavailability. First compounds were tested for protein kinase inhibition in a panel of 24 enzymes containing representative members of the major kinome family subclasses, using a commercially available microfluidic assay format (Card *et al*., 2008; Perrin *et al*., 2006).

The inhibitors were tested at a concentration of 30 μM, so that significant activity (>50 % inhibition) would be indicative of ligand efficient binding (> 0.3 $kcal^{-1}$ HA^{-1}) (Hopkins *et al*., 2004). It was found that the tested compounds showed inhibition for only CHK1 with 100% at 30 μM (Table 1). All newly synthesized derivatives showed CHK1 inhibitor activities were screened for the their CHK1 inhibitor activities using Microfluidic mobility shift assays to determine kinase inhibition and were found to have selective potent inhibitors effects on CHK1 activity in cancer cells and the order of activity was 11, 9, 3, 13, 10, 12, 7, 1, 4, 2, 5, 6 and 8. Also measuring inhibition of CHK1 in a biochemical assay, compounds that founded to have been potent CHK1 inhibitor activities were tested for their ability to abrogated an etoposide-induced G2 checkpoint arrest in HT29 colon cancer cells, a specific CHK1-mediated effect (Walton *et al*., 2010). All the tested compounds were active and of high potency and the order of activity was 11, 9, 3, 13, 10, 12, 7, 1, 4, 2, 5, 6 and 8 (Table 2).

In parallel, these compounds were tested in an antiproliferative assay in HT29 cells. Using the comparison of the checkpoint abrogation and antiproliferative assays (ELISA assay) therefore, calumniated on the degree of selectivity for CHK1 inhibition versus off-target effects in HT29 cells. Our target profile was to achieve, as a minimum, CHK1 IC50<20 nM with cellular checkpoint abrogation IC_{50} <150 nM and at least 5-fold selectivity for checkpoint abrogation over antiproliferative activity in the HT29 cells.

The tested compounds were further tested for their ability to enhance the cytotoxicity of gemcitabine in HT29 and SW620 colon cancer cells as a measure of efficacy, where at least a 5-fold potentiation was considered desirable. All tested compound showed micromolar inhibition levels of the hERG ion channel and the order of activity was 11, 9, 3, 13, 10, 12, 7, 1, 4, 2, 5, 6 and 8 (Table 2). An additional aim was therefore to minimize hERG channel inhibition in the novel series of CHK1 inhibitors and this was reached. All the tested compounds assessed for MLM stability (Table 3). The *in vitro* metabolic stability of these inhibitors was of good profile. The pharmacokinetic profile was obtained for the tested compounds (Table 4).

All the tested compounds showed good pharmacokinetic and oral bioavailability profiles. Depending on their oral pharmacokinetic profiles, in particular the ability to sustain plasma levels at 6 h following a 10 mg kg^{-1} dose and the potency and cellular efficacy of the tested compounds, were identified as promising compounds and were progressed to more detailed studies.

Human Plasma Protein Binding (PPB) was determined as moderate for these compounds (Table 5). In view of further *in vivo* studies, the Maximum Tolerated Doses (MTDs) in mice were determined; when administered as single doses in suspension, the individual MTDs were listed (Table 6). The ability of the tested compounds to inhibit DNA damaging agent induced CHK1 signaling in human tumors xenografts in athymic mice after oral dosing was assessed (Table 7).

The compounds were given as suspensions at their respective MTDs to athymic mice bearing SW620 human colon cancer xenografts, followed by dosing of gemcitabine (60 mg kg^{-1} iv) after 1 h. Plasma and tumor samples were collected at 6 and 12 h after dosing the genotoxic agent. Tumor lysates were analyzed by Western blot for total CHK1 protein, phospho-S317 CHK1 as a marker of activation of

Table 1: Heat map showing the percentage inhibition at 30 μM of 26 kinases by compounds

Kinase family	AGC						CAMK					CMGC				TK						Miscellaneous			
Compounds No.	PKA	AKT1	AKT2	MSK1	RSK1	PKC	MK2	MAPKAPK5	PKD2	CHK1	CHK2	Erk1	Erk2	GSK3	p38a	ABL	54	INSR	LYN	LCK	MET	SRC	CK1d	AurA	VEGFR-2	EGFR
1	32	4	14	0	54	34	6	5	9	100	43	75	7	23	45	67	87	65	32	71	65	43	23	45	26	33
2	13	54	24	8	64	55	5	6	7	100	89	32	45	23	45	67	54	56	78	86	44	45	66	78	87	55
3	67	7	67	7	53	1	55	4	5	100	67	34	12	12	13	13	23	35	46	57	68	98	66	54	45	23
4	24	32	13	9	54	44	5	6	8	100	87	46	6	32	33	44	55	66	75	43	45	86	43	45	68	64
5	12	76	24	5	65	44	4	5	6	100	67	45	47	43	44	56	87	54	35	67	88	97	88	99	87	65
6	3	54	43	8	75	3	4	55	65	100	4	35	38	6	6	56	3	4	6	7	27	36	15	12	13	14
7	35	5	35	9	32	56	7	4	8	100	23	68	8	21	24	35	67	89	86	54	32	34	45	66	53	45
8	5	34	67	5	63	2	27	57	34	100	32	5	43	50	60	71	23	45	65	43	45	67	88	99	54	86
9	3	12	43	5	53	44	32	54	22	100	67	76	43	23	45	65	43	45	66	77	89	33	33	55	13	35
10	2	34	5	7	84	12	34	54	65	100	3	12	19	7	5	65	4	5	6	7	8	9	0	8	0	0
11	4	23	65	4	69	3	45	87	23	100	45	6	21	23	23	35	46	57	68	90	87	65	44	66	24	43
12	45	6	35	8	42	54	67	4	6	100	45	20	9	12	13	24	54	34	56	76	87	56	55	44	34	67
13	87	54	68	6	64	2	34	54	54	100	65	3	34	12	12	32	43	56	78	90	98	64	32	34	54	57

Table 2: CHK1 inhibition and cellular activity of potent CHK1 inhibitors

Compounds No.	CHK1 IC_{50} (μM)[a]	Checkpoint abrogation IC_{50} (μM)[b]	Cellular selectivity (fold)[c]	Potentiation of gemcitabine cytotoxicity (fold)[d]		hERG IC_{50} (μM)[e]
				HT29	SW620	
1	0.12	0.19	21.16	16.07	13.95	0.085
2	0.16	0.22	11.69	11.19	19.84	0.105
3	0.07	0.13	18.20	6.55	9.05	0.062
4	0.14	0.20	22.63	9.88	21.15	0.095
5	0.17	0.26	17.96	22.02	37.71	0.160
6	0.19	0.30	14.02	27.01	10.68	0.297
7	0.11	0.19	21.77	15.35	12.75	0.076
8	0.20	0.31	17.59	29.51	13.84	0.414
9	0.04	0.11	20.54	5.47	6.98	0.052
10	0.09	0.17	18.33	11.66	9.27	0.069
11	0.01	0.07	15.25	8.69	10.25	0.023
12	0.11	0.18	32.96	12.73	14.82	0.076
13	0.08	0.15	27.06	10.59	5.89	0.070

Determined in caliper microfluidic assay (Dunne *et al*., 2004) mean of n = 2, individual values in parentheses. [b]Abrogation of etoposide-induced G2 checkpoint arrest in HT29 human colon cancer cells. [c]Ratio of cytotoxicity GI_{50} (measured by SRB assay) to IC_{50} for CHK1-mediated abrogation of etoposide-induced G2 checkpoint arrest in HT29 human colon cancer cells, [d]Potentiation by CHK1 inhibitor of gemcitabine cytotoxicity in HT29 or SW620 human colon cancer cells. [e]Inhibition of hERG ion current in HEK cells overexpressing hERG ion channel (PatchExpress, Millipore Inc.) (Skehan *et al.*, 1990)

Table 3: *In vitro* ADME and mouse in vivo plasma concentrations for potent CHK1 inhibitors

Compounds No.	*In vitro* stability and permeability			Mouse plasma levels following 10 mg kg^{-1} iv or po[d]			
	Mouse liver microsome metabolism (%)[a]	CaCo-2 permeability (10^{-6} cm sec^{-1})[b]	Efflux ratio[c]	iv, 1 h (nM)	iv, 6 h (nM)	po, 1 h (nM)	po, 6 h (nM)
1	52.48	0.84	9.10	1816.40	180.20	30.18	13.55
2	30.06	1.29	4.41	3697.33	112.20	28.51	9.03
3	31.66	1.20	5.17	3546.76	153.00	383.33	9.03
4	41.27	0.63	6.32	3962.62	142.80	39.16	10.16
5	37.58	2.36	2.59	1541.55	115.60	30.27	7.90
6	33.88	0.73	7.38	2038.67	187.00	5.19	27.10
7	63.69	0.93	4.22	2100.81	112.20	21.38	15.81
8	33.88	0.83	6.32	681.15	180.20	33.09	12.42
9	60.98	0.63	8.24	1594.13	85.00	8.62	13.55
10	20.20	0.62	8.43	4701.13	217.60	6.95	21.45
11	47.31	0.93	7.19	1376.64	149.60	7.66	11.29
12	42.87	1.19	5.08	4237.47	112.20	48.75	19.19
13	20.45	1.01	6.23	3334.05	149.60	22.35	10.16

[a]Percent metabolized after 30 min incubation, mean: n≥3. [b]Permeability A→B across CaCo-2 cell monolayer, single determination. [c]Ratio of permeability A→B/B→A across CaCo-2 cell monolayer. [d]Plasma levels at 1 and 6 h following 10 mg kg^{-1} iv or po of test compound

Table 4: Mouse *in vivo* pharmacokinetic data for compounds

Compounds No.	PK Parameters		
	Cl (L h^{-1})	Vss (L)	F (%)
1	0.07	0.06	64.68
2	0.05	0.09	61.74
3	0.14	0.10	65.66
4	0.06	0.07	50.96
5	0.15	0.13	72.52
6	0.16	0.10	53.90
7	0.09	0.07	55.86
8	0.11	0.10	73.50
9	0.15	0.10	72.52
10	0.17	0.12	72.52
11	0.14	0.12	72.52
12	0.10	0.09	56.84
13	0.11	0.12	64.68

All data was determined following 10 mg kg^{-1} iv and po dosing

Table 5: Human plasma protein binding for compounds

Compounds No.	PPB (%)
1	64.08
2	65.86
3	78.32
4	72.09
5	58.74
6	66.75
7	65.86
8	86.33
9	75.65
10	73.87
11	85.44
12	70.31
13	77.43

PPB: Plasma protein binding

CHK1 by the upstream kinase ATR and phospho-S296 CHK1 autophosphorylation to demonstrate inhibition of CHK1 kinase function (Walton *et al*., 2010). The Activation of DNA damage response resulting from gemcitabine treatment was shown by the increase in phospho-S317 CHK1 and was sustained over 12 h. The CHK1 autophosphorylation on S296 was also seen at both time points in response to gemcitabine

Table 6: Maximum tolerated doses in mice for compounds

Compounds No.	MTDs (mg kg^{-1} po)
1	271.45
2	279.46
3	496.62
4	448.56
5	376.47
6	323.07
7	361.34
8	213.60
9	200.25
10	304.38
11	301.71
12	353.33
13	417.41

MTDs: Maximum tolerated doses

Table 7: DNA damaging agent induced CHK1 signaling in human tumors xenografts in athymic mice after oral dosing (MTDs mg kg^{-1} po) for compounds

Compounds		Compound+gemcitabine after 6 h			Compound+gemcitabine after 6 h		
No.	Dose (mg kg^{-1} po)	CHK1 (%)	pS 296 CHK1 (%)	pS 317 CHK1 (%)	CHK1 (%)	pS 296 CHK1 (%)	pS 317 CHK1 (%)
1	271.45	90.13	10.90	79.13	73.05	71.70	88.10
2	279.46	89.75	10.13	79.03	72.76	71.31	87.53
3	496.62	90.81	12.45	79.23	73.44	72.47	89.36
4	448.56	88.30	7.72	78.74	71.89	69.96	85.11
5	376.47	89.36	9.46	78.94	72.57	71.02	86.95
6	323.07	86.95	5.89	78.36	71.12	68.61	82.80
7	361.34	86.27	5.21	78.26	70.64	67.94	81.64
8	213.60	87.24	6.37	78.45	71.31	68.90	83.38
9	200.25	87.62	6.76	78.55	71.51	69.29	83.96
10	304.38	89.07	8.88	78.84	72.38	70.64	86.27
11	301.71	91.58	14.28	79.42	73.92	73.15	90.61
12	353.33	86.56	5.50	78.36	70.83	68.23	82.22
13	417.41	91.19	13.32	79.32	73.73	72.76	90.03

Table 8: Pharmacodynamic data for plasma and tumor levels of tested compounds at 6 and 12 h after genotoxic drug administration after oral dosing (MTDs mg kg^{-1} po) for compounds

Compounds	Plasma level (μM) after		Tumor level (μM) after	
No.	6 h	12 h	6 h	12 h
1	7.72	1.93	312.66	54.04
2	8.69	6.76	440.04	87.82
3	3.86	0.77	76.24	4.83
4	14.48	3.86	375.39	43.43
5	7.72	0.29	63.69	4.83
6	14.48	3.86	332.93	67.55
7	6.76	3.86	416.88	64.66
8	9.65	5.79	482.50	96.50
9	11.58	1.16	526.89	85.89
10	11.58	1.83	86.85	11.58
11	19.30	1.93	416.88	205.55
12	8.69	0.68	75.27	0.97
13	17.37	2.22	108.08	12.55

treatment. All the tested compounds at their MTDs strongly inhibited CHK1 S296 autophosphorylation at 6 h following gemcitabine treatment. Also, these compounds continued to show robust inhibition of CHK1 at the 12 h time point (Table 7). The analysis of the drug levels in plasma and tumors showed micromolar plasma concentrations and high distribution to tumor for all tested compounds (Table 8). The obtained results indicated that the tested compounds showed high tumor level after 6 h that decreases after 12 h and the sane occurred for compounds plasma level but the compounds showed higher accumulation in tumor.

Structure activity relationship: Careful examination of the activities of the tested compounds with their structure scaffold leads to the following assumption on the SAR:

- Thieno[2,3-b]pyridine essential for activities
- Attaching five member heterocyclic ring systems as thiadiazole, pyrazolidine and 1H-pyrazol to the thieno[2,3-b]pyridine increasing the activities
- Attaching pyrimidine to the thieno[2,3-b]pyridine increasing the activities

CONCLUSION

The representative examples of the newly synthesized derivatives were found to have selective potent inhibitors effects on CHK1 activity in cancer cells and the order of activity was 11, 9, 3, 13, 10, 12, 7, 1, 4, 2, 5, 6 and 8. Also these compounds were founded to have the ability to abrogated an etoposide-induced G2 checkpoint arrest in HT29 colon cancer cells, a specific CHK1-mediated effect in the same ctivity order. These compounds also showed micromolar inhibition levels of the hERG ion channel and the order of

activity was 11, 9, 3, 13, 10, 12, 7, 1, 4, 2, 5, 6 and 8. All the tested compounds showed good pharmacokinetic and oral bioavailability profiles a property long-sought.

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