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STUDY OF THE ANTI-TUMOR ACTIVITY OF SYNTHETIC PYRIDO [2,3-*d*]PYRIMIDINES, PYRIMIDINES AND CORRESPONDING CHALCONE DERIVATIVES

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Pyrido[2,3-*d*]pyrimidine, 2,4,6-trisubstituted pyrimidine, 4-aminochalcone, Anticancer activity, MTT assay

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ABSTRACT: Chalcones (3a-m) was prepared by condensing 4-aminoacetophenone (1) with various aromatic aldehydes (2a-m) according to Claisen-Schmidt condensation. These 4-aminochalcones on cyclization with guanidine hydrochloride under basic alcoholic conditions gave 2,4,6-trisubstituted pyrimidines 4a-m with quantitative yields. Novel 4-amino-5,7-disubstituted pyrido[2,3-*d*]pyrimidines 6a-g were synthesized via a facile regioselective cyclization followed by aromatization of 2-amino-3-cyano-4,6-disubstitutedpyridines 5a-k which were in turn prepared from 4-aminochalcones 3a-m. All the synthesized compounds were characterized by spectroscopic means (FT-IR, ^{1}H and ^{13}C NMR, LC-MS) and elemental analysis and screened for anticancer activity by *in-vitro* MTT assay against Hos, HT 29, G 361, A 549 and DU 145 cell lines. Pyridopyrimidine nucleus was found to be the best pharmacophore than chalcone and pyrimidine nucleus in cancer therapy. And also it was found that compounds which possess 2,4-dichlorophenyl (3b,4b and 6b), 4-fluorophenyl (4c and 6c) and 4-dimethylamino (3i,4i and 6g) moieties as substituent at respective position in chalcones, pyrimidines and pyrido[2,3-*d*]pyrimidines identified as lead molecules. All the molecules were found to possess better activity against Hos (bone cancer) and G 361 (human skin cancer) cell lines.

INTRODUCTION: Chalcones occurring either naturally or synthetically are known to exhibit various biological activities; hence these are very attractive starting materials in medicinal chemistry. The presence of a reactive α, β-unsaturated keto functional group in chalcones found to be responsible for their biological activity [1].

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Chalcones provide a bifunctional site for 1,3-dinucleophiles and is possibile to synthesize various heterocyclic rings. Among all, pyrimidines are the subject of interest by synthetic and medicinal chemists because this heteroaromatic ring plays an important role in many biological and pharmacological activities reported well in literature [2]. Many naturally occurring as well as synthetic compounds bearing pyridine moiety possess important biological properties, hence several efficient procedures have been reported in the literature for the synthesis of functionalized pyridines [3-5]. Among all, 2-amino-3-cyanopyridines with different alkyl, aryl and heteroaryl groups

were found to have antimicrobial [6], anti-hypertensive [7], cardiovascular [8], anti-inflammatory, analgesic and antipyretic [9], antitumor [10], anti-parkinsonism properties [11], as well as Ikk-β inhibitor properties [12]. Synthesis of the 2-amino-3-cyanopyridines from chalcones on treatment with ammonium acetate *via* Michael type condensation [13] as well as *via* a one-pot coupling reaction of four components [14] and by other methods has been reported well in the literature [15]. Hence we have synthesized the 2-amino-3-cyanopyridines by Michael type condensation reaction **Scheme 2**, **Table 2**.

Pyrido[2,3-*d*]pyrimidines are known to be pharmacophoric elements in numerous active compounds such as anticancer, antibacterial, antimicrobial, calcium channel antagonists, anti-inflammatory and analgesic, antileishmanial, antitubercular, anticonvulsant, diuretic and CNS depressant agents [16]. More specifically these are considered as inhibitors of *Pneumocystis carinii*, *Toxoplasma gondii* of tumor cell lines in culture because an orthofused nitrogen heterocycles have been widely used as inhibitors of dihydrofolate reductase (DHFR) and amongst these, methotrexate and aminopterin are classical antimetabolites that inhibit DHFR [17].

Pyrido[2,3-*d*]pyrimidines have normally been obtained via two general routes: a) formation of the pyridine ring by cyclization of suitable substituents of a pyrimidine and b) formation of pyrimidine ring by cyclization of suitable substituents of a pyridine [18]. Hence we are interested for the synthesis of pyrido[2,3-*d*] pyrimidines according to 'b' type methodology from acyclic precursors **Scheme 2**, **Table 2**.

MATERIALS AND METHODS:

Chemistry: In view of these reports, the synthesis of a new series of 2-amino-3-cyanopyridines and their pyrido[2,3-*d*]pyrimidines are now reported. The desired target compounds, 2-amino-3-cyano-4,6-disubstituted pyridines **(**5a-k) were prepared from the 4-aminochalcones (3a-m) and malono-nitrile by refluxing them together in alcoholic ammonium acetate through Michael reaction, with the elimination of 1 mol each of water and hydrogen. The condensation of the cyanopyridines (5a-k) with formamide at 170 °C (16 - 28 h) results in the formation of corresponding pyrido[2,3-*d*] pyrimidines (6a-g) **Scheme 2**, **Table 2**. Synthesis of 2,4,6-trisubstituted pyrimidines from respective 4-aminochalcones **Scheme 1** and **Table 1** and their antimicrobial, anti-inflammatory and analgesic activity studies are reported in our previous publications [2, 19]. Compounds 5d and 6d already reported in the literature [20]. The synthetic pathways followed to obtain the final compounds are shown in **Scheme 1** and **2** and the structures were confirmed by analytical and spectral data.

The literature revealed that chalcones, pyrimidines and pyridopyrimidines exhibited anti-tumor activity by various mechanisms. The commonly known mechanism is that they act against folate metabolism which has long been recognized as an attractive target for chemotherapy because of its crucial role in the biosynthesis of nucleic acid precursors [21, 22, 23]. Inhibitors of folate-dependent enzymes in cancer, microbial and protozoan cells provide compounds that have found clinical utility as antitumor, antimicrobial and antiprotozoal agents[24]. All the synthesized chalcones, pyrimidines and pyridopyrimidines **Table 1** and **2** were tested for their anticancer activity by MTT assay method[25] against Hos (Bone cancer), HT 29 (Colon cancer), G 361 (Human skin cancer), A 549 (Lung cancer) and DU 145 (Prostate cancer) cell lines.

Experimental: Melting Points (MP) were determined on a standard Boetius apparatus and are uncorrected. The IR spectra were recorded in Perkin-Elmer BXF1 FT-IR spectrophotometer using KBr disc method. ^{1}H and ^{13}C NMR spectra were recorded in the indicated solvent on a Bruker AMX 400 and 100 MHz respectively with tetra-methylsilane (TMS) as internal standard (chemical shifts in δ ppm). The LC-MS [API/ESI-MS (80 eV)] spectra were recorded on Agilent HPLC 1100 series. The elemental analyses of the synthesized compounds were recorded on Carlo Erba 1108 elemental analyzer and were within ± 0.4% of the theoretical values. Analytical TLC was performed on Silica Gel F_{254} plates (Merck) with visualization by UV (254 nm) chamber with protective filters. All the cyanopyridines and pyrido[2,3-*d*] pyrimidines have been purified by column chromatography performed on silica gel (100-200 mesh, Merck). All reagents and solvents were used as received without further purification.

General Procedure for Synthesis of Substituted 2-amino-3-cyanopyridines, 5a-k: A mixture of 4-aminochalcones (3a-m) (0.01 mol), malononitrile (0.01 mol), ethanol (5 ml), and ammonium acetate (0.015 mol) was placed in a flask fitted with a reflux condenser and a water separator. The reaction mixture was shaken to ensure mixing and then allowed to reflux for 2 to 6 h. Reaction completion was identified by TLC using silica gel-G [10, 20, 26]. Upon completion, the reaction mixture was cooled to room temperature and quenched into crushed ice with constant stirring. The solid separated was filtered and dried. It was purified by column chromatography performed on silica gel (100-200 mesh, Merck), using ethyl acetate and hexane mixture as mobile phase, to obtain pure cyano-pyridines (5a-k) **Scheme 2**.

2-amino-3-cyano-4- (4-chlorophenyl)-6-(4-amino phenyl)pyridine, 5a: Yield 37%, mp 212 ± 2°C. IR (ν_{max}, cm^{-1}): 3402, 3338, 2200, 1574, 1367 and 820. ^{1}H NMR (DMSO-d_6): δ 5.65 (2H, br s, C-4-NH_2); 6.61 (2H, d, J = 8.4 Hz, C-3 and 5-H); 6.78 (2H, br s, C-2-NH_2); 7.08 (1H, s, C-5-H); 7.60 (2H, d, J = 8.4 Hz, C-2 and 6-H); 7.66 (2H, d, J = 8.4 Hz, C-2 and 6-H); 7.87 (2H, d, J = 8.4 Hz, C-3 and 5-H). ^{13}C NMR (DMSO-d_6): δ 85.92 (C-3), 110.65 (C-5), 112.34 (C-2), 116.54 (C≡N), 117.96 (C-3 and 5), 119.37 (C-2 and 6), 125.61 (C-3 and 5), 129.24 (C-2 and 6), 136.61 (C-1), 137.35 (C-4), 148.97 (C-1), 151.19 (C-4), 155.99 (C-4), 158.75 (C-6). LC-MS *(m/z)*: 321.5 [{M+H}$^+$]. Anal. Calcd for $C_{18}H_{13}N_4Cl$: C, 67.41; H, 4.04; N, 17.49. Found: C, 67.43; H, 4.05; N, 17.49.

2-amino-3-cyano-4-(2,4-dichlorophenyl)-6-(4-am inophenyl)pyridine, 5b: Yield 20%, mp 227±2 °C. IR (ν_{max}, cm^{-1}): 3425, 3352, 2202, 1630, 1580, 1368 and 829. ^{1}H NMR (DMSO-d_6): δ 5.67 (2H, br s, C-4-NH_2); 6.60 (2H, d, J = 8.8 Hz, C-3 and 5-H); 6.85 (2H, br s, C-2-NH_2); 7.00 (1H, s, C-5-H); 7.65-7.44 (3H, m, C-3, 5 and 6-H); 7.84 (2H, d, J = 8.8 Hz, C-2 and 6-H). LC-MS *(m/z)*: 355.8 [{M+H}$^+$]. Anal. Calcd for $C_{18}H_{12}N_4Cl_2$: C, 60.90; H, 3.41; N, 15.77. Found: C, 60.84; H, 3.41; N, 15.77.

2-amino-3-cyano-4-(4-fluorophenyl)-6 -(4-amino phenyl)pyridine, 5c: Yield 22%, mp 192 ± 2 °C. IR (ν_{max}, cm^{-1}): 3466, 3425, 2200, 1605, 1574, 1371 and 1238. ^{1}H NMR (DMSO-d_6): δ 5.63 (2H, br s, C-4-NH_2); 6.61 (2H, d, J = 8.8 Hz, C-3 and 5-H); 6.76 (2H, br s, C-2-NH_2); 7.08 (1H, s, C-5-H); 7.37 (2H, dd, J = 10.2 Hz, J = 8.8 Hz, C-2 and 6-H); 7.70 (2H, dd, J = 9.2 Hz, J = 8.6 Hz, C-3 and 5-H); 7.87 (2H, d, J = 8.4 Hz, C-2 and 6-H). LC-MS *(m/z)*: 305.2 [{M+H}$^+$]. Anal. Calcd for $C_{18}H_{13}N_4F$: C, 71.11; H, 4.31; N, 18.41. Found: C, 71.05; H, 4.31; N, 18.42.

2-amino-3-cyano-4-(3-bromophenyl)-6-(4-amino phenyl)pyridine, 5d: Yield 18%, mp 237 ± 2 °C. IR (ν_{max}, cm^{-1}): 3424, 3348, 2203, 1629, 1571, 1367 and 639. ^{1}H NMR (DMSO-d_6): δ 5.65 (2H, br s, C-4-NH_2); 6.62 (2H, d, J = 8.4 Hz, C-3 and 5-H); 6.80 (2H, br s, C-2-NH_2); 7.12 (1H, s, C-5-H); 7.52-7.48 (1H, t, J = 7.1 Hz, C-5-H); 7.64 (1H, d, J = 7.6 Hz, C-4-H); 7.72 (1H, d, J = 7.6 Hz, C-6-H); 7.83 (1H, s, C-2-H); 7.90 (2H, d, J = 8.4 Hz, C-2 and 6-H). LC-MS *(m/z)*: 365 [{M+H}$^+$]. Anal. Calcd for $C_{18}H_{13}N_4Br$: C, 59.23; H, 3.59; N, 15.34. Found: C, 59.17; H, 3.59; N, 15.34.

2-amino-3-cyano - 4- (4-methoxyphenyl)- 6- (4-aminophenyl)pyridine, 5e: Yield 19%, mp 228 ± 2°C. IR (ν_{max}, cm^{-1}): 3468, 3428, 2200, 1610, 1577, 1373 and 1251. ^{1}H NMR (DMSO-d_6): δ 3.83 (3H, s, C-4-OCH_3); 5.62 (2H, br s, C-4-NH_2); 6.55 (2H, d, J = 8.4 Hz, C-3 and 5-H); 6.68 (2H, br s, C-2-NH_2); 7.05 (1H, s, C-5-H); 7.09 (2H, d, J = 8.4 Hz, C-3 and 5-H); 7.61 (2H, d, J = 8.8 Hz, C-2 and 6-H); 7.86 (2H, d, J = 8.8 Hz, C-2 and 6-H). LC-MS *(m/z)*: 317 [{M+H}$^+$]. Anal. Calcd for $C_{19}H_{16}N_4O$: C, 71.11; H, 4.31; N, 18.41. Found: C, 71.05; H, 4.30; N, 18.40.

2-amino-3-cyano -4-(3,4-dimethoxyphenyl)-6-(4-aminophenyl)pyridine, 5f: Yield 28%, mp 234 ±2 °C. IR (ν_{max}, cm^{-1}): 3465, 3430, 2205, 1614, 1582, 1368 and 1259. ^{1}H NMR (DMSO-d_6): δ 3.74 (3H, s, C-3-OCH_3); 3.80 (3H, s, C-4-OCH_3); 5.62 (2H, br s, C-4-NH_2); 6.59 (2H, d, J = 8.4 Hz, C-3 and 5-H); 6.75 (2H, br s, C-2-NH_2); 6.99 (1H, s, C-5-H); 7.55 (2H, d, J = 8.8 Hz, C-2 and 6-H); 6.93-6.79 (3H, m, C-2, 5 and 6-H). LC-MS *(m/z)*: 347 [{M+H}$^+$]. Anal. Calcd for $C_{20}H_{18}N_4O_2$: C, 69.36; H, 5.2; N, 16.18. Found: C, 69.34; H, 5.21; N, 16.18.

2-amino-3-cyano-4-(4-methylphenyl)-6-(4-amino phenyl)pyridine, 5g: Yield 35%, mp 218 ± 2 °C. IR (ν_{max}, cm^{-1}): 3462, 3363, 2202, 1622, 1583 and

1371. ^{1}H NMR (DMSO-d_6): δ 2.39 (3H, s, C-4-CH_3); 5.62 (2H, br s, C-4-NH_2); 6.55 (2H, d, J = 8.8 Hz, C-3 and 5-H); 6.70 (2H, br s, C-2-NH_2); 7.05 (1H, s, C-5-H); 7.17 (2H, d, J = 8.0 Hz, C-3 and 5-H); 7.53 (2H, d, J = 8.0 Hz, C-2 and 6-H); 7.68 (2H, d, J = 8.6 Hz, C-2 and 6-H). LC-MS *(m/z)*: 301 [{M+H}$^+$]. Anal. Calcd for $C_{19}H_{16}N_4$: C, 76.07; H, 5.37; N, 18.65. Found: C, 76.00; H, 5.36; N, 18.66.

2-amino-3-cyano-4- (4-dimethylaminophenyl)-6-(4-aminophenyl) pyridine, 5h: Yield 34%, mp 208 ± 2 °C. IR (ν_{max}, cm^{-1}): 3444, 3328, 2212, 1614, 1561 and 1360. ^{1}H NMR (DMSO-d_6): δ 3.10 [6H, s, C-4-N(CH_3)$_2$]; 5.86 (2H, br s, C-4-NH_2); 6.84 (2H, d, J = 10.2 Hz, C-3 and 5-H); 7.07 (1H, s, C-5-H); 7.26 (2H, d, J = 9.8 Hz, C-3 and 5-H); 7.83 (2H, d, J = 10.0 Hz, C-2 and 6-H); 8.02 (2H, br s, C-2-NH_2); 8.13 (2H, d, J = 9.8 Hz, C-2 and 6-H). LC-MS *(m/z)*: 330 [{M+H}$^+$]. Anal. Calcd for $C_{20}H_{19}N_5$: C, 73.01; H, 5.81; N, 21.26. Found: C, 72.94; H, 5.80; N, 21.27.

2-amino-3-cyano-4-(9-anthracenyl)- 6 - (4-amino phenyl)pyridine, 5i: Yield 33%, mp 266 ± 2 °C. IR (ν_{max}, cm^{-1}): 3468, 3335, 2217, 1630, 1601 and 1355. ^{1}H NMR (DMSO-d_6): δ 6.19 (2H, br s, C-4-NH_2); 6.63 (2H, d, J = 8.8 Hz, C-3 and 5-H); 7.61-7.55 (4H, m, Anthracenyl-H); 7.62 (1H, s, Anthracenyl-H); 7.66 (1H, s, C-5-H); 7.90 (2H, d, J = 8.4 Hz, C-2 and 6-H); 8.25-8.14 (4H, m, Anthracenyl-H); 8.66 (2H, br s, C-2-NH_2). LC-MS *(m/z)*: 387 [{M+H}$^+$]. Anal. Calcd for $C_{26}H_{18}N_4$: C, 80.90; H, 4.69; N, 14.50. Found: C, 80.86; H, 4.70; N, 14.52.

2-amino-3-cyano-4-(2-pyridinyl)-6- (4-aminophe nyl) pyridine, 5j: Yield 12%, mp 245 ± 2 °C. IR (ν_{max}, cm^{-1}): 3423, 3330, 2215, 1644, 1576 and 1352. ^{1}H NMR (DMSO-d_6): δ 5.68 (2H, br s, C-4-NH_2); 6.65 (2H, d, J = 8.2 Hz, C-3 and 5-H); 6.85 (2H, br s, C-2-NH_2); 7.05 (1H, s, C-5-H); 7.63-7.47 (4H, m, C-3, 4, 5 and 6-H); 7.89 (2H, d, J = 8.2 Hz, C-2 and 6-H). LC-MS *(m/z)*: 288.1 [{M+H}$^+$]. Anal. Calcd for $C_{17}H_{13}N_5$: C, 71.14; H, 4.56; N, 24.37. Found: C, 71.08; H, 4.56; N, 24.39.

2-amino-3-cyano-4-(4-pyridinyl)-6- (4-aminophe nyl)pyridine, 5k: Yield 13%, mp 251 ± 2°C. IR (ν_{max}, cm^{-1}): 3425, 3330, 2216, 1644, 1575 and 1352. ^{1}H NMR (DMSO-d_6): δ 5.61 (2H, br s, C-4-NH_2); 6.60 (2H, d, J = 8.8 Hz, C-3 and 5-H); 6.70 (2H, br s, C-2-NH_2); 7.10 (1H, s, C-5-H); 7.35 (2H, d, J = 9.8 Hz, C-2 and 6-H); 7.73 (2H, d, J = 9.2 Hz, C-3 and 5-H); 7.88 (2H, d, J = 8.0 Hz, C-2 and 6-H). LC-MS [API/ESI-MS (80 eV)] *(m/z)*: 288.3 [{M+H}$^+$]. Anal. Calcd for $C_{17}H_{13}N_5$: C, 71.15; H, 4.57; N, 24.39. Found: C, 71.08; H, 4.56; N, 24.40.

General Procedure for Synthesis of Substituted pyrido[2,3-*d*] pyrimidines, 6a-g: A mixture of 2-amino-3-cyano-4,6-disubstituted pyridines (5a-k) (0.001 mol), formamide (0.001 mol) and dimethyl formamide (5 mL) were placed in a reaction flask fitted with a reflux condenser and a water separator and reflux at 170°C on oil bath for 22-24 h resulted the formation of corresponding pyrido[2,3-*d*] pyrimidines [27] (**Scheme 2**). The completion of the reaction was identified by TLC using silica gel-G. The solvent was evaporated using a rotary vacuum evaporator. The residue was poured into crushed ice and the resultant precipitate was separated by suction, washed with water, dried and purified by column chromatography on silica gel, using ethyl acetate and hexane mixture as mobile phase to give pure pyridopyrimidines (6a-g).

4-amino-5-(4-chlorophenyl)- 7 - (4-aminophenyl) pyrido[2,3-*d*]pyrimidine, 6a: Yield 55%, mp 315 ± 2 °C. IR (ν_{max}, cm^{-1}): 3402, 3342, 1681, 1605, 1365 and 821. ^{1}H NMR (DMSO-d_6): δ 7.00 (2H, br s, C-4-NH_2); 7.25 (1H, s, C-6-H); 7.31 (2H, d, J = 8.0 Hz, C-3 and 5-H); 7.63 (2H, d, J = 8.4 Hz, C-2 and 6-H); 7.64 (2H, br s, C-4-NH_2); 7.70 (2H, d, J = 8.0 Hz, C-2 and 6-H); 8.12 (2H, d, J = 8.0 Hz, C-3 and 5-H); 8.33 (1H, s, C-2-H). ^{13}C NMR (DMSO-d_6): δ 106.01 (C-10), 116.89 (C-3 and 5), 118.57 (C-6), 127.66 (C-1), 128.95 (C-2 and 6), 129.28 (C-3 and 5), 129.43 (C-2 and 6), 132.49 (C-4), 134.44 (C-1), 145.85 (C-4), 148.01 (C-5), 156.15 (C-2), 158.46 (C-7), 159.79 (C-9), 162.73 (C-4). LC-MS *(m/z)*: 348.5 [{M+H}$^+$]. Anal. Calcd for $C_{19}H_{14}N_5Cl$: C, 65.67; H, 4.06; N, 20.13. Found: C, 65.60; H, 4.02; N, 20.14.

4-amino-5-(2,4-dichlorophenyl)-7-(4- aminophen yl)pyrido[2,3-*d*]pyrimidine, 6b: Yield 34%, mp 338 ± 2°C. IR (ν_{max}, cm^{-1}): 3403, 3337, 1684, 1600, 1366 and 827. ^{1}H NMR (DMSO-d_6): δ 7.07 (2H, br s, C-4-NH_2); 7.20 (1H, s, C-6-H); 7.54 (1H, s, C-3-H); 7.62-7.56 (2H, m, C-5 and C-6-H); 7.70 (2H, d, J = 8.4 Hz, C-3 and 5-H); 7.85 (2H, br s, C-4-NH_2);

8.09 (2H, d, *J* = 8.4 Hz, C-2 and 6-H); 8.33 (1H, s, C-2-H). LC-MS *(m/z)*: 383 [{M+H}$^+$]. Anal. Calcd for $C_{19}H_{13}N_5Cl_2$: C, 59.74; H, 3.43; N, 18.32. Found: C, 59.68; H, 3.43; N, 18.33.

4-amino-5-(4-fluorophenyl)- 7 - (4-aminophenyl) pyrido[2,3-*d*]pyrimidine, 6c: Yield 51%, mp 306 ± 2 °C. IR (ν_{max}, cm^{-1}): 3406, 3300, 1682, 1604, 1366 and 1239. ^{1}H NMR (DMSO-d_6): δ 6.96 (2H, br s, C-4-NH$_2$); 7.24 (1H, s, C-6-H); 7.30 (2H, d, *J* = 8.4 Hz, C-3 and 5-H); 7.42-7.37 (2H, m, C-2 and 6-H); 7.72 (2H, br s, C-4-NH$_2$); 7.75-7.73 (2H, m, C-3 and 5-H); 8.12 (2H, d, *J* = 8.4 Hz, C-2 and 6-H); 8.34 (1H, s, C-2-H). LC-MS (80 eV)] *(m/z)*: 332 [{M+H}$^+$]. Anal. Calcd for $C_{19}H_{14}N_5F$: C, 68.94; H, 4.26; N, 21.14. Found: C, 68.88; H, 4.26; N, 21.12.

4-amino-5-(3-bromophenyl)-7 - (4-aminophenyl) pyrido[2,3-*d*]pyrimidine, 6d: Yield 64%, mp 342 ± 2 °C. IR (ν_{max}, cm^{-1}): 3407, 3339, 1684, 1606, 1360 and 701. ^{1}H NMR (DMSO-d_6): δ 7.00 (2H, br s, C-4-NH$_2$); 7.28 (1H, s, C-6-H); 7.54-7.50 (1H, t, *J* = 8.8 Hz, C-5-H); 7.68 (2H, d, *J* = 8.4 Hz, C-3 and 5-H); 7.73 (2H, *d*, *J* =10.2 Hz, C-4 and 6-H); 7.87 (2H, br s, C-4-NH$_2$); 8.13 (1H, s, C-2-H); 8.15 (2H, d, *J* = 7.8 Hz, C-2 and 6-H); 8.33 (1H, s, C-2-H). LC-MS *(m/z)*: 393 [{M+H}$^+$]. Anal. Calcd for $C_{19}H_{14}N_5Br$: C, 58.21; H, 3.60; N, 17.85. Found: C, 58.16; H, 3.59; N, 17.84.

4-amino-5-(4-methoxyphenyl)-7- (4-aminopheny l)pyrido[2,3-*d*]pyrimidine, 6e: Yield 34%, mp 315±2°C. IR (ν_{max}, cm^{-1}): 3402, 3336, 1681, 1605, 1365 and 1251. ^{1}H NMR (DMSO-d_6): δ 3.76 (3H, s, C-4-OCH$_3$); 7.03 (2H, br s, C-4-NH$_2$); 7.34 (1H, s, C-6-H); 7.29 (2H, d, *J* = 8.0 Hz, C-3 and 5-H); 7.71 (2H, d, *J* = 8.4 Hz, C-2 and 6-H); 7.68 (2H, br s, C-4-NH$_2$); 7.76 (2H, d, *J* = 8.0 Hz, C-2 and 6-H); 8.06 (2H, d, *J* = 8.0 Hz, C-3 and 5-H); 8.42 (1H, s, C-2-H). LC-MS *(m/z)*: 344.2 [{M+H}$^+$]. Anal. Calcd for $C_{20}H_{17}N_5O$: C, 69.97; H, 4.95; N, 20.40. Found: C, 69.96; H, 4.95; N, 20.40.

4-amino-5-(4-methylphenyl)-7 - (4-aminophenyl) pyrido[2,3-*d*]pyrimidine, 6f: Yield 43%, mp 312 ± 2°C. IR (ν_{max}, cm^{-1}): 3403, 3300, 1681, 1607 and 1368. ^{1}H NMR (DMSO-d_6): δ 2.40 (3H, s, C-4-CH$_3$); 6.91 (2H, br s, C-4-NH$_2$); 7.22 (1H, s, C-6-H); 7.36 (2H, d, *J* = 7.8 Hz, C-3 and 5-H); 7.57 (2H, d, *J* = 7.6 Hz, C-2 and 6-H); 7.65 (2H, br s, C-4-NH$_2$); 7.70 (2H, d, *J* = 8.8 Hz, C-3 and 5-H); 8.11 (2H, d, *J* = 8.4 Hz, C-2 and 6-H); 8.33 (1H, s, C-2-H). LC-MS *(m/z)*: 328 [{M+H}$^+$]. Anal. Calcd for $C_{20}H_{17}N_5$: C, 73.46; H, 5.23; N, 21.43. Found: C, 73.39; H, 5.20; N, 21.40.

4-amino-5-(4-dimethylaminophenyl)- 7-(4-amino phenyl)pyrido[2,3-*d*]pyrimidine, 6g: Yield 24%, mp 302 ± 2°C. IR (ν_{max}, cm^{-1}): 3457, 3364, 1682, 1597 and 1356. ^{1}H NMR (DMSO-d_6): δ 2.95 {6H, s, N(CH$_3$)$_2$}; 6.98 (2H, br s, C-4-NH$_2$); 7.23 (1H, s, C-6-H); 7.33 (2H, d, *J* = 8.8 Hz, C-3 and 5-H); 7.40-7.37 (2H, *J* = 8.2 Hz, C-2 and 6-H); 7.75 (2H, br s, C-4-NH$_2$); 7.84 (2H, d, *J* = 8.2 Hz, H-3 and H-5); 8.08 (2H, d, *J* = 8.4 Hz, H-2 and H-6); 8.30 (1H, s, H-2). LC-MS *(m/z)*: 357.2 [{M+H}$^+$]. Anal. Calcd for $C_{21}H_{20}N_6$: C, 70.78; H, 5.61; N, 23.59. Found: C, 70.76; H, 5.60; N, 23.61.

Biological Assay:

***In-vitro* Anticancer Activity Using MTT Assay:** Hos (Bone cancer), HT 29 (Colon cancer), G 361 (Human skin cancer), A 549 (Lung cancer) and DU 145 (Prostate cancer) cell line was obtained from National Center for Cell Science (NCCS), Pune, India. DMEM (Dulbeccos modified eagles medium), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], Trypsin, EDTA were purchased from Sigma Chemicals Co. (st. Louis, MO), Fetal bovine serum were purchased from arrow labs and 96 well flat bottom tissue culture plates were purchased from Tarson. The reference compounds doxorubicin and methotrexate were purchased from Aldrich Company Ltd., Dorset (UK).

Hos, HT 29, G 361, A 549 and DU 145 cell lines were grown as adherent in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 µg/mL penicillin, 200 µg/mL streptomycin, 2 mM L-glutamine and culture was maintained in a humidified atmosphere with 5% CO_2. Stock solution of 10 mg/mL was prepared in dimethyl sulphoxide. From the above stock various dilutions were made with sterile water to get required concentration. The entire cell lines were seeded at a density of 1x10^4 cells (cell number was determined by trypan blue exclusion dye method) per each well in 100 µL of DMEM supplemented with 10% fetal bovine serum. After 12 h seeding, above medium was replaced with fresh DMEM supplemented with

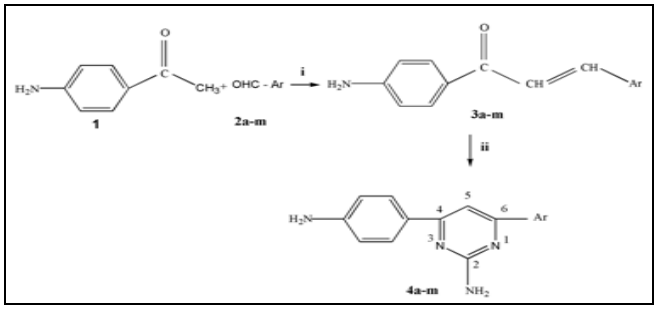
10% FBS then 10 µL sample from above stock solutions were added to each well in triplicates which gives final concentration of 200, 100, 50, 10 µg/mL. The above cells were incubated for 48 h at 37 ºC with 5% CO_2.

After 48 h incubation, the above medium was replaced with 100 µL of fresh DMEM without FBS and to this 10 µL of MTT (5 mg dissolved in 1 mL of PBS) was added and incubated for 3 h at 37ºC with 5% CO_2. After 3 h incubation the above medium was removed with multi channel pipette and then 200 µL of DMSO was added to each well and again incubated at 37 ºC for 15 min. Finally the plate was read at 570 nm using spectrophotometer (Spectra Max, Molecular devices) within 1 h of DMSO addition. The absorbance is directly proportional to the number of cells used. The readings were averaged and validity of the test samples was compared with DMSO control. The results and statistical analysis of anticancer activity of the compounds tested are shown in **Table 3**, **4** and **5**.

RESULTS AND DISCUSSION:

Synthesis: Chalcones 3a-m of 4-aminoacetophenone were obtained from 4-aminoacetophenone 1 using various aldehydes 2a-m by Claisen-Schmidt condensation reaction. Pyrimidines 4a-m were synthesized by 1,2-addition and/or 1,4 addition of the amino group of the guanidine to the oxo group followed by cyclization **Scheme 1**, **Table 1**. We already reported the establishment of structure of the products 3a-m and 4a-m spectroscopically [2, 19].

Chalcones 3a-m on treatment with malononitrile in the presence of ammonium acetate in absolute alcohol gave 2-amino-3-cyano-4,6-diarylsubstituted pyridine derivatives 5a-k **Scheme 2**, **Table 2** proceed *via* imine formation from keto group and ammonium acetate; imine reacts with malononitrile, followed by cycloaddition, isomerization and aromatization. The identity of the product was determined by IR, ^{1}H- and ^{13}C-NMR and EI-MS spectral studies. The IR spectra of the compounds exhibited absorption at 3468-3330 cm^{-1} for -NH_2, 2217-2200 cm^{-1} for -CN, 3027-3105 cm^{-1} for aromatic C-H stretchings and 1574-1400 cm^{-1} for C = C aromatic and C = N stretching of pyridine. The ^{1}H-NMR spectra of compounds 5a-k showed the absence of the olefinic protons (-HC=CH-) of respective chalcones 3a-m at δ 7.41-6.71 ppm and δ 8.05-7.66 ppm, a more characteristic signal for C-5-H at δ 7.66 - 7.00 ppm as singlet and moreover singlets at δ 6.80-5.65 ppm and multiplets at δ 6.62 -7.90 ppm appeared for amines (D_2O exchangeable) and aromatic protons respectively. The ^{13}C-NMR spectrum is in agreement with the structure assigned. All the aromatic carbons of compounds 5a-k showed signals around δ 170-108 ppm in the spectra. The signal at δ 90-84 ppm is assigned to carbon attached with carbonitrile. Besides, the structure of the compound was confirmed by its mass spectral studies gave molecular ion peaks [M^+] or [{M+H}$^+$] corresponding to molecular formula. The elemental analysis values are similar with theoretical data within ± 0.4% deviation.



SCHEME 1: SYNTHESIS OF CHALCONES 3a-m AND 2,4,6-TRISUBSTITUTED PYRIMIDINES 4a-m

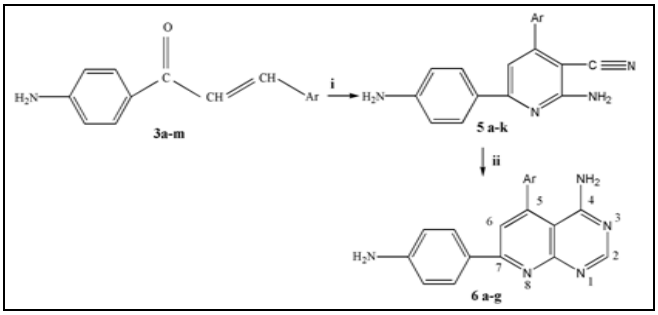
Reagents and conditions: (i) ethanol, aqueous potassium hydroxide, room temperature (16-20 h); (ii) guanidine hydrochloride, ethanol, solid KOH, reflux (2 to 6 h) at 75-80 ºC.

TABLE 1: 4-AMINOCHALCONES (3a-m) AND 2,4,6-TRISUBSTITUTED PYRIMIDINES (4a-m)

Compound	Ar
3a, 4a	4-Chlorophenyl
3b, 4b	2,4-Dichlorophenyl
3c, 4c	4-Fluorophenyl
3d, 4d	3-Bromophenyl
3e, 4e	4-Methoxyphenyl
3f, 4f	3,4-Dimethoxyphenyl
3g, 4g	3,4,5-Trimethoxyphenyl
3h, 4h	4-Methylphenyl
3i, 4i	4-Dimethylaminophenyl
3j, 4j	9-Anthracenyl
3k, 4k	2-Pyridinyl
3l, 4l	4-Pyridinyl
3m, 4m	3-Pyridinyl
3h, 4h	4-Methylphenyl

Target compounds 6a-g were synthesized by the reaction which proceeds as a sequential reaction of the condensation of amino group of cyanopyridine 5a-k with carbonyl group of formamide and intermolecular/ regioselective cyclization followed

by aromatization may take place during the formation of the pyrido[2,3-*d*]pyrimidines. The structure of the products, 6a-g were established spectroscopically. Thus, their IR spectra showed two bands in the region 1640-1610 cm^{-1} and 1375-1350 cm^{-1} are characteristics of the C=N and C-N stretching of pyrimidine as well as pyridine system and three bands in the regions 3480-3450 cm^{-1}, 3370-3300 cm^{-1} and 3200-3100 cm^{-1} (ν NH_2: free and H-bonded).



SCHEME 2: SYNTHESIS OF 2-AMINO-3-CYANOPYRIDINES 5a-k AND PYRIDO[2,3-*d*] PYRIMIDINES 6a-g

Reagents and conditions: i) malononitrile, ethanol, ammonium acetate, reflux (4 to 6 h) at 75-80 °C; (ii) formamide, DMF, reflux (24 to 30 h) at 170-180 °C.

TABLE 2: CYANOPYRIDINES 5a-k AND PYRIDO[2,3-*d*]PYRIMIDINES 6a-g

Compound	Ar
5a	4-Chlorophenyl
5b	2,4-Dichlorophenyl
5c	4-Fluorophenyl
5d	3-Bromophenyl
5e	4-Methoxyphenyl
5f	3,4-Dimethoxyphenyl
5g	4-Methylphenyl
5h	4-Dimethylaminophenyl
5i	9-Anthracenyl
5j	2-pyridinyl
5k	4-pyridinyl
6a	4-Chlorophenyl
6b	2,4-Dichlorophenyl
6c	4-Fluorophenyl
6d	3-Bromophenyl
6e	4-Methoxyphenyl
6f	4-Methylphenyl
6g	4-Dimethylaminophenyl

And also absence of band at 2217-2200 cm^{-1} for C≡N or (ν CN) further confirmed the structure. The ^{1}H-NMR spectra of these compounds gave further support for the aminopyrimidine structure, since they show a broad singlet signals at δ 7.87-7.51 ppm (C-4-NH_2, D_2O exchangeable) and δ 7.07-6.91 ppm (C-4"-NH_2, D_2O exchangeable). And also the characteristic singlet peaks observed in the range δ 8.34-8.33 ppm and δ 7.28-7.18 ppm indicates the presence of single protons at C-2 and C-6 positions further Confirmed the formation of pyridopyrimidine nucleus. The ^{13}C-NMR spectra exhibited characteristic peaks between δ 170-150 ppm for ring carbons adjacent to nitrogen atom in pyridopyrimidine nucleus, and δ 150-100 ppm for other ring carbons confirming the pyridopyrimidine structure. The mass spectra showed the corresponding molecular ion peak [M^+] or [{M+H}$^+$] as the base peaks and the fragmentation pattern was characteristic of respective pyrido- pyrimidines. The elemental analyses of all the newly synthesized compounds confirmed their structures.

Biology: The anticancer activity of all the synthesized chalcones **(**3a-m), 2,4,6-trisubstituted pyrimidines (4a-m) and pyrido[2,3-*d*]pyrimidines (6a-g) were evaluated against various human cancer cell lines Hos (bone cancer), HT 29 (colon cancer), G 361 (skin cancer), A 549 (lung cancer) and DU 145 (prostate cancer) using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. Doxorubicin and methotrexate were used as reference compounds.

Among all the Chalcones **Table 3**, 3b and 3i showed significant activity against all the cell lines with IC_{50} values ranging from 51-77 µM and 78-102 µM respectively and this may be due to the presence of chlorine at C-2 and 4 and dimethyl-amino at C-4 position on ring-B of chalcone. All the chalcones exhibited identifiable activity against Hos cell lines. Compound 3b was identified as the lead compound with potent cytotoxic activity against all the cell lines especially against Hos and DU 145 cancer cells having IC_{50} values of 51.78 µM and 51.98 µM respectively. Moreover, compound 3i also possessed moderate level of cytotoxicity against G 361 cancer cells with IC_{50} value of 78.23 µM. The remaining chalcones exhibited low cytotoxicity levels (IC_{50} values higher than 100 µM) and some were found to be inactive (IC_{50} values higher than 200 µM). This result demonstrated that the α,β-unsaturated ketone moiety of 4-aminochalcones is necessary to play an important role in thiol/enzyme-alkylation, preferentially *via* Michael addition and also the double bond is essential for chalcones as antitumor agents [28].

TABLE 3: *IN-VITRO* CYTOTOXIC ACTIVITY OF 4-AMINOCHALCONES (3a-m) AGAINST VARIOUS HUMAN CANCER CELL LINES BY MTT ASSAY

Compound	Ar	IC_{50} (µM)				
		Bone cancer (Hos)	Colon cancer (HT 29)	Human skin cancer (G 361)	Lung cancer (A 549)	Prostate cancer (DU 145)
3a	4-chlorophenyl	123.14	190.86	171.23	167.37	152.47
3b	2, 4-dichlorophenyl	51.78	52.63	76.95	76.16	51.98
3c	4-fluorophenyl	118.14	*a*	114.27	186.59	190.24
3d	3-bromophenyl	124.44	140.12	121.05	*a*	171.09
3e	4-methoxyphenyl	131.14	*a*	148.73	*a*	*a*
3f	3,4-dimethoxy phenyl	153.35	102.19	160.98	149.43	118.97
3g	3,4,5-trimethoxy phenyl	153.13	185.68	*a*	173.35	104.60
3h	4-methylphenyl	112.27	142.53	148.56	119.74	119.70
3i	4-dimethylamino phenyl	84.81	86.27	78.23	101.99	92.07
3j	9-anthracenyl	103.99	*a*	165.04	*a*	147.80
3k	2-pyridinyl	126.38	111.87	134.86	163.25	141.65
3l	4-pyridinyl	133.57	171.74	107.99	172.00	168.03
3m	3-pyridinyl	143.79	176.34	147.81	*a*	*a*
	Doxorubicin	0.22	4.32	0.08	6.54	5.76
	Methotrexate	0.31	2.5	10.87	2.13	0.35

[a] Inactive (IC_{50} values > 200 µM); IC_{50} values mean of three experiments in replicate

All the 2,4,6-trisubstituted pyrimidines 4a-m, have been evaluated for their anticancer activity and their IC_{50} values represented in **Table 4**. From the obtained IC_{50} values, it is summarized that compound 4b, 4c, 4d and 4i were found to be most active against all the cell lines tested with the range from 3 to 38 µM, 31 to 70 µM, 54 to 74 µM and 20 to 52 µM respectively which contained 2,4-dichlorophenyl, 4-fluorophenyl, 3-bromophenyl and 4-dimethylaminophenyl moiety at C-6 position of pyrimidine nucleus respectively. It is interestingly observed that compound 4b and 4i, showed maximum activity especially against bone cancer (Hos) cell lines and compound 4c and 4d possessed maximum activity against skin cancer (G 361) and colon cancer (HT 29) respectively. The remaining pyrimidines exhibited low cytotoxicity levels (IC_{50} values higher than 100 µM) and some were found to be inactive (IC_{50} values higher than 200 µM). Converting the α,β-unsaturated ketones (chalcones) to the corresponding 2,4,6-trisubstituted pyrimidines increased the cytotoxicity. This result demonstrated that the pyrimidine nucleus improves the cytotoxic activity and can be used as anticancer agents.

TABLE 4: *IN-VITRO* CYTOTOXIC ACTIVITIES OF 2,4,6-TRISUBSTITUTED PYRIMIDINES (4a-m) AGAINST VARIOUS HUMAN CANCER CELL LINES BY MTT ASSAY

Compound	Ar	IC_{50} (µM)				
		Bone cancer (Hos)	Colon cancer (HT 29)	Human skin cancer(G 361)	Lung cancer (A 549)	Prostate cancer (DU 145)
4a	4-chlorophenyl	80.50	*a*	83.23	120.87	*a*
4b	2, 4-dichlorophenyl	3.11	20.00	37.88	32.32	28.88
4c	4-fluorophenyl	69.67	43.26	30.64	53.38	*a*
4d	3-bromophenyl	73.60	54.63	65.21	*a*	61.52
4e	4-methoxyphenyl	52.67	*a*	106.02	*a*	*a*
4f	3,4-dimethoxyphenyl	93.04	103.35	161.73	*a*	109.39
4g	3,4,5-trimethoxyphenyl	151.70	119.00	150.79	178.89	192.35
4h	4-methylphenyl	159.89	*a*	158.84	103.62	145.74
4i	4-dimethylamino phenyl	20.91	37.11	34.03	28.52	51.70
4j	9-anthracenyl	180.44	*a*	*a*	147.87	119.77
4k	2-pyridinyl	89.04	98.93	92.69	123.61	125.34
4l	4-pyridinyl	111.25	183.19	194.22	118.89	151.97
4m	3-pyridinyl	119.35	*a*	*a*	*a*	*a*
	Doxorubicin	0.31	5.47	0.16	5.36	5.04
	Methotrexate	0.52	2.96	9.52	3.85	1.28

[a] Inactive (IC_{50} values > 200 µM); IC_{50} values mean of three experiments in replicate

In addition, all pyrimidine derivatives showed fairly good activity against bone cancer (Hos) cell lines as well as increased activity about two to ten times than the respective chalcones.

All the seven synthesized new pyrido[2,3-*d*] pyrimidine derivatives (6a-g) have been evaluated for their anticancer activity against five aforementioned cell lines by MTT assay method and the IC_{50} values are summarized in **Table 5**. Compounds 6b, 6c and 6g showed significant anticancer activity against all the tested cancer cell lines with IC_{50} values ranges from 2 to 16 µM, 4 to 27 µM, and 5 to 14 µM respectively and this may be due to the presence of 2,4-dichlorophenyl, 4-fluorophenyl and 4-dimethylaminophenyl moieties at C-5 position of pyrido[2,3-*d*]pyrimidine nucleus. It is interestingly noticed that the compound 6b, 6c and 6g exhibited maximum activity against Hos cancer cells with IC_{50} values 2.38 µM, 4.04 µM and 4.38 µM respectively whereas compound 6b and 6g possessed moderate activity against G 361 cancer cells with respective IC_{50} values of 3.31 µM and 6.12 µM. The remaining pyridopyrimidines exhibited low cytotoxicity levels (IC_{50} values higher than 25 µM) and also all were found to be active against all the tested cancer cells.

TABLE 5: *IN-VITRO* CYTOTOXIC ACTIVITIES OF PYRIDO[2,3-*d*]PYRIMIDINES (6a-g) AGAINST VARIOUS HUMAN CANCER CELL LINES BY MTT ASSAY

Compound	Ar	IC_{50} (µM)				
		Bone cancer (Hos)	Colon cancer (HT 29)	Human skin cancer (G 361)	Lung cancer (A 549)	Prostate cancer (DU 145)
6a	4-chlorophenyl	51.78	62.50	50.34	79.45	46.92
6b	2, 4-dichlorophenyl	2.38	9.71	3.31	11.88	16.09
6c	4-fluorophenyl	4.04	11.14	14.86	17.25	26.45
6d	3-bromophenyl	20.06	32.57	41.12	53.34	26.65
6e	4-methoxyphenyl	70.46	43.46	50.69	89.85	27.25
6f	4-methylphenyl	37.06	96.36	55.56	58.13	75.32
6g	4-dimethylamino phenyl	4.38	10.30	6.12	11.79	13.67
	Doxorubicin	0.32	5.64	0.22	6.98	6.76
	Methotrexate	0.54	3.12	9.46	3.04	1.03

IC_{50} values mean of three experiments in replicate

Overall, compounds which possess 2,4-dichloro-phenyl, 4-fluorophenyl and 4-dimethylamino moieties as substituent at proper position in chalcones, pyrimidines and pyridopyrimidines identified as lead anticancer agents. From the obtained results, it is concluded that pyridopyrimidines exhibited more anticancer activity when compared with pyrimidines which are cyclized from the same chalcones as well as chalcones themselves. The present study identified novel substituted chalcones, pyrimidines and pyrido[2,3-*d*]- pyrimidines.

Anticancer activity was greatly enhanced after conversion of chalcones to respective pyrido-pyrimidines. This inference demonstrated that the pyridopyrimidine nucleus improves the anticancer activity and also pyridopyrimidines are better lead molecules as anticancer agents compared with chalcones and pyrimidines especially against Hos and G 361 cell lines. Further studies are required to elucidate the exact mechanism of action for their therapeutic potential as anticancer agents.

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CONFLICT OF INTEREST: The authors declare to have no financial/commercial conflict of interest.

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