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Synthesis and Lung Cancer Cell Line Study of Pyrrolo[2,3-*d*]pyrimidine Analogs

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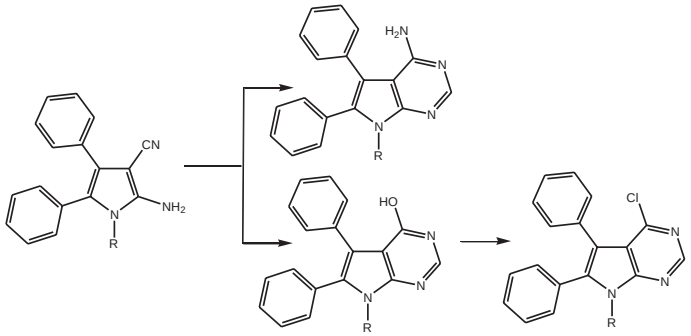
ABSTRACT Three series of new pyrrolo[2,3-*d*]pyrimidines having diverse groups at position C4 and N7 of the pyrrolo[2,3-*d*]pyrimidine core were synthesized and their cell line study on the National Cancer Institute (NCI) H1 299 lung cancer cell line was performed. The details of the synthetic methods and characterization data of the synthesized compounds have been presented in this study. Compounds **08a, 08h, 08j, 09h, 09i, 09j, 09m, 09n, and 09o** showed excellent anticancer activity compared to standard doxorubicin half maximal inhibitory concentration value on NCI H1 299 (lung cancer cell line) which was nontoxic to normal Vero cell line.



KEYWORDS Doxorubicin, Pyrrolo[2,3-d]pyrimidine, Anticancer.

INTRODUCTION

Cancer is characterized by uncontrolled growth of abnormal cells.[1] The abnormal growth can also be referred to as a neoplasm. Neoplasms can be benign (noncancerous) or malignant (cancerous). Cancerous growths can occur in any organ of the body and are characterized by three distinct properties: The cells replicate rapidly with reduced growth control, the cells lose contact inhibition *in-vitro*, and the resulting neoplasm invades surrounding tissues and may spread to other parts of the body.[2,3] Cells of benign tumors have reduced growth control but do not invade surrounding tissues or spread to other parts of the body. Other important aspects of cancer cells include the ability to be self-sufficient and generate local angiogenesis while resisting antigrowth and apoptosis signals. If the spread of a malignant neoplasm is not controlled, it can result in death.[4,5]

Cancer is caused by both external factors (tobacco, chemicals, radiation, and infectious organisms) and internal factors (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism).[6] As the humanistic and economic burdens of cancer continue to

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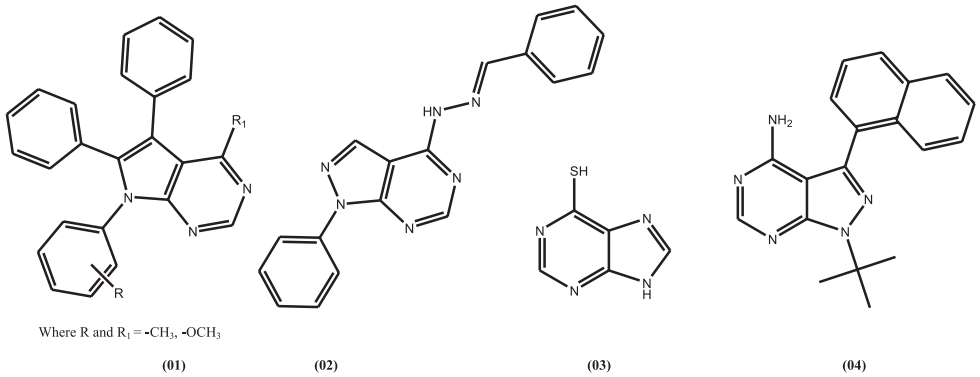


Where R and R_1 = -CH_3, -OCH_3

(01) **(02)** **(03)** **(04)**

rise, prevention of the development of cancer becomes more important. Over half of all cancer cases may be preventable. Reducing or avoiding exposure to external factors such as tobacco may help to reduce the number of preventable causes of cancer.[7,8]

Increasing interest in biological studies of pyrrolo[2,3-*d*] pyrimidine in the last decade is a consequence of their wide usage as a pharmaceutically important class of compounds. Pyrrolo[2,3-*d*]pyrimidine derivatives have considerable potential in the field of chemotherapy, as they were found to exhibit their antitumor activity by inhibiting different types of enzymes such as cyclin-dependent kinase, *Src* and *Abl* tyrosine kinase, glycogen synthase kinase-3, adenosine deaminase, and epidermal growth factor receptor protein tyrosine kinase.[9] The derivatives of pyrrolo[2,3-*d*]pyrimidine have already been discovered as antitumor agents by the National Cancer Institute (NCI, USA) on HCT116 and other cell lines. Some pyrrolo[2,3-*d*]pyrimidines **(1)** structurally related with allopurinol **(2)**, 6-mercaptopurine **(3),** and 1-(1,1-dimethylethyl)-3-(1-naphthalenyl)-1*H*-pyrazolo[3,4-*d*] pyrimidin-4-amine (1-NA-PP1) **(4)** have also been reported as potent inhibitors of growth of several human tumor cell lines.[10]

In view of these observations, we undertook synthesis and lung cancer cell line study of three series of pyrrolo[2,3-*d*] pyrimidine analogs **(8-10)**.

RESULTS AND DISCUSSION

We synthesized 5,6-diphenyl-7-substituted-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl-amines **(08a-08o)**, 5,6-diphenyl-7-substituted-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-ols **(09a-09o)**, and 4-chloro-5,6-diphenyl-7-substituted-7*H*-pyrrolo[2,3-*d*] pyrimidines **(10a, 10c, 10f, 10m, 10n, and 10o)** as depicted in **Scheme 1**. Intermediate 2-amino-4,5-diphenyl-1-(substituted)-1*H*-pyrrole-3-carbonitriles **(07a-07o)** were prepared from reaction of benzoin with respective amines followed by treatment of *in situ* generated initially formed intermediate **(6)** with malononitrile in the presence of sodium ethoxide. 5,6-Diphenyl-7-substituted-7*H*-pyrrolo[2,3-*d*] pyrimidin-4-yl-amines **(08a-08o)** were prepared by refluxing 2-amino-4,5-diphenyl-1-(substituted)-1*H*-pyrrole-3-carbonitrile **(07a-07o)** with formamide. Another series of compounds containing 4-hydroxy group were prepared by refluxing **07a-07o** with formic acid. Some of these 4-hydroxy derivatives **(09a, 09c, 09f, 09m, 09n, and 09o)** were converted to the corresponding chloro derivatives **10a, 10c, 10f, 10m, 10n, and 10o** by refluxing with phosphorus oxychloride.

In the present investigation, all the compounds were evaluated against cell lines named NCI H1 299 (lung cancer cell line) and Vero cell line (normal cell line) for each tested compound as well as standard anticancer drug doxorubicin, dose response curve (DRC) against all cell lines was plotted with 10 analysis point, i.e., with 10 different drug concentrations. The concentration causing 50% cell growth inhibition (half maximal inhibitory concentration [IC_{50}]) was determined from DRC using GraphPad Prism Software (Ver. 5.04) (GraphPad Software, Inc., USA) and Microsoft excel 2007 (Microsoft Corporation, USA) application **[Table 1]**.

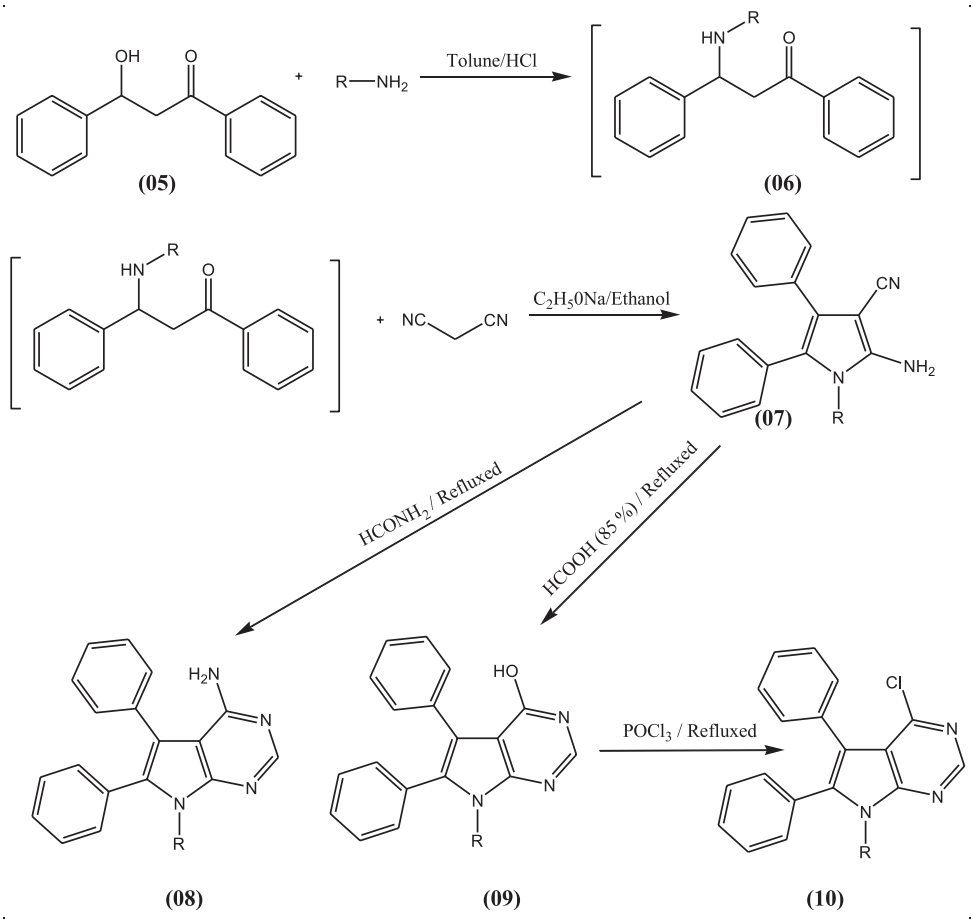
NCI H1 299 (lung cancer cell line) study

Among all the tested compounds **08a, 08g, 08h, 08i, 08j, 08k, 08h, 09i, 09j, 09k, 09m, 09n, 09o, and 10f** showed highest potential effect on NCI H1 299 cell line compared to standard doxorubicin IC_{50} value. Compounds **08b, 08e, 08l, 08m, 08n, 08o, 09a, 09d, 09e, 09f, 09g, 09l, 10a, and 10d** possessed good anticancer/cytotoxicity activity. Compounds **08c, 08d, 08f, 09b, 09c, 10m, 10n, and 10o** did not show any activity as their IC_{50} values are higher than 100. All compounds were also check for their normal cell line activity with Vero cell line. Compounds **08a, 08b, 08d, 08e, 08h, 08j, 08l, 08m, 08o, 09a, 09c, 09d, 09e, 09f, 09h, 09i, 09j, 09m, 09n, 09o, and 10d** were found to be nontoxic to Vero normal cell line. Compounds **08a, 08h, 08j, 09h, 09i, 09j, 09m, 09n, and 09o** showed higher anticancer activity than the standard doxorubicin IC_{50} value on NCI H1 299 (lung cancer cell line) cell lines with nontoxic to Vero normal cell line. Further, the activity of potent/lid compounds can be confirmed by performing their DNA fragmentation study, which may be followed by tunnel assay, flow cytometry, DNA fragmentation assay, or CASPACASE assay **[Figure 1]**.

(Blue line indicates that the compounds are toxic to Vero normal cell line and red line indicates that the compounds are nontoxic to Vero normal cell line) against standard – doxorubicine

EXPERIMENTAL

All the melting points were determined in open capillaries and are uncorrected **[Table 2]**. Infrared (IR) spectra of all



Scheme 1: Synthesis of pyrrolo[2,3-*d*]pyrimidine analogs
Where R= a) C_6H_5NH, b) 2-CH_3-C_6H_5NH, c) 3-CH_3-C_6H_5NH, d) 4-CH_3-C_6H_5NH, e) 2-OCH_3-C_6H_5NH, f) 3-OCH_3-C_6H_5NH, g) 4-OCH_3-C_6H_5NH, h) 2-NO_2-C_6H_5NH, i) 3-NO_2-C_6H_5NH, j) 4-NO_2-C_6H_5NH, k) 2-Cl-C_6H_5NH, l) 3-Cl-C_6H_5NH, m) 4-Cl-C_6H_5NH, n) 2,4-Cl-C_6H_5NH, o) 2,6-Cl-C_6H_5NH

compounds were recorded in Fourier transform IR 8400S Shimadzu spectrophotometer using KBr. Mass spectra were obtained using 2010EV liquid chromatography–mass spectrometry Shimadzu Instrument at 70 eV. ^{1}H nuclear magnetic resonance (NMR) spectra were obtained in dimethyl sulfoxide (DMSO) on BRUKER Avance-II 400 MHZ instrument and chemical shift was measured as parts per million downfield from tetramethylsilane as internal standard.

General method for synthesis of 2-amino-4,5-diphenyl-1-(substituted)-1*H*-pyrrole-3-carbonitriles (07a-07o)

A mixture of benzoin (2 g, 0.01 mol), the appropriate amine (aniline [0.93 g, 0.01 mol], *o*-toluidine or *m*-toluidine or *p*-toluidine [1.17 g, 0.01 mol], or *o*-anisidine or *m*-anisidine or *p*-anisidine [1.23 g, 0.01mol], or *o*-chloroaniline, or *m*-chloroaniline, or *p*-chloroaniline [1.25 g, 0.01 mol], or *o*-nitroaniline, or *m*-nitroaniline, or *p*-nitroaniline [1.36 g, 0.01 mol], 2,4-dichloroaniline, 2,6-dichloroaniline [1.61 g, 0.01 mol]), and concentration HCl (6–8 drops) in toluene (50 mL) was heated under reflux for 6 h and cooled. The reaction mixture was filtered and the resulting residue was dissolved in 30 mL absolute alcohol. To thus obtain solution, added malononitrile (0.66 mg, 0.01 mol) followed by sodium ethoxide (2 g sodium metal in 20 mL absolute alcohol) as catalyst. The mixture was refluxed until a solid was formed. The solvent was evaporated under reduced pressure and the residue was recrystallized from methanol to give pure yellow crystalline product.

General method for synthesis of 5,6-diphenyl-7-substituted-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl-amines (08a-08o)

A mixture of the appropriate aminopyrrole (**07**, 0.01 mol) and formamide (30 mL, 0.066 mol) was heated under reflux for 6 h, cooled, and poured into crush ice to give precipitates which were filtered, dried, and recrystallized from ethanol to yield yellow crystalline pure product.

General method for synthesis of 5,6-diphenyl-7-substituted-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-ols (09a-09o)

A mixture of the appropriate aminopyrrole (**07**, 0.01 mol) and formic acid (20 mL, 85%) was heated under reflux for 3 h, cooled, and poured onto crush ice to give precipitates which were filtered, dried, and recrystallized from ethanol to yield yellow crystalline pure product.

Table 1: Celle line study of pyrrolo[2,3-*d*]pyrimidine analogs

Compounds	NCI-H1-299 cell line study		Vero cell line study (normal cell line)	
	IC_{50} (µM/ml)	R^2	IC_{50} (µM/ml)	R^2
08a	2.72	0.9728	254.4	0.9862
08b	18.09	0.9948	631	0.9891
08c	47.2	0.9967	19.04	0.9891
08d	28.45	0.9925	>100	0.9595
08e	14.65	0.9754	>1000	0.992
08f	37.11	0.9653	15.72	0.9765
08g	06.57	0.9570	93.32	0.9957
08h	2.17	0.9759	>100	0.9738
08i	3.37	0.9839	8.03	0.9767
08j	2.12	0.9909	143.8	0.9778
08k	5.4	0.9750	20.73	0.9726
08l	12.94	0.9628	144.92	0.9869
08m	13.11	0.9834	162	0.9946
08n	12.15	0.9668	133	0.9933
08o	23.69	0.9519	>1000	0.9823
09a	10.27	0.9878	209.1	0.9986
09b	100.8	0.9892	5.74	0.9905
09c	59	0.9604	>100	0.9764
09d	9.51	0.9987	144.9	0.9978
09e	23.05	0.9884	>100	0.9553
09f	21.11	0.9842	487.4	0.9822
09g	11.55	0.9693	6.9	0.9606
09h	7.24	0.9564	>1000	0.9955
09i	5.79	0.9449	299	0.8944
09j	4.23	0.9762	>100	0.9988
09k	4.93	0.9556	79.71	0.9902
09l	11.79	0.9535	17.06	0.9783
09m	2.05	0.9897	>100	0.9798
09n	2.11	0.9838	410.4	0.9923
09o	4.2	0.9617	257.5	0.9948
10a	9.66	0.9899	>100	0.9645
10c	25.39	0.9891	94.89	0.9908
10f	4.62	0.9692	23.84	0.9625
10m	247.9	0.9741	57.06	0.9911
10n	89.04	0.9955	69.66	0.9755
10o	100	0.9736	24.43	0.9044
Standard	**6.17**	**0.9229**	**314.6**	**0.9921**

IC_{50}: Half maximal inhibitory concentration

General method for synthesis of 4-chloro-5,6-diphenyl-7-substituted-7*H*-pyrrolo[2,3-*d*]pyrimidines (10a, 10c, 10f, 10m, 10n, and 10o)

An appropriate pyrrolopyrimid-4-ol (**09**, 0.01 mol) in phosphorus oxychloride (30 mL) was heated under reflux for 4 h, cooled, and poured onto crush ice to give precipitates which were filtered, dried, and recrystallized from ethanol to yield yellow crystalline pure product.

2-Amino-1,4,5-triphenyl-1H-pyrrole-3-carbonitrile (07a)

Yellow crystalline solid, yield 80%, mass (m/e) 336.15 (M + 1), IR (cm^{-1}) CN – 2201, NH_2 – 3385 1H, NMR (δ ppm, DMSO-d6) 5.75 (br-s, 2H, NH_2), 6.93–7.38 (m, 15H, Ar-H).

2-Amino-4,5-diphenyl-1-p-tolyl-1H-pyrrole-3-carbonitrile (07d)

Yellow crystalline solid, yield 60%, mass (m/e) 350.16 (M + 1), IR (cm^{-1}) CN – 2207, NH_2 – 3322 1H, NMR

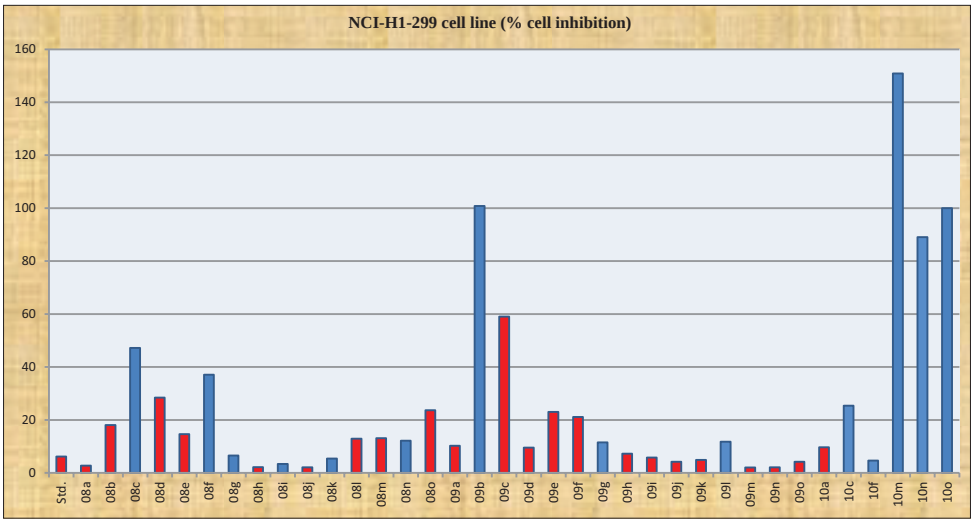


Figure 1: Graphical representation of cell line activity of pyrrolo[2,3-*d*]pyrimidine analogs

Table 2: Physical properties of 2-amino-4,5-diphenyl-1-(substituted)-1*H*-pyrrole-3-carbonitriles (07a-07o), 5,6-diphenyl-7-substituted-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl-amines (08a-08o), 5,6-diphenyl-7-substituted-7*H*- pyrrolo[2,3-*d*]pyrimidin-4-ols (09a-09o), and 4-chloro-5,6-diphenyl-7-substituted-7*H*-pyrrolo[2,3-*d*]pyrimidines (10a, 10d, 10f, 10m, 10n, and 10o)

Compound code	Melting point (°C)	Compound code	Melting point (°C)	Compound code	Melting point (°C)
07a	170–172	**08c**	235–237	**09e**	233–235
07b	228–230	**08d**	230–232	**09f**	210–212
07c	136–138	**08e**	226–228	**09g**	>300
07d	170–172	**08f**	260–262	**09h**	211–211
07e	174–177	**08g**	>300	**09i**	260–262
07f	164–166	**08h**	273–275	**09j**	>300
07g	123–125	**08i**	235–237	**09k**	240–242
07h	145–147	**08j**	>300	**09l**	269–271
07i	160–122	**08k**	231–333	**09m**	280–282
07j	182–184	**08l**	232–234	**09n**	>300
07k	181–183	**08m**	245–247	**09o**	288–290
07l	160–162	**08n**	>300	**10a**	>300
07m	136–138	**08o**	>300	**10d**	290–292
07n	210–112	**09a**	199–201	**10f**	280–282
07o	200–202	**09b**	213–215	**10m**	>300
08a	262–264	**09c**	180–182	**10n**	>300
08b	218–220	**09d**	190–192	**10o**	291–293

(δ ppm, DMSO-d6) 2.29 (s, 3H, CH_3), 5.70 (br-s, 2H, NH_2), 6.93–7.26 (m, 14H, Ar-H).

5,6,7-Triphenyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine (08a)

Yellow crystalline solid, yield 40%, mass (m/e) 363.15 (M + 1), IR (cm^{-1}) NH_2 – 3463 1H, NMR (δ ppm, DMSO-d6) 8.30 (s, 1H, C-2H), 7.06 (br-s, 2H, NH_2), 7.15–7.52 (m, 15H, Ar-H).

5,6-Diphenyl-7-m-tolyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine (08c)

Yellow crystalline solid, yield 60%, mass (m/e) 378.00 (M + 1) IR (cm^{-1}) NH_2 – 3488 1H, NMR (δ ppm, DMSO-d6) 2.36 (s, 3H, CH_3), 8.33 (s, 1H, C-2H), 5.02 (br-s, 2H, NH_2, D_2O exchangeable), 6.98–7.40 (m, 14H, Ar-H).

7-(2-Chlorophenyl)-5,6-diphenyl-7H-pyrrolo[2,3-d] pyrimidin-4-amine (08k)

Yellow crystalline solid, yield 30%, mass (m/e) 397.12 (M + 1), 399.11 M + 2 IR (cm^{-1}) NH2 – 3465 1H, NMR (δ ppm, DMSO-d6) 8.33 (s, 1H, C-2H), 6.96–7.38 (m, 14H, Ar-H), 5.11 (br-s, 2H, NH_2, D_2O exchangeable).

5,6,7-Triphenyl-7H-pyrrolo[2,3-d]pyrimidin-4-ol (09a)

Yellow crystalline solid, yield 40%, mass (m/e) 364.00 (M + 1), IR (cm^{-1}) OH – 3241 1H, NMR (δ ppm, DMSO-d6)

8.20 (s, 1H, C-2H), 10.20 (s, 1H, C-OH), 6.92–7.38 (m, 15H, Ar-H).

5,6-Diphenyl-7-m-tolyl-7H-pyrrolo[2,3-d]pyrimidin-4-ol (09c)

Yellow crystalline solid, yield 60%, mass (m/e) 379.0 (M + 1), IR (cm^{-1}) OH – 3234 1H, NMR (δ ppm, DMSO-d6) 2.34 (s, 3H, CH_3), 8.21 (s, 1H, C-2H), 8.60 (s, 1H, C-OH), 6.92–7.37 (m, 14H, Ar-H).

7-(2-Chlorophenyl)-5,6-diphenyl-7H-pyrrolo[2,3-d] pyrimidin-4-ol (09k)

Yellow crystalline solid, yield 30%, mass (m/e) 398.10 (M + 1), 400.10 M + 2 IR (cm^{-1}) OH – 3308 1H, NMR (δ ppm, DMSO-d6) 7.80 (s, 1H, C-2H), 12.25 (s, 1H, C-OH, D_2O exchangeable), 6.90–7.38 (m, 14H, Ar-H).

4-Chloro-5,6,7-triphenyl-7H-pyrrolo[2,3-d]pyrimidine (10a)

Yellow crystalline solid, yield 50%, mass (m/e) 382.11 (M + 1), 384.11 (M + 2), IR (cm^{-1}) Cl – 695 1H, NMR (δ ppm, DMSO-d6) 6.91–7.20 (m, 15H, Ar-H), 7.90 (s, 1H, C-2H).

4-Chloro-5,6-diphenyl-7-p-tolyl-7H-pyrrolo[2,3-d] pyrimidine (10d)

Yellow crystalline solid, yield 60%, mass (m/e) 396.11 (M + 1), 398.11 (M + 2), IR (cm^{-11}) Cl – 697 1H, NMR (δ ppm, DMSO-d6) 2.51 (s, 3H, CH_3), 7.18–7.84 (m, 14H, Ar-H) 8.60 (s, 1H, C-2H).

General method for cell line study (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide [MTT] assay)[11,12]

It is a laboratory test and a standard colorimetric assay for measuring cellular growth. It can also be used to determine cytotoxicity of potential medicinal agents and other toxic materials.

This assay is a sensitive, quantitative, and reliable colorimetric assay that measures viability, proliferation, and activation of cells. The assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate MTT into a dark blue formazan product which is insoluble in water. The amount of formazan produced is directly proportional to the cell number in range of cell lines.

CONCLUSION

From this cell line study, it can be concluded that compounds series with electron-withdrawing groups at N_7 and electron-donating groups at C_4 give excellent cytotoxic activity. The compounds with electron-donating OR no groups at N_7 and electron-donating groups at C_4 give good cytotoxic activity, whereas compounds containing electron-withdrawing groups at N_7 and C_4 do not give cytotoxic activity on NCI H1 299.

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