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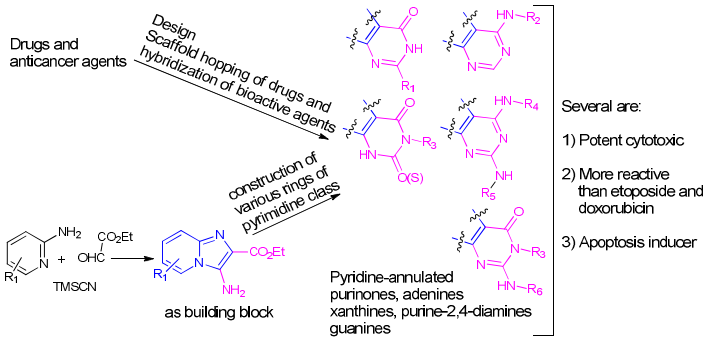

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ARTICLE TYPE

Scaffold-hopping and hybridization based design and building block strategic synthesis of pyridine-annulated purines: Discovery of novel apoptotic anticancer agents

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A set of novel pyridine-annulated analogs of purinones, adenines and their oxo/thio congeners, xanthines, guanines, and purine-2,4-diamines as potential anticancer agents was considered based on the scaffold-hopping and hybridization of known anticancer agents/drugs, purine derivatives and our recently developed imidazo-pyridine derivatives. Towards synthesis of these compounds, a new approach involving convenient preparation of 3-amino-2-carboxyethyl substituted imidazo[1,2-*a*]-pyridine and its use as building block for construction of the fused rings was developed. The approach enabled the preparation of a number of compounds with relevant substitutions for each class. Several of pyridine-annulated adenine and its oxo/thio analogs, xanthine and purine-2,4-diamine were found to possess significant anticancer activities in kidney cancer cells and relatively less cytotoxicities to normal cells. They were relatively more active than anticancer drugs, etoposide and doxorubicin. A representative compound pyridine-annulated adenine derivative (**22**) was found to exert significant apoptosis.

Introduction

Cancer is the second major cause of deaths worldwide after cardiovascular diseases.[1] Towards development of new chemical entities (NCEs), various approaches are followed in medicinal chemistry research. Among them, scaffold/template hopping[2] has been recognized as a valuable approach. The scaffold hopping offers an opportunity to explore new chemotypes that can possess potential similar biological activities and are outside the coverage of existing patents. There are several marketed drugs and clinical trial agents discovered by this strategy. For example, vardenafil drug, a PDE5-inhibitor, has been discovered from heterocyclic scaffold hopping of another drug sildenafil, likewise, cyclooxygenase-inhibiting anti-inflammatory drugs etoricoxib and valdecoxib from celecoxib. A selective DPP-4 inhibitor imigliptin developed by scaffold hopping-based design on alogliptin and structure-activity guided lead optimization is currently under clinical trial.[3] Another important approach is molecular hybridization of chemotypes of known drugs and therapeutic agents.[4] A well-known example is azatoxin derived by structural hybridization of topoisomerase II–targeting drugs etoposide and ellipticine.[5] Azatoxin with relevant substitutions showed better topoisomerase II inhibitory activity than etoposide or ellipticine. Therefore, an amalgamation of template-hopping and molecular hybridization on drugs and known bioactive agents can be an important design approach towards discovery of new chemical entities (NCEs).

Purine class of compounds are known for their wide range of anticancer activities. They interfere with various biochemical pathways, such as, inhibition of CDK,[6] PDE[7] and Hsp90,[8] and disruption[9] of microtubule dynamics. Various marketed drugs[10] also possess purine nucleus (Figure 1). QAP 1, an adenine derivative that shows ATP-competitive catalytic inhibition of topoisomerase II is in clinical trial.[11] In 2014, the USFDA approved a drug idelalisib, an adenine derivative, for the treatment of various leukemia and lymphoma.[12] For some specific pharmacological activities, purine derivatives have been found to be less potent, while their analogs with an annulated ring have been found to possess enhanced pharmacological activities and better pharmacokinetic profiles.[13] These features incited medicinal and organic chemists to generate an area of biologically important class of compounds, heterocyclic-condensed purines. These compounds have been shown to posses various bioactivities[14] such as, antihypertension,[15] anti-inflammatory,[13a] human A3 adenosine receptor antagonism,[13c] and inhibition of PDE1/5[16] and tyrosine kinase EphB4.[17]

Recently, we developed imidazo-pyridines, designed based on scaffold-hopping of existing drugs and known agents, as novel anticancer agents that induced apoptosis in G1/S phase.[18a] As a part of our program aimed at anticancer drug discovery,[18] employing the amalgamation of scaffold-hopping and molecular hybridization design approaches on these structural classes of anticancer agents/drugs, purines (Figure 1), annulated purines and imidazo-pyridines, we considered a series of novel pyridine-annulated purine class of compounds as potential anticancer agents (Figure 2).

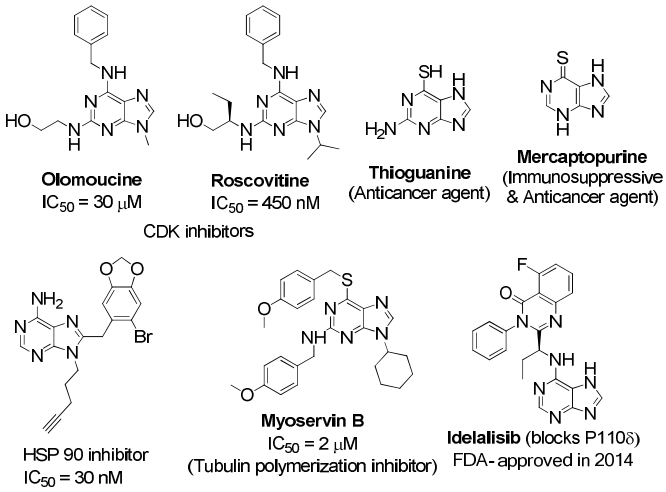


Figure 1: Pharmacological activities of purine-based compounds and drugs

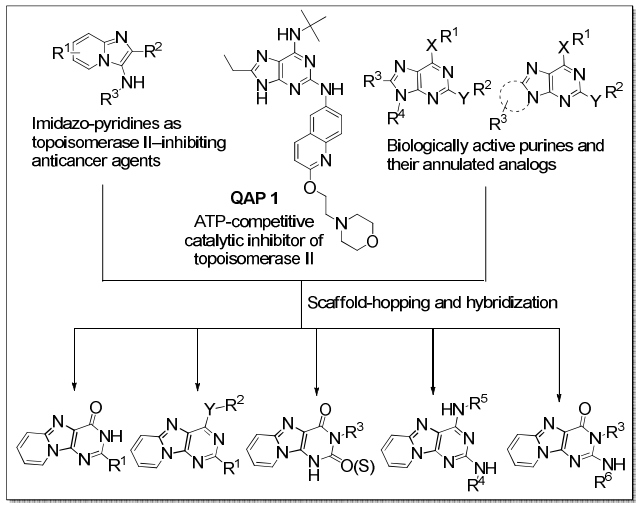


Figure 2: Design of target compounds for potential anticancer activity

Accordingly, the studies for establishment of diversity-feasible building block approach for synthesis of pyridine-annulated analogs of purinone, adenine and its oxo/thio congener, xanthine, purine-2,4-diamine and guanine derivatives, preparation of relevant substituted compounds, and evaluation of anticancer activities were undertaken. Several of these new compounds were found to show significant anticancer activities, lower cytotoxicities to normal cells, and apoptosis in cancer cells.

Results and Discussion

Chemistry

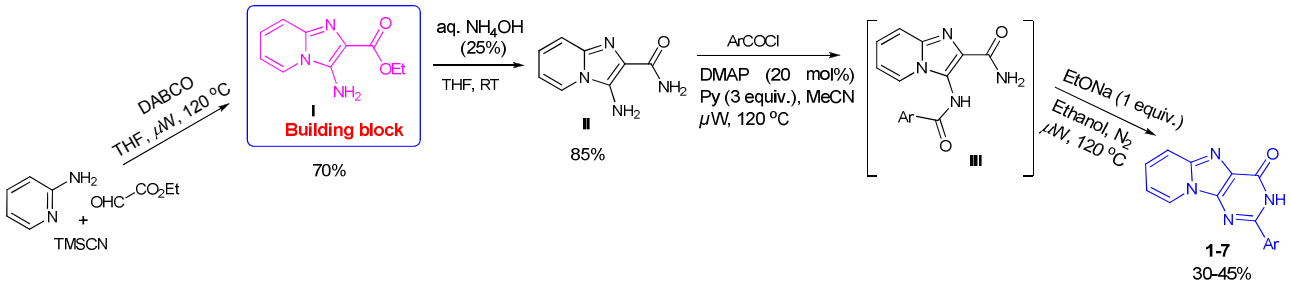
The known methods for preparation of heterocyclic-annulated purine derivatives involve mostly the construction of annulated new rings on purines.[13a,c,d,15,19] Following these methods, the preparation of pyridine-condensed purinones, adenines, xanthines, and purine-2,4-diamines with a variety of relevant substitutions would require for each compound the separate reaction-routes involving construction of the annulated pyridine ring on these purine class of motifs. In addition, construction of annulated pyridine ring is difficult. We considered a building block synthetic strategy via a suitably functionalized compound that could enable the construction of fused pyrimidine ring and follow up derivatization towards preparation of all designed series compounds (Scheme 1a, 1b and 2). In this direction, we have recently developed a method for preparation of 3-amino-2-carboxyethyl substituted imidazo[1,2-*a*]-pyridine (**I**) in one step and its use as building block in the synthesis of pyridine-fused purinones and adenines (Scheme 1a and 1b).[20] In the present work, the synthesis of pyridine-annulated xanthines, guanines and purine-2,4-diamines has been explored by the building block strategy. Some relevant pyridine-fused adenines (ten compounds) have also been prepared.

The development of a method towards preparation of pyridine-annulated xanthines was initially investigated. The preparation of fused pyrimidine-dione ring on 2-aminobenzenecarboxamide by its reaction with urea at 190 ^{o}C is known.[21] Following this method, the reaction of the amide derivative (**II**) of the building block (**I**) with urea was performed. However, the reaction did not undergo. Variation in conditions including addition of acid/base promoters also did not help. These reveal the poor amine-nucleophilicity of the C2-carboxyethyl substituted imidazopyridinyl-3-amine towards urea. A set of reactions of the amide derivative (**II**) were then performed using various reactants, such as, di-ethyl/methyl carbonate, ethyl chloroformate, and Boc-anhydride, which could promote the domino conversions of C3-amine to carbamate and follow up trans-amidation. However, none of these reactions provided the construction of fused pyrimidine-dione. In each case, either the reaction did not undergo or a mixture of non-isolable products was obtained on prolonging the reaction or enhancing the reaction temperature. The mass spectroscopic studies of the crude mixture obtained under higher temperature indicated the formation of desired product as well as the unwanted product generated from intermolecular trans-amidation of the derivative **II**. Therefore, this domino reaction is associated with chemoselectivity issue also. The construction of fused pyrimidine-dione ring directly from the building block (**I**) by reaction with urea, ethyl carbamate or isocyanate was then investigated. Gratifyingly, the reaction of the building block with isocyanate was found to be promising. In the reaction, bases as promoters were found to be more efficient than acids. In evaluation of various bases, to our delight, NaOEt-mediated reaction under microwave irradiation was found to be most effective and afforded pyridine-annulated xanthine in good yield (Scheme 2). This developed method enabled the preparation of various pyridine-annulated xanthines (**33-41**). The protocol was also suitable for construction of thioxanthine (**42**) by reaction of the building block with isothiocyanate.

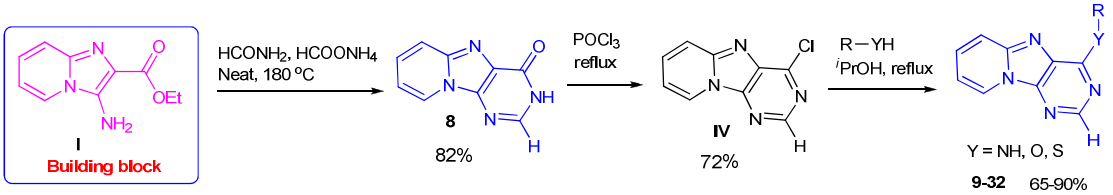
Towards synthesis of pyridine-condensed guanines starting from the building block, condensed thioxanthine (**42**) was considered as suitable substrate. The thio-methylation and S_NAr with amines produced pyridine-enlarged guanines (**47-49**).

Purine-2,6-diamines are well known for their inhibitory activities against various kinases.[22] This incited us to investigate also the use of the building block in the synthesis of pyridine-enlarged

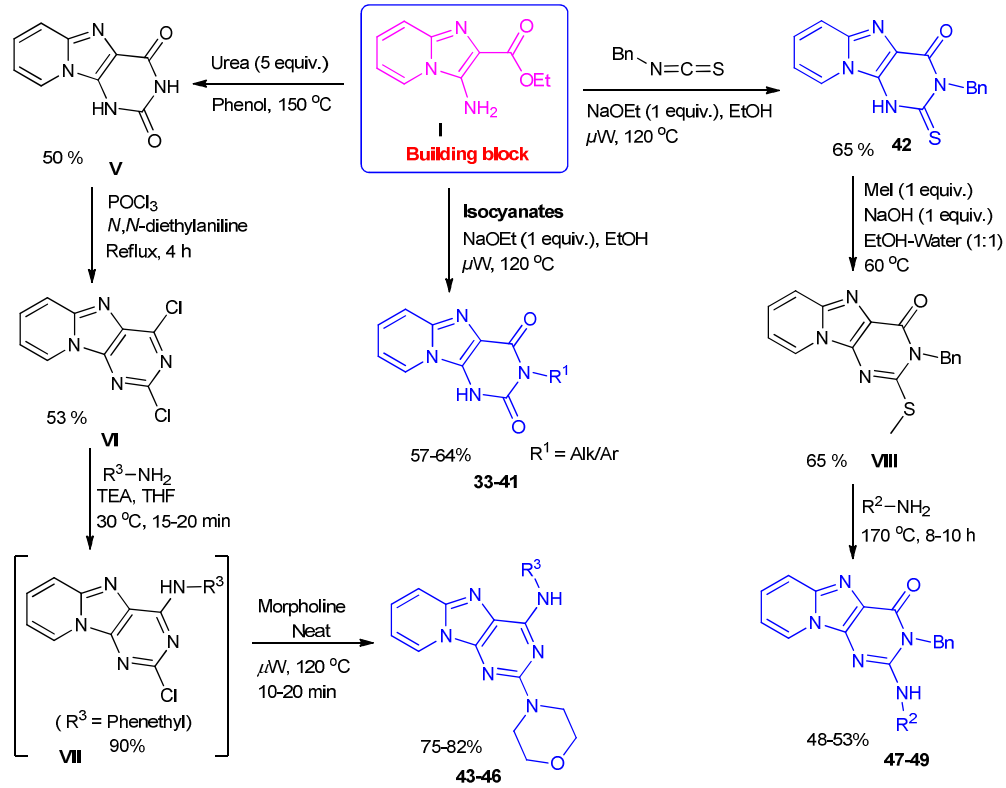
purines with 2,4-differential amine substitutions. In this aspect, we required to obtain chemoselective differential amination of dichloro derivative of purine-annulated xanthine (**VI**), prepared by deoxychlorination of pyridine-condensed xanthine (**V**). Compared to 2-Cl group, 4-Cl functionality was found to be more reactive for S_NAr and underwent rapid reaction at room temperature, whereas the reaction of 2-Cl with amine required microwave irradiation at higher temperature and neat condition. This differential reactivity of 4-Cl vs 2-Cl group enabled the preparation of desired annulated purine-2,6-diamines (**43-46**) in moderate to good yields (Scheme 2)**.** Using the developed building block synthetic strategy, we have prepared new pyridine ring-condensed analogs of all known classes of purine scaffolds, purinone, adenine and its oxo/thio-congeners, xanthine, guanine, and purine-2,6-diamine. This approach is convenient, concise, and flexible for incorporation of various substituents/functionalities including those are therapeutically important into the products (Figure 3). This approach bears potential application in the synthesis of various annulated purines.



Scheme 1a: Synthesis of pyridine-condensed purinones (**1-7**).[20]



Scheme 1b: Synthesis of pyridine-condensed adenines and their oxo/thio analogs (**9-32**).[20]



Scheme 2: Synthesis of pyridine-condensed xanthines (**33-41**), thioxanthine (**42**), purin-2,4-diamines (**43-46**) and guanines (**47-49**).

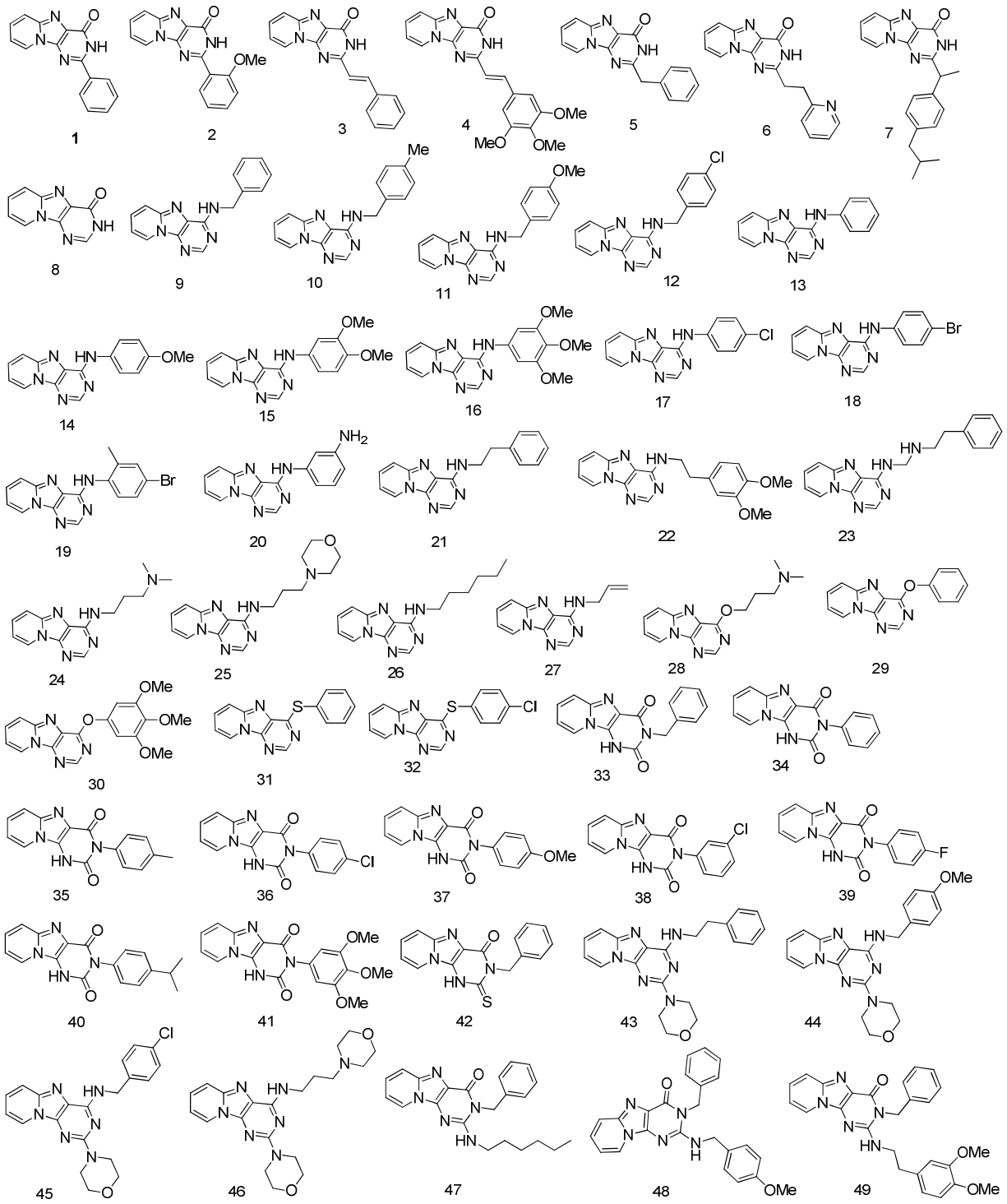


Figure 3: Synthesized pyridine-condensed purinones, adenines and their oxo/thio analogs, xanthines, guanines, and purine-2,4-diamines as potential anticancer agents.

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Table 1 IC_{50} (μM) values for cytotoxicities in HEK 293T and Vero cells

Compounds	IC_{50} (μM) HEK 293T	IC_{50} (μM) Vero	Compounds	IC_{50}(μM) HEK 293T	IC_{50} (μM) Vero
1	49 ± 2.0	60 ±1.9	**28**	8 ± 1.7	40 ± 1.7
2	60 ± 1.5	50 ± 1.0	**29**	2.5 ± 1.1	44 ± 2.0
3	60 ± 1.8	50 ± 1.1	**30**	2.5 ± 0.5	50 ± 1.5
4	29 ± 3.0	55 ± 2.1	**31**	3 ± 1.6	38 ± 2.2
5	25 ± 2.0	49 ± 2.0	**32**	3 ± 1.5	60 ± 1.7
6	30 ± 1.5	55 ± 1.8	**33**	10 ± 2.0	50 ± 2.5
7	28 ±1.7	58 ± 2.5	**34**	4 ± 0.7	56.5 ± 1.0
8	12 ± 1.8	48 ± 1.8	**35**	4 ± 1.8	35 ± 1.5
9	5 ± 2.0	49 ± 1.1	**36**	12 ± 1.3	42 ± 2.0
10	8 ± 1.6	30 ± 1.2	**37**	3 ± 0.5	45 ± 2.2
11	5.2 ± 3.1	28 ± 1.0	**38**	4 ± 2.0	55 ± 1.1
12	7 ± 3.0	29 ± 2.1	**39**	18 ± 2.5	60 ± 2.5
13	6 ± 2.5	25.5 ± 1.0	**40**	12 ± 1.8	58 ± 1.2
14	6.2 ± 2.2	27 ± 1.5	**41**	4 ± 2.1	38 ± 0.7
15	3.0 ± 2.1	29 ± 2.1	**42**	5 ± 2.0	36 ± 0.5
16	6.0 ± 2.0	24.2 ± 0.5	**43**	4 ± 2.2	24 ± 0.2
17	1.5 ± 1.8	29.5 ± 0.8	**44**	4 ± 1.8	18 ± 0.5
18	4 ± 2.0	30 ± 1.1	**45**	6 ± 1.0	24 ± 1.1
19	8 ± 1.9	22 ± 1.2	**46**	2.5 ± 1.5	40 ± 2.0
20	4 ± 2.2	60 ± 1.0	**47**	2.5 ± 0.5	8 ± 2.1
21	5 ± 2.5	38 ± 0.8	**48**	8.5 ± 1.1	14 ± 2.5
22	2.5 ± 1.0	58 ± 0.5	**49**	6 ± 1.0	10 ± 2.0
23	3 ± 1.1	54 ± 1.1	**IV**	8 ± 1.9	44 ± 2.0
24	4 ± 1.7	50 ± 1.2	**V**	10 ± 2.1	60 ± 2.0
25	4 ± 2.0	52 ± 1.0	**VI**	8 ± 1.0	42 ± 2.1
26	5 ± 2.2	25 ± 0.5	**VII**	6 ± 1.1	50 ± 1.1
27	5 ± 1.5	44 ± 0.2	**VIII**	6.5 ± 1.5	60 ± 2.0
Doxorubicin	20 ± 1.1	60 ± 1.1	**Etoposide**	22 ± 1.5	50 ± 1.5

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Biological studies

Cytotoxicity assay:

The cytotoxic activities of the synthesized compounds (**1-49,** Figure 3) were measured by a well-known colorimetric-based MTT assay. A representative cancer cell, kidney cancer cell line (HEK 293T) and its corresponding normal cell (Vero) were considered for testing cytotoxic activities. Doxorubicin (DOX) and etoposide (clinically used anticancer drugs) were used as positive controls. The cells were treated with doxorubicin, etoposide and test compounds for 48 h, each of increasing concentrations, following a protocol described in experimental section. The percent viability was measured. IC_{50} (concentration of compound to effect 50% cell growth inhibition in culture) of all **22**, **23**, **30**, **32**, **34**, **37** and **46** were found to be relatively more active and showed remarkably low IC_{50} (2.5, 3, 2.5, 3, 4, 3 and 2.5 μM, respectively) in HEK 293T as compared to doxorubicin (20 μM) or etoposide (22μM). In Vero cell line these compounds exhibited relatively higher IC_{50} (58, 54, 50, 60, 56.5, 45 and 40 μM, respectively) (Figure 4**).** To further confirm the cytotoxicity, a clonogenic assay was performed according to the protocol described in experimental section. Table 2 demonstrates the LC_{50} (concentration of compound caused 50 percent death of cells) values. For compounds **22**, **23**, **30**, **32**, **34**, **37** and **46**, LC_{50} values were found to be 1.4, 1.1, 1.7, 1.2, 3.4, 1.4 and 2.2 μM respectively. A dose dependent decrease in colony formation was observed (Figure 5).

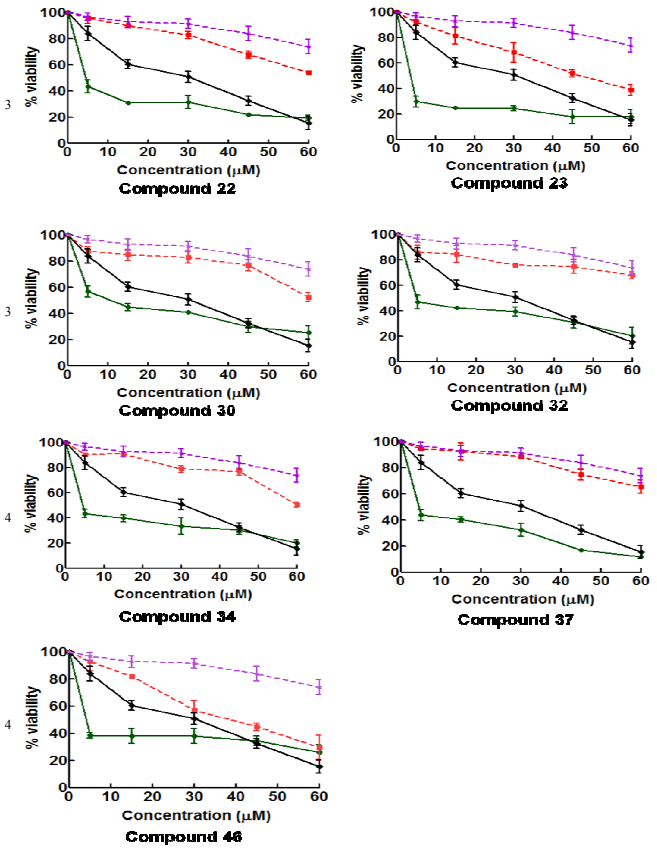

Figure. 4 Cell survivals in MTT assay for the selected compounds 22, 23, 30, 32, 34, 37 and 46. Cells were treated with investigated compounds and doxorubicin of various concentrations for 48h. Symbols , , , represent HEK 293T/compound, Vero/compound, HEK 293T/DOX and Vero/DOX, respectively. Data presented is the mean ± SD of three independent set of experiments

Table 2: LC_{50} (μM) values in clonogenic assay using HEK 293T cells

Compounds	LC_{50} (μM) HEK 293T	Compounds	LC_{50}(μM) HEK 293T
22	1.4 ± 0.5	**34**	3.4 ± 1.5
23	1.1 ± 0.8	**37**	1.4 ± 0.5
30	1.7 ± 1.1	**46**	2.2 ± 1.0
32	1.2 ± 0.2		

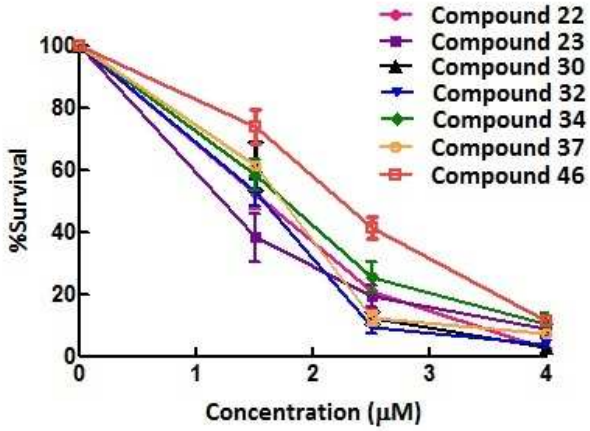

Figure 5: Clonogenic cell survival in HEK 293T cells. , , , , , , Symbols represent compound **22, 23, 30, 32, 34, 37 and 46**, respectively. Data presented is the mean ±SD of three individual set of experiments.

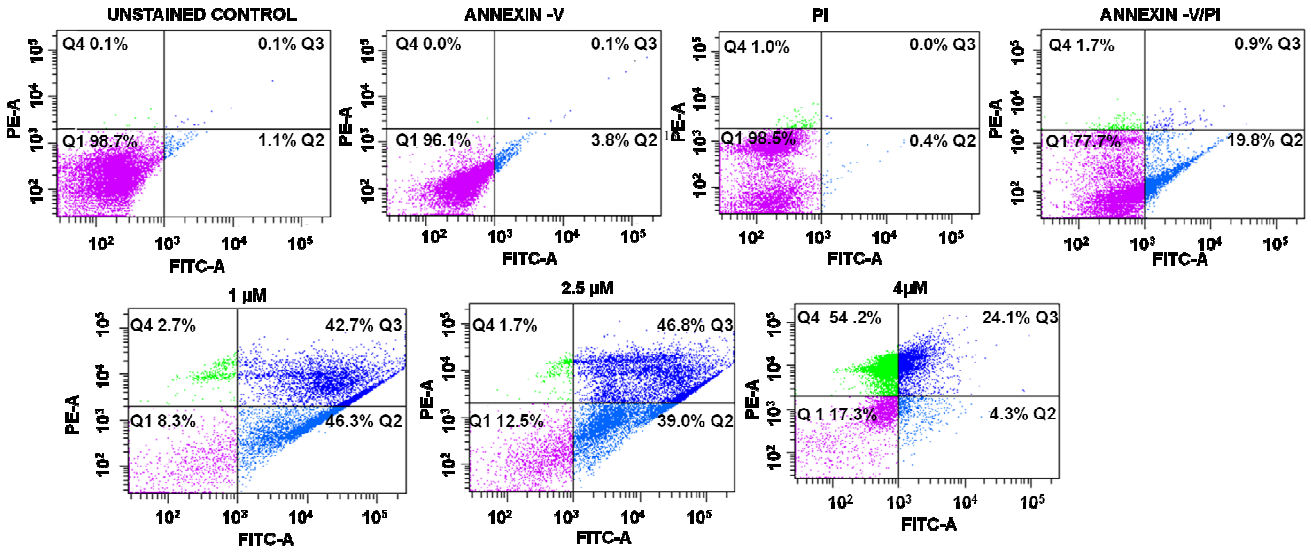

Figure 6: Measurement of apoptosis by Annexin -V/FITC method. Cells were treated with compound 22 of various concentrations for 48 h and experiment was carried out as described in the experimental section. Data given here is the one of three separate experiments.

Measurement of apoptosis in HEK 293 cells by Annexin V/FITC assay:

Apoptosis measurement was done by Annexin -V/FITC staining followed by FACS after treatment with compound 22 for 48 h. Figure 6 demonstrated the distribution of cells in various phases of apoptosis. Approximately 46.8 percent apoptotic cells (Q3) at 2.5 µM and more than 54 percent necrotic cells (Q4) were observed at 4 µM compound treated cells. Thus, the result indicated that cells were moving towards necrotic phase followed by true apoptotic phase with increase in concentrations of compound 22 (Figure 6).

Expressions of CASPASE 3: Increased expression of CASPASE 3, an important marker, is indication of apoptosis. To check whether exposure to compound **22** caused the apoptosis in HEK 293T cells, we measured the CASPASE 3 expression by immunostaining the CASPASE 3 after exposure to compound 22. HEK 293T cells were treated with compound 22 of increasing concentrations and probed with CASPASE-3 antibody (cat #9662 from cell signaling, MA, USA). It was re-probed with secondary anti-rabbit conjugated to TRITC (Tetramethylrhodamine, a red fluorescent dye) followed by counterstained with DAPI and visualized under fluorescence microscope. Increasing expression of CASPASE-3 (red fluorescence) was noted with increasing dose of the compound (Figure 7).

Measurement of apoptosis using DAPI stain:

To further confirm the apoptotic effect of compound **22**, a nuclear staining experiment using HEK 293T cell was performed. Cells were treated with compound **22** of increasing concentrations and fluorescence was observed after staining with DAPI. Significantly higher chromatin condensation and nuclear fragmentation in treated cells compared to untreated cells were observed and these were found to enhance with increasing concentrations of the compound (Figure 8A and 8B). Approximately, 4, 13 and 15 fold increase in apoptotic nuclei in cells treated with compound of 1, 2.5 and 4 μM concentration compared to untreated cells were observed.

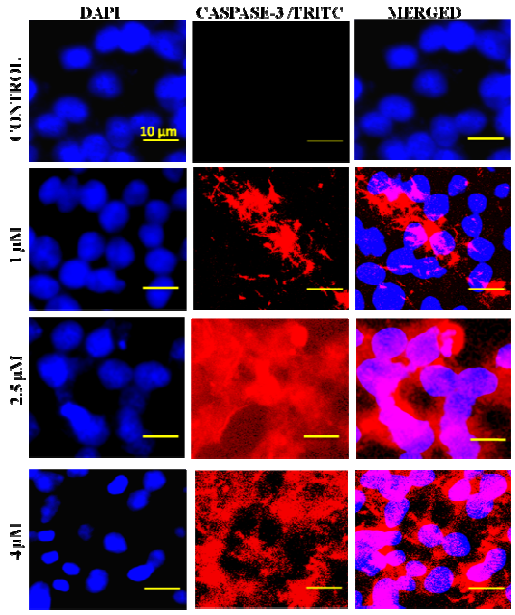

Figure 7: Compound **22** increased the CASPASE 3 expression in HEK 293T cells. Left and middle panel represent the cells stained with DAPI, CASPASE 3 / TRITC (Tetramethylrhodamine, a red fluorescent dye) and the right panel represents the MERGED image of both. Images were taken under florescence microscope, 40X magnification. Images presented here are the best of three individual set of experiments (scale bar 10 µm).

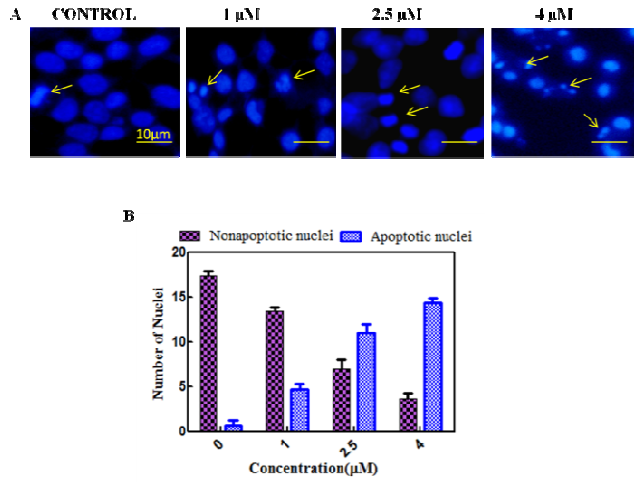

Figure 8: A. DAPI nuclear staining: Images were taken under florescence microscope at 40X magnification (scale bar 10 µm). B. Bar-diagram presentation is the number of apoptotic and non-apoptotic nuclei taken from A.

Experimental Section

Chemistry

General considerations: The starting materials and solvents were used as received from commercial sources without further purification. The ^{1}H and ^{13}C spectra were recorded in $CDCl_3$/DMSO-d_6/CD_3OD solvents on 400 MHz spectrometer using TMS as internal standard. HRMS was measured using TOF analyzer. Melting points determined are uncorrected. Microwave assisted reactions were carried out in sealed reaction vessel using Biotage Initiator.

Ethyl 3-aminoimidazo[1,2-*a*]pyridine-2-carboxylate (**I**), 3-Aminoimidazo[1,2-*a*]pyridine-2-carboxamide (**II**), *N*-benzoylimidazo[1,2-*a*]pyridine-3-amine-2-carboxamide (**IIIa**), Pyrido[1,2-*e*]purin-4(3*H*)-one (**1-7**), Pyrido[1,2-*e*]purin-4(3*H*)-one (**8**), 4-Chloropyrido[1,2-*e*]purine (**IV**), Pyrido[1,2-*e*]purin-4-amines (**9**, **11**, **12**, **14**, **17**, **19**, **20**, **22**, **25**, **27**, **28**, **29**, **31** and **32**) were prepared by our reported procedures.[20] Some more relevant pyridine-fused adenines (**10**, **13**, **15**, **16**, **18**, **21**, **23**, **24**, **26** and **30**) were prepared by following our reported procedures.[20]

Representative experimental procedure for synthesis of 3-Benzylpyrido[1,2-*e*]purine-2,4(1*H*,3*H*)-dione (33): To a solution of ethyl 3-aminoimidazo[1,2-*a*]pyridine-2-carboxylate (**I**, 205 mg, 1 mmol) in anhyd. ethanol (2 mL) taken in a vial, were added subsequently benzyl isocyanate (200 mg, 1.5 mmol) and sodium ethoxide (68 mg, 1mmol) under flow of N_2. Reaction mixture was heated at 120 °C under microwave irradiation. After completion of the reaction (monitored by TLC, 20 min.), solvent was evaporated under vacuum. The column chromatographic purification of crude mass on neutral alumina provided 3-benzylpyrido[1,2-*e*]purine-2,4(1*H*,3*H*)-dione (**33**, 181 mg, 62%). The other compounds (**34-42**, Scheme 2) were prepared following this procedure.

Experimental procedure for synthesis of 3-Benzyl-2-(methylthio)pyrido[1,2-*e*]purin-4(3*H*)-one (VIII, Scheme 2): 3-Benzyl-2-thiopyrido[1,2-*e*]purin-4(1*H*)-one (**42**) was taken in ethanol-water mixture (1:1, 2 mL) in a round bottom flask. Sodium hydroxide (40 mg, 1 mmol) and methyl iodide (142 mg, 1 mmol) were subsequently added to it. Reaction mixture was heated at 60 °C in a closed system. After completion of the reaction (monitored by TLC, 1 h) solvent mixture was evaporated under vacuum. The column chromatographic purification of crude mass on neutral alumina provided 3-benzyl-2-(methylthio)pyrido[1,2-*e*]purin-4(3*H*)-one (**VIII**, 209 mg, 65%).

Representative experimental procedure for synthesis of 3-Benzyl-2-(hexylamino)pyrido[1,2-*e*]purin-4(3*H*)-one (47, Scheme 2): 3-Benzyl-2-(methylthio)pyrido[1,2-*e*]purin-4(3*H*)-one (**VIII**, 322 mg, 1 mmol) was taken in a sealed tube and hexylamine (2 mL) was added to it and the tube was closed. Reaction mixture was heated at 170 °C. After maximum conversion in the reaction (monitored by TLC, 8 h), the column chromatographic purification of crude mass on neutral alumina provided 3-benzyl-2-(hexylamino)pyrido[1,2-*e*]purin-4(3*H*)-one (**47**, 199 mg, 53%).

The products **48** and **49** were prepared following this procedure.

Experimental procedure for the synthesis of pyrido[1,2-*e*]purine-2,4(1*H*,3*H*)-dione (V): To a solution of ethyl 3-aminoimidazo[1,2-*a*]pyridine-2-carboxylate (**I**, 205 mg, 1 mmol) in phenol (2 mL) in a round bottom flask, urea (315 mg, 5 mmol) was added. Reaction mixture was heated at 150 °C. After completion of the reaction (monitored by TLC, 24 h), the column chromatographic purification of crude mass on neutral alumina provided pyrido[1,2-*e*]purine-2,4(1*H*,3*H*)-dione (**V**, 102 mg, 50%).

Experimental procedure for synthesis of 2,4-Dichloropyrido[1,2-*e*]purine (VI): Pyrido[1,2-*e*]purine-2,4(1*H*,3*H*)-dione (**V**, 203 mg, 1 mmol) was taken in a round bottom flask. *N*,*N*-Diethylaniline (149 mg, 1 mmol) and $POCl_3$ (2 mL) were added subsequently to it under flow of N_2 . Reaction mixture was heated at reflux. After reaction completion (monitored by TLC, 4 h), reaction mixture was poured into ice cold water (30 mL). 20% aq. NaOH solution was added dropwise till pH 8. Then the mixture was extracted with EtOAc (2 × 40 mL). The combined organic layer was washed with water, dried with anhyd. Na_2SO_4, and concentrated under vacuum. The column chromatographic purification of crude mass on silica gel provided 2,4-dichloropyrido[1,2-*e*]purine (**VI**, 127 mg, 53%).

Representative experimental procedure for synthesis of 2-Morpholino-*N*-phenethylpyrido[1,2-*e*]purin-4-amine (43, Scheme 2): To a solution of 2,4-dichloropyrido[1,2-*e*]purine (**VI**) in THF (1 mL) in a round bottom flask, phenethylamine (121 mg, 1 mmol) and triethylamine (101 mg, 1 mmol) were added. Reaction mixture was stirred at room temperature (25-27 °C). After completion of the reaction (monitored by TLC, 10 min.), solvent was evaporated under vacuum. To this crude reaction mixture morpholine (1 mL) was added and the mixture was further heated at 120 °C under microwave irradiation. After completion of the reaction (monitored by TLC, 10 min.), the column chromatographic purification of crude mass on neutral alumina provided 2-morpholino-*N*-phenethylpyrido[1,2-*e*]purin-4-amine (**43**, 307 mg, 82%).

The other products (**44-46**, Scheme 2) were prepared following this procedure. 2-Chloro-*N*-phenethylpyrido[1,2-*e*]purin-4-amine (**VII**) was obtained only by 4-amination of compound **VI**. The column chromatographic purification of crude mass on neutral

alumina provided **VII** in 90% isolated yield.

Biology:

Cell line and culture conditions: HEK 293T (cat #CRL-11268) a kidney cancer cell line and Vero, normal kidney cells (Cat. #CCL-81) were grown in DMEM medium supplemented with 10% FBS,1% antibiotic (100 units of penicillin and 10 mg streptomycin per mL in 0.9% normal saline) and maintained in 5% carbon dioxide in humidified condition at 37 °C. All cell culture reagents were purchased from Hi Media, Mumbai, India .

MTT assay: Cell proliferation ability of the investigated compounds (1-49) was measured by performing a colorimetric based assay by using MTT reagent. Approximately 10,000-12,000 cells per well were seeded in a 96 well tissue culture plate and was incubated for 24 h. After 24 h of incubation the cells were exposed to different concentrations of the investigated compounds for 48 h. Then cells were washed with 1X PBS followed by addition of 100 μL of 0.05% MTT reagent to each well. The plate was incubated for overnight at 37 °C. Purple color formazan crystals were formed which was dissolved by using 10% NP-40 with 4 mM HCl. Plate was incubated for 1 h at 37 °C and then the intensity of developed color was measured at 570 nm using a microplate reader (Barthold, Germany). The data was calculated and a graph representing percent viability was plotted in comparison to the control. Data was the mean ± SD of at least three experiments.

Clonogenic assay: Colony forming abilities of the cells after treatment with the investigated compounds were tested by performing clonogenic assay. This assay is done to check the anti-proliferating or colony forming ability of the selected investigated compounds. Approximately 500 cells per well of HEK 293T a kidney cancer cell line was seeded in 12 well plates. The plate was incubated for 24 h. The cells were treated with various concentrations of the selected compounds for 48 h. Then the media was aspirated and replaced with fresh media. The plates were incubated further to allow the cells to grow for 5-6 doublings by changing the media every 72 h. Then media was removed and cells were washed with 1X PBS followed by addition of 0.2% crystal violet stain to each well of the plate. It was kept for 1 h and the excess stain was removed by washing with 1X PBS. The plates were air dried and the colonies were counted under a gel documentation system (UVP, Germany). The colonies formed in the treated wells were counted against the untreated well that served as control (100% survival) and a graph was plotted representing the percent survival of the cells. Data presented here is the mean ±SD of three different experiments.

Annexin -V/FITC Staining:

Annexin -V/FITC staining is used to detect the distribution of cells in early, apoptotic and late apoptotic (necrosis) phase. For this assay cells were seeded in a 6 well cell culture plate in a density of $1x10^5$ cells per well. 70-80 Percent confluent grown cells were treated with varying concentrations of compound 22 for 48h. Then cells were harvested, washed and stained with Annexin -V/FITC and PI according to manufacturer protocol (Sigma). Finally, stained cells were sorted by FACS using PE-A (capture the PI stained cells) and FITC-A (capture the Annexin – V stained cells) channel. According to principle, Annexin-V dye stains living and apoptotic cells, whereas PI stains all cells including necrotic cells. The percentage of cells population in Q1, Q2, Q3 and Q4 represent the normal, early apoptotic, apoptotic and necrotic cells, respectively.

Immunofluorescence of CASPASE 3: Immunofluorescence of CASPASE 3 was done to check the expression of CASPASE 3 after treatment of the cells with compound 22. HEK 293T cells ($1x10^4$) were seeded on sterile coverslips and allowed to attain 50-60% confluency. The cells were treated with various concentrations of the drug for 48 h. Drug treated media was removed and the cells were washed with 1X PBS. It was then fixed with acetone-methanol (1:1) for 15 min in -20 °C. The fixative solution was removed and the cells were blocked for 30 min in 37 °C with 2% BSA in 0.02% triton X 100 in 1X PBS. Cells were stained with CASPASE 3 antibody (cat #9662 from cell signaling, MA, USA) and incubated for 3 h. Cells were washed with 1X PBS followed by immunostaining with secondary antibody (anti-rabbit) conjugated with TRITC and incubated for 1 h in 37 °C. After completion of incubation time cells were washed with 1X PBS and stained with DAPI. Cover slips containing cells were washed properly with 1X PBS, air dried and mounted on slides. Images were taken using florescence microscope.

DAPI nuclear staining assay: This assay helps in detection of apoptosis and nuclear fragmentation. HEK 293T cells were seeded in a 96 well tissue culture plate (1x104/well) and incubated for 24 h. Cells were treated with various concentrations of compound 22 and incubated for another 48 h. Then plates were washed with 1X PBS and fixed with acetone-methanol (1:1) and then kept in -20 °C for 15 min. After removing the fixative the cells were washed with 1X PBS and stained with 4,6-diamidino-2-phenylindole (DAPI). It was again incubated for another 30 min in dark in 37 °C. After end of the incubated times slides were once washed with 1X PBS and the visualized under fluorescence microscope. Data provided here is the one of three independent experiments.

Conclusions

A set of novel pyridine/pyrazine annulated purinones, adenines and their oxo/thio analogs, xanthines, guanines, and purine-2,4-diamines as potential anticancer agents were considered based on scaffold-hopping and hybridization of known drugs and bioactive agents. They, with relevant substitutions, were synthesized conveniently via a building block strategy with 3-amino-2-carboxyethyl substituted imidazo[1,2-*a*]-pyridine as building block. Their MTT-cytotoxicity and clonogenic cell survival studies revealed several of pyridine-annulated purines significantly active in anticancer properties and relatively less cytotoxic to normal cells. They were found to be relatively more potent than anticancer drugs, etoposide and doxorubicin. A representative compound (**22**) exerted significant apoptotsis. The present work has potential application in the synthesis of annulated purine class of compounds via building block strategy and will incite for investigation towards exploration of bioactive heterocyclic-annulated purines. Further optimization of developed new anticancer agents is being underway.

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Notes and references

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Electronic supplementary information (ESI) available: General Information, Spectral data, and NMR Spectra (^{1}H and ^{13}C) of compounds

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