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Urease inhibition and anticancer activity of novel polyfunctional 5,6-dihydropyridine derivatives and their structure-activity relationship

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ABSTRACT

A novel series of tricyano substituted polyfunctional 5,6-dihydropyridine **8a-n** bearing functionalized aromatic rings at C-4 and C-6 position have been prepared from (α-methylbenzylidene) malononitriles in good to excellent yields (52-98%) in solvent free conditions. All the synthesized compounds (**8a-n**) were evaluated for their *in vitro* urease inhibition and anticancer activity against prostate cancer (PC3) and Hela cell lines. Compound **8k** (4,6-*bis*(4-methoxyphenyl)-5,6-dihydropyridin) showed slightly better urease inhibitory potential (IC_{50} = 20.47 μM) as compared to standard thiourea (IC_{50} = 21 μM). Whilst in the case of anticancer studies the compound **8a** 2-(4,6-*bis*(4-bromophenyl)-6-methyl-5,6-dihydropyridin found to be most active (IC_{50} = 4.40 and 8.80 μM) among the series when compared with standard doxorubicin 4 (IC_{50} = 0.91 and 3.1 μM) in both cell lines respectively. A structure-activity relationship of this series has been established on the basis of electronic effects and position of different substituents (H, Br, Cl, I, F, Me, OMe, OH, and NO_2) present on the C-4 and C-6 phenyl rings. The anticancer activity evaluation of these pyridine derivatives envisage that the compound **8a** could be putatively linked with doxorubicin IV to developed new anticancer prodrugs for multidrug resistant (MDR) cancer cells. All the synthesized compounds were characterized by spectroscopic techniques.

1. Introduction

Pyridine derivatives are important class of *N*-heterocyclic compounds exhibiting a wide range of biological activities, e.g. antifungal [1], antibacterial [2] and anticancer [3], *etc*.. In this perspective, a synthesized series 5,6-dihydropyridine derivatives **8a-n** were evaluated as urease inhibitor and anticancer agents [4]. The development of urease inhibitors, usually considered as antiulcer agents, carry a significant interest for medicinal chemists. Urease is an enzyme that is clinically used as diagnostic to determine the presence of pathogens in the gastrointestinal and urinary tracts. It has been described that the bacterial urease causes many clinically harmful infections, like stomach cancer, infectious stones and peptic ulcer formation in human and animal health [5]. Urease is also involved in the other pathogenesis like hepatic come, urolithiasis, urinary catheter encrustation and oral cavity infections by hydrolyzing the salivary urea [6]. Cancer, an uncontrolled aggregation of abnormal cells is one of the prominent cause of human mortality in the world. Currently available chemotherapies i.e. doxorubicin IV and related compounds etc. encounter some major issues such as cardiotoxicity [7] and multidrug-resistant. Multiple drug resistant (MDR) mainly occurs due to the over expression of P-glycoprotein (multidrug resistance protein) efflux pump which extrude the drugs from the cell [8-10]. Toyota *et. al*. [11] described that the multiple resistant cells accelerate the membrane transports, especially the excretion process which lower the drug concentration in the cells. They prepared 1,4-dihydropyridine, calcium channel antagonist, (Structure 1, Figure 1) derivatives which were used in combination with commercially available drugs e.g. doxorubicin IV etc. to increase the anticancer drug concentration in cancel cells by inhibiting extracellular excretion process of cell. Abadi A. H. *et al*. [12] reported compound **2** as inhibitor of oncogene serine/threonine kinase PIM-I, an enzyme over expressed in many cancer cells line. In the view of these antitumor investigations in drug resistant cancer cells, we have screened our polyfunctional dihydropydridine compounds (Structure 3) for anticancer activity (Figure 1).

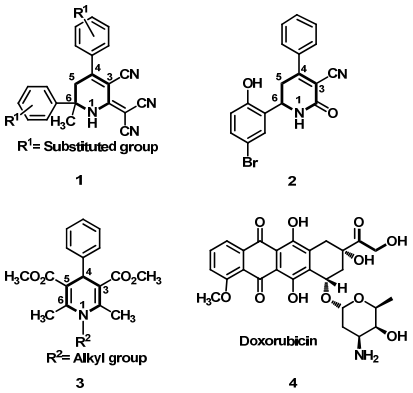

Figure 1. 5,6-Dihydropyridine, **1**, and its related compounds.

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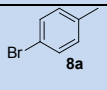
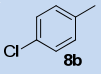
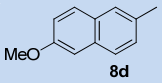
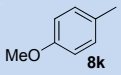
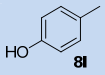
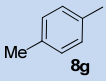
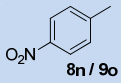
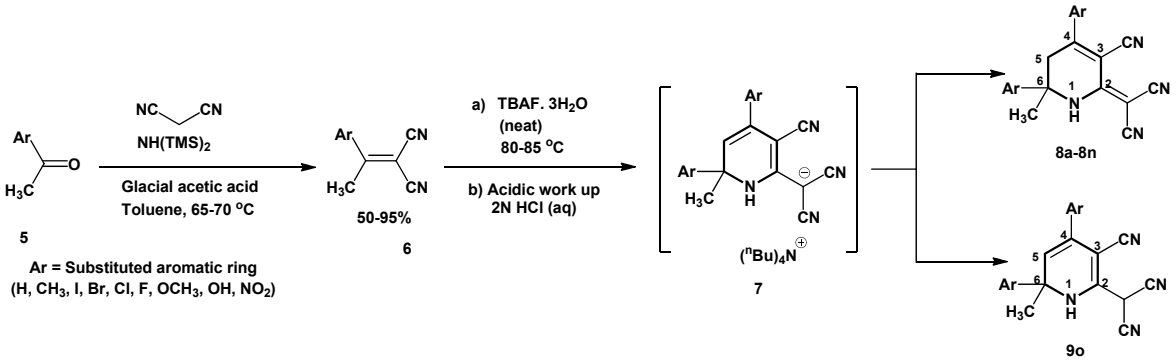
Table 1. Different substituted phenyl groups on core dihydropyridine ring.

Entry	Aryl group (Ar-)	Yield (%)	Entry	Aryl group (Ar-)	Yield (%)
1	 8a	90	8	 8h	80
2	 8b	90	9	 8i	75
3	 8c	98	10	 8j	75
4	 8d	65	11	 8k	95
5	 8e	89	12	 8l	75
6	 8f	90	13	 8m	93
7	 8g	95	14	 8n / 9o	52 (2:1 ratio)



Scheme 1

To pursue the interest, we have developed a solvent-free, cost-effective and scalable method for the preparation of desired compounds **8a-n** (Scheme 1) [4]. The highly functionalized 5,6-dihydropyridine derivatives **8a-n** were synthesized from corresponding alkylidenemalononitrile **6** by dimerization in good to excellent yield (52-95%). The reaction was promoted with neat tetrabutylammonium fluoride trihydrate (TBAF.3H_2O) under solvent free conditions to develop an ecofriendly methodology. The final compounds (**8a-n**) were evaluated for antiurease and anticancer activities. The results showed a wide range of both activities depending upon the substituents present on the phenyl rings.

2. Experimental

2.1. General procedure for 5,6-dihydropyridine derivatives synthesis (8a-n)

To an oven dried screw-capped vial, corresponding alkylidenemalononitrile (1.0 equiv) and neat TBAF.3H_2O (1.0 equiv) was added along with magnetic stirrer bar. The resulting mixture was heated at 85-90 °C for 15 h. Then, the reaction mixture was cooled to room temperature and further diluted with dioxane (2-3 mL). 1.0 M HCl (100 mL) aqueous solution was then added to remove excess TBAF reagent. The resulting suspension was stirred for additional 5 min for complete precipitation. The precipitate was filtered and washed with more 1.0 M HCl to give the pure compounds **8a-n** good to excellent yield (52-95%). The structure of all dihydropyridine derivatives (Table 1) was confirmed with different spectroscopic techniques including ^{1}H NMR, ^{13}C NMR, EI-HRMS and IR spectroscopy [4]. All the synthesized compounds were then subject to biological evaluation.

2.2. Urease Inhibition assay (In vitro)

The mixture consists of the solution of 25 µL enzyme (Jack bean urease) and 55 µL of buffers having 100 mM urea. It was then incubated with 5 µL of test compounds (0.5 mM concentration) at 30 °C for 15 min in 96-well plates. The urease inhibitory activity was determined by determining ammonia production by utilizing indophenol method [13]. Concisely, to each well 45 µL each phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprussside) and 70 µL of alkali reagent (0.5% w/v NaOH and 0.1% active chloride NaOCl) were added.

The increasing absorbance at 630 nm was measured after 50 min, using a microplate reader (Molecular Device, USA). All the reactions were performed in triplicate in a final volume of 200 µL. The results (change in absorbance per min) were processed by using softMax Pro software (molecular Device, USA). The pH was maintained 6.8 in all assays. Percentage inhibitions were calculated from the formula 100-(OD test well/OD control)×100. Thiourea was used as the standard inhibitor of urease (Table 2).

Table 2. *In vitro* urease activity of compounds **8a-n**.

Entry	Compounds	IC_{50} (μM ± SEM [a])
1	8a	NA [b]
2	8b	NA [b]
3	8c	NA [b]
4	8d	NA [b]
5	8e	NA [b]
6	8f	NA [b]
7	8g	244.66 ± 9.37
8	8h	NA [b]
9	8i	NA [b]
10	8j	NA [b]
11	8k	20.48 ± 0.66
12	8l	84.87 ± 6.99
13	8m	NA [b]
14	8n/9o	NA [b]
Thiourea [c]		21.0 ± 0.01

[a] SEM is the standard error of the mean.
[b] NA: Not active.
[c] Thiourea.

2.3. Anticancer activity assay (In vitro)

The anticancer activity of all the compounds in the series (**8a-n**) was estimated in 96-well flat-bottomed micro plates by using the standard MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide) colorimetric assay [14]. Cells were cultured in Dulbecco's Modified Eagle Medium, supplemented with 5% of fetal bovine serum (FBS), 100 IU/mL of penicillin and 100 µg/mL of streptomycin in 75 cm^2 flasks, and kept in 5% CO_2 incubator at 37 °C. The rapidly growing cells were collected, counted with haemocytometer and diluted with a particular medium. Cell cultures ($1x10^5$ cells/mL) was prepared and introduced (100 µL/well) into 96-well plates. The medium was removed after overnight incubation and 200 µL of fresh medium was added with different concentrations of compounds (5-50 µM). After 48 h, 200 µL MTT (0.5 mg/mL) was added to each well and incubated further for 4 h. Consequently, 100 µL of DMSO was added to each well. The reduction in MTT to formazan within cells was calculated by measuring the absorbance at 570 nm, using a micro plate reader (Spectra Max plus, Molecular Devices, CA, USA). The cytotoxicity was recorded as concentration causing 50% growth inhibition (IC_{50}) for cell lines. The percent inhibition was calculated by using the following formula: % inhibition = 100-((mean of O.D of test compound – mean of O.D of negative control)/ (mean of O.D of positive control-mean of O.D of negative control)*100). Doxorubicin was used as the standard anticancer drugs.

3. Results and discussion

The synthesis of polyfunctional 5,6-dihydropyridine derivatives **8a-n** have been shown in the Scheme 1. The structure of these compounds **8a-n** was confirmed by using different spectroscopic techniques [4]. ^{1}H-NMR spectra of the synthesized dimers **8a-n** showed two distinctive doublets at 3.49 ppm and 3.15 ppm for C-5 methylene (-CH_2-) group protons.

The proton signals for other groups such as methyl, methoxy and hydroxy group of new derivatives were also observed within the range. Infrared spectroscopy of phenyl derivative **8a** showed absorption bands for characteristic NH group at 3448 cm^{-1} of dihydropyridine ring. Characteristic absorption bands for bromo, chloro, iodo, methyl, methoxy, hydroxyl and nitro group of the corresponding synthesized compounds **8a-n** were observed on infrared spectrum.

All synthetic polyfunctional 5,6-dihydropyridine derivatives **8a-n** (Table 1) were screened for their urease inhibitory potential [12] (Table 2) and anticancer activity against prostate cancer (PC3) and Hela cell lines according to literature protocol [14] (Table 3). The urease inhibitory analysis showed that most of the dihydropyridine derivatives are inactive in the series. However, the compound **8k** (IC_{50} = 20.4 ± 0.66 µM) showed comparable urease inhibitory potential, while compounds **8l** (IC_{50} = 84.8 ± 6.99 µM), demonstrated moderate and compounds **8g** (IC_{50} = 244.6 ± 9.37 µM) weak inhibition potential respectively.

Table 3. *In vitro* anticancer activity of compounds **8a-n** against PC3 and Hela Cell lines.

Entry	Compounds	PC3 Cell line	Hela Cell line
		IC_{50} (μM ± SEM [a])	IC_{50} (μM ± SEM [a])
1	8a	4.40 ± 0.13	8.81 ± 0.25
2	8b	6.85 ± 0.34	8.81 ± 0.63
3	8c	8.03 ± 0.22	23.06 ± 0.10
4	8d	8.36 ± 0.48	14.67 ± 0.75
5	8e	8.87 ± 0.59	9.67 ± 0.20
6	8f	9.62 ± 0.55	11.26 ± 0.85
7	8g	10.48 ± 0.24	18.84 ± 0.87
8	8h	11.51 ± 0.45	20.18 ± 0.39
9	8i	13.10 ± 0.62	23.09 ± 0.03
10	8j	14.02 ± 0.42	26.41 ± 1.97
11	8k	14.55 ± 0.38	14.73 ± 0.14
12	8l	> 30	> 30
13	8m	> 30	> 30
14	8n/9o	> 30	> 30
Doxorubicin [b]	-	0.91 ± 0.12	3.1 ± 0.20

[a] SEM is the standard error of the mean.
[b] Doxorubicin standard anticancer drug for many type of cancer cell lines.

Compound **8k** (4,6-dimethoxy-5,6-dihydropyridine derivative) demonstrated excellent inhibitory potential among the series with IC50 values 20.4 ± 0.66. While compound **8l** (4,6-dihydoxy-5,6-dihydropyridine derivative) having an IC_{50} value 84.8 ± 6.99 µM showed moderate activity. The activity difference between these analogs suggests that the electronic effects and substituents position on phenyl rings affect the urease inhibitory activity. The 4,6-dimethyl-5,6-dihydro-pyridine derivative **8g** with also IC_{50} value 244.66 ± 0.01 µM exhibit week inhibition due to electron denoting effects. In this series, it has been observed that most of the compounds are inactive due to electron withdrawing effect of substituents and position of electron denoting groups in the series **8a-n**. Thus, it has been concluded that position and electronic effects of functional group on phenyl ring significantly affect the urease inhibitory activity. This study identifies compound **8k** as lead compound for further development.

We have also explored our dihydropyridine series (**8a-n**) against prostate cancer cell line and Hela cell line. The anticancer activity results on both cell lines showed that most of the compounds **8a-n** are active and have a varying degree of anticancer potential having IC_{50} values ranging between 4.40 ± 0.12 and 8.80 ± 0.25 to >30 µM, if compared with standard doxorubicin (IC_{50} value 0.91 ± 0.12 and 3.1 ± 0.20 µM). Compounds **8a** (IC_{50} = 4.40 ± 0.13 and 8.80 ± 0.25 µM), **8b** (IC_{50} = 6.85 ± 0.34 and 8.81 ± 0.63 µM) showed highest cytoxicity among the series in both cell lines as compared to doxorubicin. The compounds **8c** (IC_{50} = 8.03 ± 0.22 µM), **8d** (IC_{50} = 8.36 ± 0.48 µM), **8e** (IC_{50} = 8.87 ± 0.59 µM), **8f** (IC_{50} = 9.62 ± 0.55 µM) and **8g** (IC_{50} = 10.48 ± 0.240 µM), **8h** (IC_{50} = 11.51 ± 0.45 µM), **8i** (IC_{50} = 13.1 ± 0.62 µM), **8j** (IC_{50} = 14.0 ± 0.42 µM) and **8k** (IC_{50} = 14.5 ± 0.38 µM) demonstrated moderate cytoxicity, while compounds **8i**, **8j** and mixture of compounds **8n/9o** (IC_{50} > 30) exhibited a weak activity towards PC3 Cells line. However, in the case of hela cell line the compounds **8e** (IC_{50} = 9.67 ± 0.20 µM), **8f** (IC_{50} = 11.26 ± 0.85 µM), **8k** (IC_{50} = 14.73 ±

0.14 μM), **8d** (IC_{50} = 14.67 ± 0.75 μM) demonstrated moderate cytoxicity, while the compound, **8c**, **8g**, **8h**, **8i**, **8j**, **8k**, **8l, 8m**, **8n/9o** exhibit very low anticancer activity (IC_{50} = 20.18 ± 0.14 to > 30 μM) against hela cell line (Table 3).

The structure activity relationship showed that varying in the anticancer activity in both cell lines again depends upon position and nature of the substituents on the phenyl ring. It has been found that the 4-bromo derivative **8a** (IC_{50} = 4.40 ± 0.13 μM and 8.80 ± 0.25 μM) and 4-chloro derivative **8b** (IC_{50} = 6.85 ± 0.34 μM and 8.81 ± 0.63 μM) are most cytotoxic compounds in both cell lines when compared with doxorubicin value (IC_{50} = 0.91 ± 0.12 μM and 3.1 ± 0.20 μM).

The higher cytoxicity of these compounds **8a** and **8b**, as compared to other compounds in the series (**8a**-**n**), can be explained on the basis of electronic effect and leaving group ability of substituents on the aromatic ring. The derivatives **8a** and **8b** bears electron withdrawing substituents at 4-position of the aromatic ring, so if this is only factor of cytotoxicity then 4-nitro **8n/9o** (IC_{50} > 30 μM) would be most active than the former derivatives in the activity Table 2. The lower activity of this compound can be explained on the basis of poor leaving group ability of nitro group as compared to bromo and chloro group. The position of the substituent is also play a significant role in the anticancer activity. The activity of 3-bromo derivative **8f** (IC_{50} = 9.62 ± 0.55 and 11.26 ± 0.85 μM) is lower than activity of 4-bromo derivative **8a** (IC_{50} = 4.40 ± 0.13 and 8.80 ± 0.25 μM), whilst in the case of 3-methoxy derivative **8h** (IC_{50} = 11.51 ± 0.45 and 20.19 ± 0.39 μM) activity is slightly higher than 4-methoxy derivative **8k** (IC_{50} = 14.55 ± 0.38 and 14.73 ± 0.14 μM). An addition of phenyl ring i.e. 6-methoxynaphthalene dihydropyridine derivative **8d** also showed significant sensitivity (IC_{50} = 8.36 ± 0.48 and 14.67 ± 0.75 μM) towards PC3 cell line and Hela cell line respectively. A future study on such naphthalene derivatives will carry out to explore their anticancer potential. These anticancer results concluded that electron withdrawing group as well as their position on the phenyl ring significantly enhanced the anticancer activity of compounds. This study identifies compounds **8a** and **8b** which may serve as lead compounds for the further research towards to new therapeutic agents or linked with available drugs to increase their cytotoxic effects in cancer resistance cells.

4. Conclusion

A series of 5,6-dihydropyridine derivatives have been prepared under solvent free conditions in good to excellent yields (52-95%). This series has been tested for urease inhibitory activity and anticancer activity against PC3 and Hela cell lines. The results of these biological assays have been present in Table 2 and 3. The compound **8k** was found to be most active ureases inhibitor among the series which could potentially be used as lead for further development of drug. The anticancer assays explored that compounds **8a** and **8b** are most active cytotoxic agents in the series. These compounds could be possibly linked with doxorubicin chemically to overcome drug resistance offered by over expression of P-glycoprotein in cancer cells. These compounds **8a** and **8b** can also be used as lead compounds for further development of drugs.

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