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Research Article

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In vitro Anticancer Activity of Pyrazole Fused Triazole Hybrids as Schiff and Mannich Bases through MTT Assay and Flow Cytometry

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ABSTRACT

A novel series of substituted 4-({[3-Substituted-1H-pyrazol-4-yl] methylidene}amino)-5-[(substituted) methyl]-1,2,4-triazole-3-thiols (Schiff bases) (3a-l) , 4-({[3-Substituted-1H-pyrazol-4-yl] methylidene}amino)-2-[(diphenylamino)-methyl-5-[(substituted) methyl]-1,2,4-triazole-3-thiones (Mannich bases) (4a-e) and 4-({[3-(4- Substituted-1H-pyrazol-4-yl] methylidene}amino)-2-(morpholin-4-yl-methyl)-5-{(4-methylphenoxy) methyl}-1,2,4-triazole-3-thione (Mannich bases) (5a-e) were evaluated for their liver cancer inhibitory activity. Meanwhile, the activity of compounds containing 1,2,4-triazoles was higher than that of reference compounds. Out of these all, compound 3e showed the most notable anticancer activity against liver cancer cell lines with IC50 (µg/mL). The hepatocellular carcinoma cells, HepG2 were used for assessing the anticancer potential of Schiff and Mannich bases. Few of the newly synthesized Schiff and Mannich bases were screened for their anticancer activities. Thirteen compounds were selected for anticancer assay: eight Schiff bases and five Mannich bases. Among the five Mannich bases, two were diphenylamine Derivatives and the remaining three were morpholine derivatives. Doxorubicin is used as the standard drug to find out the chemoselectivity of Cancer Cells. In MTT assay the Schiff bases were found to be more active than Mannich bases. MTT assay of Schiff bases (Mean Values). Minimum dose required to reduce 50% of the Cell population of replication pool3e in Hep G2 cell showed a dose-dependent reduced. The treatment for 3a, 3d, 3g, and 3h did not show a dose-dependent reduction in cell proliferation when labeled with titrated thymidine. MTT assay of Mannich bases 5b was founded to be the most potent. Flow cytometry is a laser or impedance-based biophysical technology through hydrodynamic focusing employed in cell counting, cell storage, and protein engineering. Flow cytometry is used in the diagenesis of health disorders like blood cancer, research, and clinical trials. Flow cytometry graph of 4-({[3-(4-fluorophenyl)-1H-pyrazol-4-yl] methylidene}amino)-5[(2methylphenoxy) methyl]-1,2,4-triazole-3-thiol (3e).

Keywords: 1,2,3-triazoles; Anticancer activity; MTT assay; Flow cytometry

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INTRODUCTION

Human liver carcinoma is the fifth most common cancer in the world and accounts for more than six lakhs of deaths yearly. The majority of patients diagnosed with hepatocellular carcinoma die within one year. Presently, the treatment mainly includes surgery and chemotherapy, but the curative effects of the existing chemotherapeutic drugs are not good enough and they have s side effects. Our understanding of the biological processes which govern carcinogenesis is growing rapidly and provides the basis for identifying novel cellular targets for anticancer drug development. Therefore, searching for highly efficient anti-tumor drug remains a hot research area. Schiff base, named after Hugo Schiff, is a compound that contains the -C=N-(azomethine) group, synthesized by the condensation of primary amines and active carbonyl groups. The reaction to Schiff bases and secondary amine in presence of catalytic amount of formaldehyde produces the corresponding Mannich base. Schiff bases and Mannich bases have attracted much interest in the development of pharmacologically active compounds. They are well

known for their biological activity as antibacterial, antifungal, anticancer and antiviral agents [1,2]. Schiff bases appear to be important intermediates in a number of enzymatic reactions involving an interaction of the enzyme with an amino or a carbonyl group of the substrate. Schiff bases derived from aromatic amines and aromatic aldehydes have a great utility in important fields such as medicine, agriculture, cosmetic products and a wide variety of applications in inorganic and analytical chemistry [3]. Some Schiff bases bearing aryl groups or heterocyclic residues like triazole and pyrazole possess excellent biological activities. Triazoles constitute an important class of nitrogen heterocyclic, which display an ample spectrum of biological activities and are widely employed as pharmaceuticals and agrochemicals. 1,2,3-triazoles play a key role in many bioactive molecules/drugs and are bioisosteres of amide bonds due to structural and electronic similarity and are more stable against metabolic degradation [4,5]. Lung cancer is among the most common cancers and has resulted in the highest mortality rate in the world [6].

EXPERIMENTAL SECTION

Materials and Methods (Figures 1-3 and Table 1)

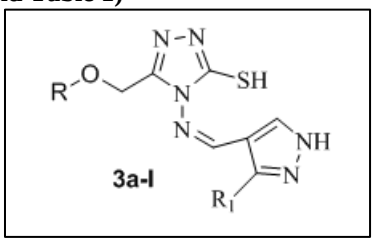

Figure 1: 4-({[3-Substituted-1H-pyrazol-4-yl] methylidene}amino)-5-[(substituted)methyl]-1,2,4-triazole-3-thiols (Schiff bases) (3a-l)

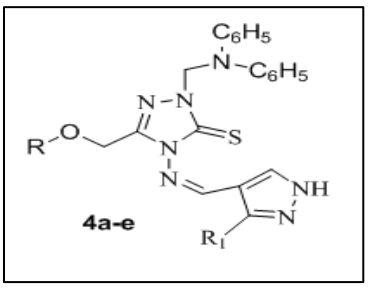

Figure 2: 4-({[3-Substituted-1*H*-pyrazol-4-yl] methylidene}amino)-2-[(diphenylamino)-methyl-5-[(substituted)methyl]-1,2,4-triazole-3-thiones (Mannich bases) (4a-e)

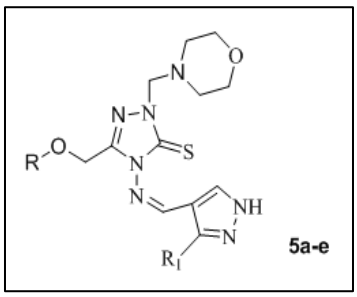

Figure 3: 4-({[3-(4- Substituted-1H-pyrazol-4-yl] methylidene}amino)-2-(morpholin-4-yl-methyl)-5-{(4-methylphenoxy)methyl}-1,2,4-triazole-3-thione(Mannich bases) (5a-e)

In vitro Anticancer Studies

The hepatocellular carcinoma cells, HepG2 were used for assessing the anticancer potential of Schiff and Mannich bases. Few of the newly synthesized Schiff and Mannich bases were screened for their anticancer activities at CDRI, Lucknow, India. Thirteen compounds were selected for anticancer assay: eight Schiff bases and five Mannich bases. Among the five Mannich bases, two were diphenylamine derivatives and the remaining three were morpholine derivatives. Doxorubicin is used as the standard anti-cancer drug.

Table 1: List of substituent's from Schiff bases (3a-l) and Mannich bases (4a-e) and (5a-e)

Entry	R	R_1	Entry	R	R_1
3a	C_6H_5	4-ClC_6H_4	**4a,5a**	4-$CH_3C_6H_4$	4-ClC_6H_4
3b	C_6H_5	4-FC_6H_4	**4b,5b**	4-$CH_3C_6H_4$	4-FC_6H_4
3c	C_6H_5	C_2H_5	**4c,5c**	2-$CH_3C_6H_4$	4-FC_6H_4
3d	2-$CH_3C_6H_4$	4-ClC_6H_4	**4d, 5d**	2-$CH_3C_6H_4$	4-ClC_6H_4
3e	2-$CH_3C_6H_4$	4-FC_6H_4	**4e,5e**	C_6H_5	4-ClC_6H_4
3f	2-$CH_3C_6H_4$	C_2H_5			
3g	4-$CH_3C_6H_4$	4-ClC_6H_4			
3h	4-$CH_3C_6H_4$	4-FC_6H_4			
3i	4-$CH_3C_6H_4$	C_2H_5			
3j	2-ClC_6H_4	4-ClC_6H_4			
3k	2-ClC_6H_4	4-FC_6H_4			
3l	2-ClC_6H_4	C_2H_5			

MTT assay [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt] assay:

In vitro anti-cancer activity of the test, compounds were tested using MTT assay [7-11] as per ATCC protoCol For each test sample, the IC50 value was determined from the dose-response curves. The assay was performed in triplicate for each of the test samples and mean IC50 values were calculated. IC50 is nothing but minimum dose required to reduce 50% cell population in replication pool.

In MTT assay the Schiff bases were found to be more active than Mannich bases. The % survival of HepG2 cells on the treatment of Schiff bases and Mannich bases and initially incubated for 3-4 hr at 37°C. They were given in Tables 2 and 3 respectively. It is used to find out the chemoselectivity of cancer cells.

Cytotoxic effect of Schiff bases:

Interestingly, Schiff bases 3a, 3d, 3e, 3g and 3h containing fluoro and chloro substituents showed dose-dependent cytotoxic activity in MTT assay with low IC50 values. 3i which had ethyl substituent was found to be the least active among all the Schiff bases.

The replacement of aromatic phenyl group connected to pyrazole ring by ethyl substituent must have been the cause for the reduced activity. The IC50 value of the standard drug, Doxorubicin was 21 µg/ mL. The Mannich bases 5a, 5b, and 5d which were derived from morpholine showed better activity compared to that derived from diphenylamine. Among the Mannich bases, 5b was the most potent. Since above study suggested that the Schiff bases affected the cell viability, it was interesting to know whether they could inhibit cell division.

[^{3}H]-Thymidine incorporation assay:

3a, 3d, 3e, 3g, and 3h were taken for the [3H]-Thymidine incorporation assay. Treatment of 3e in HepG2 cells showed a dose-dependent reduced in replication pool. However, it was also possible that in addition to its effect on cell division, 3e could also induce apoptosis.

The treatment of 3a, 3d, 3g, and 3h did not show a dose-dependent reduction in cell proliferation when labeled with titrated thymidine. The graphs representing the results of mean values of different concentrations were obtained in the thymidine incorporation assay are given in Figure 4.

Table 2: MTT assay of Schiff bases

Comp No.	Vehicle Control (Doxorubicin)	0.1 µg/mL	1 µg/mL	10 µg/mL	100 µg/mL	IC_{50} µg/mL
3a	80.75 ± 2.04	90.92 ± 0.69	74.33 ± 2.39	51.64 ± 2.30	36.46 ± 1.09	18
3b	80.75 ± 1.94	82.94 ± 2.45	88.26 ± 4.07	92.00 ± 1.06	30.04 ± 2.34	71
3d	94.64 ± 1.06	86.27 ± 3.24	76.92 ± 2.45	67.57 ± 2.21	23.57 ± 1.76	44
3e	78.07 ± 2.12	71.18 ± 0.96	85.38 ± 1.36	77.03 ± 1.96	13.15 ± 0.88	48
3g	80.75 ± 4.03	66.82 ± 2.09	69.48 ± 2.20	61.03 ± 2.45	36.46 ± 1.09	28
3h	80.75 ± 0.36	77.15 ± 2.13	77.15 ± 1.07	66.51 ± 3.05	24.88 ± 1.87	45
3i	78.07 ± 1.89	99.60 ± 0.89	99.00 ± 0.57	93.94 ± 2.46	75.78 ± 5.08	>100
3k	78.07 ± 2.09	99.00 ± 1.85	98.00 ± 1.01	97.00 ± 1.98	28.18 ± 1.01	71

Table 3: MTT assay of Mannich bases

Comp No.	Vehicle Control (Doxorubicin)	0.1 µg/mL	1 µg/mL	10 µg/mL	100 µg/mL	IC_{50} µg/mL
4a	95.31 ± 0.56	92.60 ± 1.32	89.64 ± 3.29	85.00 ± 3.98	49.21 ± 1.57	97
4b	95.31 ± 3.92	97.53 ± 3.40	96.35 ± 4.51	84.61 ± 2.21	45.71 ± 3.28	90
5a	95.31 ± 3.09	94.82 ± 2.10	90.58 ± 2.03	80.25 ± 1.38	18.88 ± 2.01	54
5b	94.64 ± 2.54	81.90 ± 1.89	76.84 ± 1.57	66.44 ± 2.76	5.65 ± 0.98	34
5d	94.64 ± 0.98	85.74 ± 3.67	82.57 ± 1.29	70.28 ± 0.54	29.48 ± 1.07	54

Mean of MTT values are described at different concentrations

Table 4: Flow cytometry data of 3e

Concentration	Sub-G1 (%)	G1 (%)	S (%)	G2/M (%)
Control	3.56	50.64	32.82	12.94
4 µg/mL	7.02	53.56	27.1	12.27
6 µg/mL	7.44	59.31	25.12	8.05

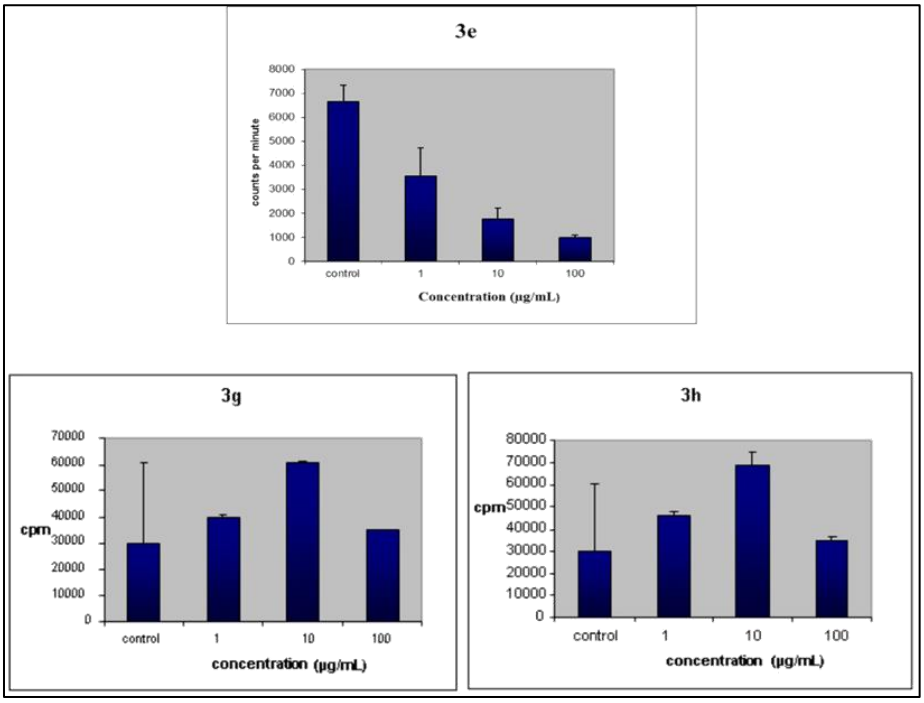

Figure 4: [^{3}H]-thymidine incorporation assay

Flow Cytometry

Flow cytometry is a laser-based, a biophysical technology employed in cell counting, cell storage and also used in the diagnosis of health disorders like blood cancer and clinical trials.

3e affected cell cycle profile upon treatment with appearance of sub-G1 cells:

As 3e induced a reduction in the number of viable cells in MTT assay and also exhibited a reduction in the cell proliferation in titrated thymidine assay, it was interesting to study the cell cycle distribution by fluorescence-activated cell sorting analysis of PI-labeled cells. The bar diagram quantifying the percentage of cells in the different phases of the cell cycle in control and treated group is shown in Figure 5.

The data is represented in Table 4. The histogram of DMSO treated cells showed a standard cell cycle pattern, which included 'G1 ' and 'G2/M ' peaks separated by 'S ' phase peak. The sub-G1 peak showed very less percentage of dead cells, treated with DMSO. Interestingly upon addition of 3e, a concentration-dependent change was observed in the percentage of cells in each phase of the cell cycle. There was a remarkable dose-dependent increase in the percentage of cells in the sub-G1 phase.

More cells in the 'G1 ' phase were observed when compared to 'S ' and 'G2/M ' phase, indicating a cell cycle arrest probably in the 'G1 ' phase of the cell cycle allowing fewer cells to enter into the S phase, confirming the results obtained in the [3H]-thymidine incorporation assay.

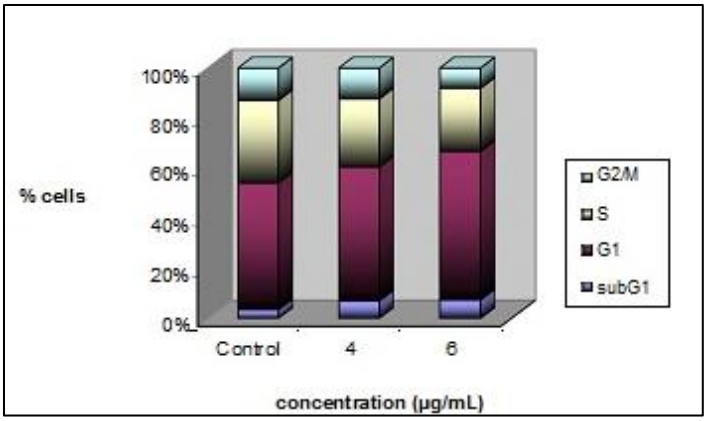

Figure 5: Flow cytometry graph of 4-({[3-(4-fluorophenyl)-1*H*-pyrazol-4-yl]methylidene}amino)-5-[(2-methylphenoxy)methyl]-1,2,4-triazole-3-thiol (3e)

The concentration-dependent change was observed in the percentage of cells in each phase of the cell cycle. There was a remarkable dose-dependent increase in the percentage of cells in the sub-G1 phase. More cells in the 'G1 ' phase observed when compared to 'S ' and 'G2/M ' phase, indicating a cell cycle arrest probably in the 'G1 ' phase of the cell cycle allowing fewer cells to enter into the S phase, confirming the results obtained in the [3H]-thymidine incorporation assay.

CONCLUSION

The Schiff bases showed better results when compared to Mannich bases on cytotoxicity determining MTT assay in Hep G2 cells. The Mannich bases derived from morpholine were found to be more effective cytotoxic agents than those obtained from diphenylamine. Compound 3e has fluorophenyl and 2-tolyl substituent's exhibited very high potency against Hep G2 cells through an apoptotic pathway. The highly electronegative fluorine atom might have added to the potency of the compound as it induces an increase in lipophilicity. The fluorine atom might also have favored the passage of biomembranes. The steric hindrance generated by methyl group present at the ortho position of the phenyl ring could have imposed particular conformation of the molecule that might have favored better interactions responsible for its excellent anticancer activity.

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