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Research Article

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SYNTHESIS, CHARACTERIZATION AND *IN-VITRO* CYTOTOXIC ACTIVITY OF N-ALKYL DERIVATIVES OF ISATIN

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ABSTRACT

In the present study, **3,3'-(5,5'-methylene bis(3-mercapto-4*H*-1,2,4-triazole-5,4-diyl) bis (azan-1-yl-1-ylidene)diindolin-2-ones** compounds were prepared by conjugation of 4, 5, 7 substituted isatin derivatives with 5, 5'-methylene –diyl-bis (4-amino-4H, 1,2,4 triazole – 3-thiol) derivatives. The new series of isatin and triazole derivatives was synthesized and characterized by spectral data and screened for i*n vitro* cytotoxic activity (MTTassay, Tryphan blue dye exclusion method, antimitotic activity)against two human cancer cells lines including HT-29 (Colon cancer) and A549 (Lung cancer) and DNA binding activities.

Keywords: Antitumor activity, triazole derivative, HT-29, A549 Cell lines.

INTRODUCTION

Cancer is a group of diseases in which cells are aggressive (grow and divide without respect to normal limits), invasive (invade and destroy adjacent tissues), and sometimes metastatic (spread to other locations in the body). It is the second leading cause of death worldwide. Hence, improvements in cancer detection, diagnosis, and treatment have increased the survival rate for many types of cancer.[1, 2, 3]

Nearly, all cancers are caused by abnormalities in the genetic material of the transformed cells. These abnormalities may be due to the effects of carcinogens, such as tobacco smoke, radiation, chemicals, or infectious agents. New aspect of the genetics of cancer pathogenesis, such as DNA methylation, and micro RNAs are increasingly being recognized as important.[4, 5]

In view of the above implication, there has been significant progress in the development of targeted therapy drugs that act specifically on detectable molecular abnormalities in certain tumors, and thus minimize damage to normal cells.[6] In search of chemotherapeutic agents for selectively interfering with the growth of neoplastic cells as compared to normal cells, the idea of incorporating alkylating function into a derivative of indole has attractive implications.

It was therefore, strongly suggested that tryptophan pyrrolase or of the other enzymes associated with tryptophan (or indole) metabolism may be available to detoxify an indole-N-mustard in normal cells while the toxic properties of the N-mustard remain substantially undiminished in tumor system.

On the basis of this hypothesis, Mannich bases with nitrogen mustards and isatins were prepared and tested. They showed antineoplastic activity to some extent.[7, 8] It was later found that some β- thiosemicarbazone derivatives of isatin also exhibit antitumor activity.[9]

Among, the series of 3-orthonitrophenyl hydrazone derivatives of isatin were synthesized and tested for their antineoplastic activity, only one derivative was found effective against Walker carcinosacroma – 256.[10] However, in search for new isatin derivatives, Dobrynin et al.[11] reported the synthesis and anti-tumor activity of 3-substituted 1-glycosyl isatins. Also, Maysinger et al.[12] reported the preparation of 5-haloisatins, Mannich bases and hydrazones of 5-chloro and 5-iodoisatin. Among the various derivatives, antimitotic activities of iodinated isatin derivatives were found highly effective as growth inhibitors of Hela cell cultures and *L. sativum*.

Further, Frank D. Popp et al.[13] reported the synthesis of 3-O-nitrophenyl hydrazones of isatin by the condensation of isatin and o-nitrophenyl hydrazine, which was found to be active against Walker carcinoma 256 but inactive against L1210 lymphoid leukemia.

In view of biological significance of isatin and triazole moieties, it was planned to synthesize some new derivatives containing bis isatin and triazole molecule to get the more potent compounds and evaluate their potential *in vitro* cytotoxic activity (MTTassay, Tryphan blue dye exclusion method, antimitotic activity) against two human cancer cells lines including HT-29 (Colon cancer) and A549 (Lung cancer) and DNA binding activities.

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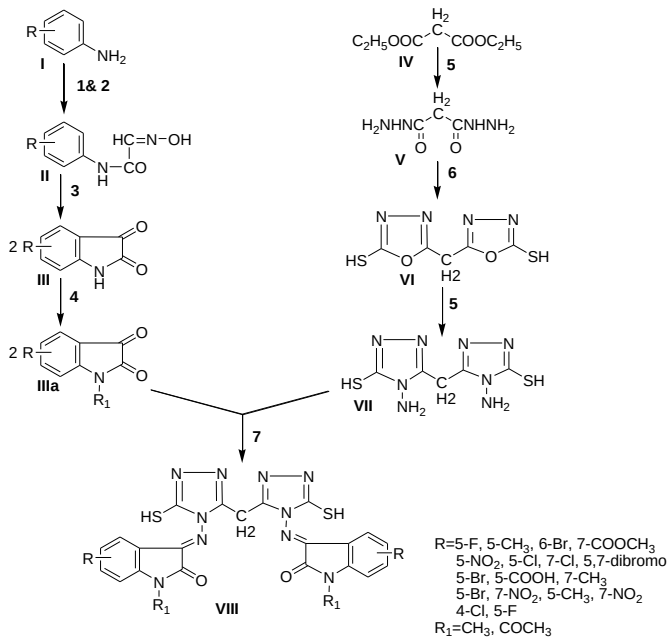
CHEMISTRY

It involves (VI) combination of crucial basic skeletons of N-alkyl isatin derivatives (IIIa) and 5, 5'methene diyl bis (4-amino-4H) 1, 2, 4 – triazole – 3- thiol) VII. Isonitrasoacetanilide (II) was prepared from selective aniline derivative by using Sandmeyer method. Isonitasoacetanlide on cyclisation with H_2SO_4 at 70, Isatin (III) was obtained. N-methyl and N-acetyl isatins (IIIa) was prepared by using alkylating agents like dimethyl sulfate and acetyl-ing agent like acetic-anhydride. 5, 7, di-bromoisatin was prepared by bromination with Bromine water (Lindwall method). 5-nitro-isatin was prepared by nitration with KNO_3 and H_2SO_4. 5-Bromo, 7-Nitroisatin was prepared by nitrating the 5-Bromoisatin with nitration minture (KNO_3 + H_2SO_4). All the above compounds were purified and crystallized by using appropriate solvent.

The crucial basic sketeton 5,5'- methylene diyl bis (4-amino 4H, 1, 2, 4 trazole-3 – thio) (VII),was prepared from malonic acid hydrazide (V). Malonic acid hydrazide was prepared by treating dimethyl malonate with hydrazine hydrate. Malonic acid hydrazide on treating with carbondisulphide in presence of alcoholic KOH, 5, 5'-melthlene diyl bis (1, 3, 4 – oxadiazole – 2- thiol) VI was formed. The resulting compounds was neutralized with HCl and purified by column chromatography. Compound (VII) is formed by treating the compound (VI) with hydrazine hydrate in excess alcohol for 12hrs. The required compound is synthesized by combining the H-alkyl isatin derivatives with compound (VII) in methanol for 2hrs. The crude products isolated were recrystallized from methanol to get pure products. The synthesized compounds were characterized by IR, NMR, and Mass spectroscopy. (Scheme **1**)

SCHEME-I



1. Chloral hydrate, 2. Hydroxylamine HCl, 3. Conc Sulphuric acid, 4. Dimethylsulphate/ Acetic acid, 5. Hydrazine hydrate, 6. CS_2/ Alc KOH, 7. Ethanol containing trace of Acetic acid

PHARMACOLOGY

Twenty compounds of 3,3'-(5,5'-methylene bis(3-mercapto-4*H*-1,2,4-triazole-5,4-diyl) bis (azan-1-yl-1-ylidene)diindolin-2-ones compounds have been synthesized by conjugation of 4, 5, 7 substituted isatin derivatives with 5, 5'-methylene –diyl-bis (4-amino-4H, 1,2,4 triazole – 3-thiol) derivatives. The antitumor activity of these compounds was examined against two different human tumor cell lines i.e. HT-29 (Colon cancer) and A549 (Lung cancer). Among 20 compounds, only five exhibited good cytotoxic activity.

RESULTS AND DISCUSSION

Biological assay

All the compounds (VIII) were evaluated for cytotoxicity properties on human lung and liver carcinoma cell lines with cisplastin as a standard. Inhibition of cell-proliferation was measured by MTT, antimitotic, viable cell count and DNA binding assays. The IC50 values are the average of at least three independent experiments.

MTT (Microculture Tetrazolium Assay)

The cytotoxic activity of 3, 3'-(5, 5'-methylene bis (3-mercapto-4H-1, 2,4-triazole-5,4-diyl) bis (azan-1-yl-1-ylidene) di indolin-2-ones (VIII) is presented in **Table 1.** Almost all the 20 compounds except two exhibited good activity against A-549 and HT-29 cell lines. Among the test compounds, compound VIIIp (R=4-Cl- 5-F, R^1=H) showed comparatively good cytotoxic activity with an IC_{50} values as 39.82 and 46.73 µM against A-549 (lung cancer) and HT-29 (colon cancer) cell lines respectively. Compounds VIIIn (R=5-Cl-7-NO_2, R^1=H) VIIIi (R=5,7-dibromo, R^1=H) and VIIIg (R=5-Cl, R^1=H), VIIIh (R=7-Cl, R^1=H) showed the IC_{50} values as 58.23µM, 67.23µM 68.20 µM and 95.00 µM against A-549 cell lines and 64.7µM , 82.34µM, 52.7µM, 75 µM against HT-29 cell lines respectively. The assay revealed that all the compounds except VIIIr (R=H, R^1=$COCH_3$), and VIIIk (R=5-COOH, R^1=H) exhibited cytotoxicity against A-549(lung cancer) and HT-29(Colon cancer) cell lines.

Viable cell assay by dye exclusion method

The cytotoxic activity data of 3,3'-(5,5'-methylene bis(3-mercapto-4H-1,2,4-triazole-5,4-diyl) bis (azan-1-yl-1-ylidene) di indolin-2-ones (VIII) by dye exclusion method is shown in **Table 2**. Almost seventeen compounds, out of twenty exhibited good cytotoxic activity against HT-29 cell lines. Among the test compounds, compound VIIIp (R=4-Cl- 5-F, R^1=H) showed comparatively good cytotoxic activity with IC_{50} value of 9.34 µM against HT-29 cell lines. Compounds VIIIf (R=5-NO_2, R1=H) and VIIIt (R=7-NO_2, R^1=H) were shown cytotoxic activity with IC_{50} values of 10.23 µM and 11.90 µM against HT-29(Colon cancer) cell lines respectively.

DNA binding study

The results of DNA binding study of compounds (VIII) using Herring sperm DNA is presented in **Table 3**. Cisplatin was used as the standard drug. Among the test compounds, Compound VIIIp (R=4-Cl- 5-F, R^1=H) showed comparatively good DNA binding capacity with percentage inhibition of 72.67.

Anti mitotic activity (using germinating Bengal gram seeds)

Antimitotic activity of 3,3'-(5,5'-methylene bis(3-mercapto-4H-1,2,4-triazole-5,4-diyl) bis (azan-1-yl-1-ylidene) di indolin-2-ones (VIII) by using germinating Bengal gram seeds is presented in **Table 4**. Compound VIIIp (R=4-Cl- 5-F, R^1=H) showed 82.18% growth inhibition of germinating Bengal gram seeds. Compounds VIIIi(R=5, 7-dibromo, R^1=H), VIIIn (R=5-Cl-7-NO_2, R^1=H) and VIIId(R=6-bromo, R^1=H), showed good antimitotic activity with growth inhibition percentages of 79.23%, 77.82% and 71.87% respectively.

Table 1. cytotoxic activity of 3, 3'-(5, 5'-methylene bis (3-mercapto-4H-1, 2,4-triazole-5,4-diyl) bis (azan-1-yl-1-ylidene) di indolin-2-ones (VIII)

S.No.	Compound	Substituent		A549 Lung cancer cell lines IC_{50} values (µM)	HT-29 Colon cancer cell lines IC_{50} values (µM)
		R	R^1		
1	**VIIIa**	H	H	225.00	232.00
2	**VIIIb**	5-F	H	112.00	123.00
3	**VIIIc**	5-CH_3	H	154.00	164.00
4	**VIIId**	6-Br	H	141.28	154.32
5	**VIIIe**	7-$COOCH_3$	H	NA	NA
6	**VIIIf**	5-NO_2	H	152.72	138.30
7	**VIIIg**	5-Cl	H	68.20	52.70
8	**VIIIh**	7-Cl	H	95.00	75.00
9	**VIIIi**	5,7-dibromo	H	67.23	82.34
10	**VIIIj**	5-Br	H	102.45	90.95
11	**VIIIk**	5-COOH	H	NA	NA
12	**VIIIl**	7-CH_3	H	172.32	204.98
13	**VIIIm**	5-Br-7-NO_2	H	116.00	110.92
14	**VIIIn**	5-Cl-7-NO_2	H	58.23	64.70
15	**VIIIo**	5-CH_3- 7-NO_2	H	108.00	148.24
16	**VIIIp**	4-Cl-5-F	H	39.82	46.73
17	**VIIIq**	H	CH_3	154.00	168.00
18	**VIIIr**	H	$COCH_3$	NA	NA
19	**VIIIs**	5-Br	$COCH_3$	128.00	156.29
20	**VIIIt**	7-NO_2	H	102.00	110.23
21	**Cisplatin**			25	25

NA - No Activity

Table 2. Cytotoxic activity of 3,3'-(5,5'-methylene bis(3-mercapto-4H-1,2,4-triazole-5,4-diyl) bis (azan-1-yl-1-ylidene) di indolin-2-ones (VIII) by dye exclusion method

S.No.	Compound	Substituent		IC_{50} value (µM)
		R	R^1	
1	**VIIIa**	H	H	19.65
2	**VIIIb**	5-F	H	14.45
3	**VIIIc**	5-CH_3	H	15.78
4	**VIIId**	6-Br	H	12.98
5	**VIIIf**	5-NO_2	H	10.23
6	**VIIIg**	5-Cl	H	14.14
7	**VIIIh**	7-Cl	H	13.76
8	**VIIIi**	5,7-dibromo	H	12.76
9	**VIIIj**	5-Br	H	14.32
10	**VIIIl**	7-CH_3	H	17.00
11	**VIIIm**	5-Br-7-NO_2	H	18.65
12	**VIIIn**	5-Cl-7-NO_2	H	13.43
13	**VIIIo**	5-CH_3- 7-NO_2	H	15.67
14	**VIIIp**	4-Cl- 5-F	H	9.34
15	**VIIIq**	H	CH_3	16.78
16	**VIIIs**	5-Br	$COCH_3$	18.67
17	**VIIIt**	7-NO_2	H	11.90
18	**Cisplatin**			6.63

Table 3. DNA binding study of compounds (VIII) using Herring sperm DNA

S.No.	Compound	Substituent		% growth Inhibition
		R	R^1	
1	**VIIIa**	H	H	42.96
2	**VIIIb**	5-F	H	68.30
3	**VIIIc**	5-CH_3	H	53.82
4	**VIIId**	6-Br	H	71.87
5	**VIIIf**	5-NO_2	H	50.00
6	**VIIIg**	5-Cl	H	62.13
7	**VIIIh**	7-Cl	H	58.78
8	**VIIIi**	5,7-dibromo	H	79.23
9	**VIIIj**	5-Br	H	48.90
10	**VIIIl**	7-CH_3	H	39.26
11	**VIIIm**	5-Br-7-NO_2	H	66.89
12	**VIIIn**	5-Cl-7-NO_2	H	77.82
13	**VIIIo**	5-CH_3- 7-NO_2	H	49.86
14	**VIIIp**	4-Cl- 5-F	H	82.18
15	**VIIIq**	H	CH_3	30.56
16	**VIIIs**	5-Br	$COCH_3$	38.67
17	**VIIIt**	7-NO_2	H	28.98
18	**Cisplatin**			97.5

Table 4. Antimitotic activity of 3,3'-(5,5'-methylene bis(3-mercapto-4H-1,2,4-triazole-5,4-diyl) bis (azan-1-yl-1-ylidene) di indolin-2-ones (VIII) by using germinating Bengal gram seeds

S.No.	Compound	Substituent		% Inhibition
		R	R^1	
1	**VIIIb**	5-F	H	48.56
2	**VIIIg**	5-Cl	H	52.56
3	**VIIIi**	5,7-dibromo	H	43.45
4	**VIIIo**	5-CH_3-7-NO_2	H	43.28
5	**VIIIp**	4-Cl-5-F	H	72.67
6	**Cisplatin**			82.90

CONCLUSION

We have synthesized novel N-alkyl derivatives of isatin by molecular conjugation with triazoles and tested for their anticancer activity on A549 and HT-29 cell lines. Compounds VIIIp was identified as good cytotoxic agent having IC_{50} values of 39.82 and 46.73 µM against A-549 (lung cancer) and HT-29 (colon cancer) cell lines respectively. Also, Compound VIIIp (R=4-Cl- 5-F, R^1=H) showed comparatively good DNA binding capacity of 72.67. From the above results, we conclude that 4-Chloro substituted isatin derivatives (VIIIp, VIIIg, VIIIn, VIIIh) shown more cytotoxic activity compared to bromo, nitro, methyl, carboxyl, carbomethoxy substituted derivatives of Isatin. Among the various substitution, dihalogen substituted derivatives of Isatin mainly 4Cl, 5F substituted isatin (VIII) shown more cytotoxic and DNA binding activity.

EXPERIMENTAL

Chemistry

All the starting materials and reagents were obtained from commercial sources and were used without further purification. IR spectra were recorded on a Digital Spectra Max FT-IR spectrometer. PMR spectra were recorded in CDCl3/DMSO-d6 at 400 MHz on a spectrometer. All the chemical shifts were reported in δ units and downfield from TMS as internal standard. High-resolution mass spectra were recorded on a double focusing magnetic sector mass spectrometer at 70 eV. Melting points were recorded on melting point apparatus (Acro Steel Pvt. Ltd.) and are uncorrected. The residues were purified by flash chromatography (230–400 mesh). Elemental analyses were recorded using a rapid elemental analyzer.

Synthesis of indole-2, 3-diones (Isatins, III) ***Isonitrosoacetanilides (II)*****:** In a 5 lit. R.B. Flask were placed chloral hydrate (0.54 mol) and 1200 ml of water. To this solution, were then added crystallized sodium sulphate (1300 gm) followed by a solution of appropriate aniline (I) in 300 ml of water and concentrated hydrochloric acid (0.52 mol). Finally, a solution of hydroxylamine HCl (1.58 mol) in 500 ml of water was added. The contents of the flask were heated over a wire-guage by a mecker burner so that vigorous boiling began in about 45 minutes. After 1 to 2 minutes of vigorous boiling the reaction was completed. During the heating period itself the crystals of isonitrosoacetanilides started separating out. On cooling under the current of water, the entire product was solidified. It was filtered under suction, air dried and purified by recrystallization from suitable solvent(s).

Indole-2, 3-diones[14, 15, 16, 17] ***III*****:** Sulphuric acid (600 g, d 1.84, 326 ml) was warmed at 50^{0}C in a one liter R.B. flask fitted with an efficient mechanical stirrer and to this finely powdered appropriate isonitrosoacetanilide (II, 0.46 mol) was added at such a rate so as to maintain the temperature between 60°C to 70°C but not higher. External cooling was applied at this stage so that the reaction could be carried out more rapidly. After the addition of isonitroso compound was completed the temperature of the solution was raised to 80°C and maintained at that temperature for 10 minutes to complete the reaction. Then the reaction mixture was cooled to room temperature and poured on to crushed ice (2.5 kg) while stirring. After standing for about half-an-hour, the product separated was filtered, washed several times with small portions of cold water and dried. Purification of the compound was effected by the recrystallization from methanol.

Synthesis of N-methylisatin (IIIa R^1 =CH_3 , R=H): To a solution of isatin (0.1 mol, 14.7 g) in methanol (10ml), 10% sodium hydroxide (0.12mole) was added until the violet colour did not persist and equimolar amount of dimethyl sulfate (0.12 mole, 12.6 g) was added and the reaction mixture was shaken for 2 hrs in a magnetic stirrer and kept a side for half an hour. On adding water reddish orange needle shaped crystals of N-methylisatin was separated out. The crystals were filtered off, dried and further recrystallised from ethanol, m.p. 131°C (lit [18] Yield 76.8%).

Synthesis of N-acetylisatin (IIIa R^1=CH_3CO, R=H): N-Acetylation of isatin was carried out by taking equimolar amount of isatin (0.1 mol, 11.7 g) with acetic anhydride (0.12mole, 8.16 g) and sodium acetate(0.12mole, 8.16g) . The reaction mixture was kept aside for 24 hrs and it was poured in to ice-cold water and yellowishorange precipitate was obtained immediately. It was filtered dried and crystallized from ethanol, m.p. 140°C (Lit[19] yield 74.8%).

Synthesis of 5, 7-dibromoisatin (III R=5,7-dibromo, R^1=H): The synthesis of 5, 7-dibromoisatin was based on the method of Lindwall.[20] Isatin (0.1mole, 5.0 gm, 1 Equiv) was warmed in Ethanol (95%, 100 ml) with stirring until it dissolved. Bromine (0.1mole, 16.3 gm, 5.2 ml, 3.0 Equiv) was added drop wise to the stirred isatin solution while maintaining the temperature of the reaction mixture between 70 and 75°C. The solution was cooled to room temperature and placed on ice for 30 min. The resulting precipitate was washed with water and cold ethanol and then recrystallized from ethanol to yield bright orange-red crystals of 5, 7-dibromoisatin m.p. 253-255°C.

Synthesis of 5-nitro isatin (III R=5-NO_2,R^1=H): General method for the nitration of the isatin was based on the method of calvery.[21] A solution of isatin (0.1mole, 500 mg) in conc, H_2SO_4 (3.2ml) was added drop wise to a solution of KNO_3 (0.1mole, 344 mg) over a period of 1 hour while maintaining the temperature between 0 and 4°C. The reaction mixture was poured in to 25 ml of ice cold water and the precipitate was washed with cold water. The crude 5-nitroisatin was purified by flash chromatography. The product was a bright yellow/orange solid (350 mg, 47%), m.p. 252-254°C.

Synthesis of 5-bromo-7-nitroisatin: (III R=5-bromo-7-nitro,R¹=H): This compound was prepared by the nitration of 5-bromoisatin (0.1mole, 1.0 gm) adopting the method for the preparation of 5-nitroisatin. The crude product was purified using flash chromatography, m .p. 246-248°C.

Synthesis of malonic acid hydrazide (V): Diethylmalonate (IV, 0.1 mole) in alcohol (10ml) was refluxed with hydrazine hydrate (99.9%, 0.04 mole, and 10ml) for 15 minutes. The resulting compound was cooled and the solvent was removed by distillation. The product thus obtained was recrystallized from ethanol. m.p. 155-157 °C.

Synthesis of 5, 5¹-methylene diyl bis (1, 3, 4-oxadiazole-2-thiol) (VI): A mixture of malonic acid hydrazide (V, 0.1mole), 10% alcoholic potassium hydroxide (0.12mole, 10ml) and carbon disulfide (in excess) was refluxed for 4 hours. The solvent was removed and digested with water, and neutralized with dilute hydrochloric acid. The resulting compound was filtered washed several times with cold water, dried, recrystallized from alcohol and purified by column chromatography. m.p. 143-145°C. The compound was characterized by IR (KBr), PMR (DMSO-d_6), Mass spectrum and Elemental analysis – IR (in cm $^{-1}$): 2927.06 (C-H), 2361.09 (S-H), 2344.62 (S-H), 1514.77(C=N), 1508.42 (C=N), 1161.77 (C-O-C). 1113.04 (C-O-C); ^{1}H NMR (MEOH-D^4, 400 MHZ) in δ ppm: 2.5(s, 2H, CH_2), 4.627 (s, 2H, 2SH); MS (m/z): 234 (M^+);Elemental analysis of the compound showed Nitrogen- 22.47%, Carbon-30.94%, Hydrogen-4.10%, Sulphur-4.41%.

Synthesis of 5, 5¹-methylene diyl bis (4-amino-4H- 1,2,4-triazole- 3-thiol) (VII): A mixture of 5, 5¹-methylene diyl bis (1, 3, 4-oxadiazole-2-thiol) (VI, 0.01 mole) and hydrazine hydrate (in excess) in alcohol was refluxed for 12 hours. The solvent was distilled off and resulting white solid was dried and purified by recrystallization from suitable solvent(s) and column chromatography m.p. 244 -246°C; IR (in cm $^{-1}$): 3330.69(NH_2), 3194.77 (NH_2), 2927.66(C-H), 2379.05(S-H), 2347.69(S-H), 1498.35(C=N); ^{1}H NMR (MEOH-D, 400 MHZ in δ ppm): 2.6 (s,2H, CH_2), 4.62 (s, 2H, 2SH), 8.64 (s, 4H, 2NH_2); MS(m/z): 246.4 (M^+).

Synthesis of 3, 3'-(5, 5'-methylene bis (3-mercapto-4H-1, 2, 4-triazole-5, 4-diyl) bis (azan-1-yl-1-ylidene) diindolin-2-ones (VIII): A mixture of an appropriate indole-2,3-dione (III, 0.02 mol) and 5, 5¹-methylene diyl bis (4-amino-4H- 1, 2, 4-triazole-3-thiol) (VII, 0.01 mol) in methanol (50 ml) was refluxed for 12 hours. The solvent was removed by distillation and resulting white solid was dried and recrystallized from methanol, purified by column chromatography. IR (in cm $^{-1}$):3274.28(NH), 3215.50(NH), 2921.97 (C-H), 2378.65 (S-H), 2346.31 (S-H),1690.02(C=O), 1650.80(C=O), 1487.94(C=N), 1427.94(C=N); ^{1}H NMR (MEOH-D, 400 MHZ) in δ ppm: 2.89(s, 2H, CH_2), 4.2(s, 2H, 2SH). 6.5-7.9 (m, 8H, Ar-H), 11.16(s, 1H, indole NH),11.22(s, 1H, indole NH), MS(m/z): 503.6(M^+). It also showed peak at m/z 209.7(30%), 155.4(10%) due to the fragmentation of molecule.

Biological Assays

The new molecules containing both isatins and triazoles were screened for cytotoxicity (MTT assay, Tryphan blue dye exclusion method, Antimitotic activity) and DNA binding studies.

Antiproliferative study by Microculture tetrazolium (MTT) assay:[22, 23, 24, 25] Both HT-29 (Colon cancer), A549 (Lung cancer) cell lines, obtained from National Center for Cell Science) (NCCS) Pune, India. were grown as adherent cells in DMEM media supplemented with 10% fetal bovine serum, 100 µg/ml penicillin, 200 µg/ml streptomycin, 2mM L-glutamine, and culture was maintained in a humidified atmosphere with 5%CO_2 at 37^{0}C for 72 hr. The cytotoxicity was determined by the microculture tetrazolium (MTT) assay**,** based on the metabolic ability of mitochondrial dehydrogenase enzyme to reduce 3-(4, 5-dimethylthiazol-2, 5-diphenyl) tetrazolium bromide (MTT) to water insoluble formazan crystals, which gives direct correlation of viable cells. The required dilutions were made with sterile water to get required concentrations from stock solution of 10 mg/ml in DMSO. HT-29 (Colon cancer), A549 (Lung cancer) cell line were seeded at a density of 1 x 10^4 cells (cell number was determined by Trypan blue exclusion dye method) per each well in 100 µl of DMEM supplemented with 10% FBS.12 hrs after seeding, above media was replaced with fresh DMEM supplemented with 10% FBS then 10µl sample from above stock solutions were added to each well in triplicates which gives final concentration of 200, 100, 50, 10 µg/well. The above cells were incubated for 48 hrs at 37°C with 5% CO_2. After 48 hrs incubation the above media was replaced with 100 µl of fresh DMEM without FBS and to this 10 µl of MTT (5mg dissolved in 1 ml of PBS) was added and incubated for 3 hrs at 37°C with 5% CO_2.. After 3 hrs incubation, the above media was removed with multi channel pipette, and then 200 µl of DMSO was added to each well and then incubated at 37°C for 15 min. Finally, the plate was read at 570 nm using spectrophotometer (Spectra Max, Molecular devices). In all experiments, cisplatin (IC_{50} 25 µM) was used as the positive control. The results are expressed as the average of triplicate assays.

Viable cell assay by dye exclusion method: This method is particularly recommended for assays in suspension cultures and based on the principle that live (viable) cells actively pump out the dye by efflux mechanism, where as dead (non-viable) cells do not. Hence, white transparent cells are viable cells and blue cells are dead cells.

The contents of the culture flask were transferred into a centrifuge tube aseptically and then centrifuged at 2000 rpm for 2 minutes. Supernatant was discarded and the pellet was resuspended in fresh medium and mixed thoroughly to get a uniform cell suspension. 0.3 ml of the cell suspension was added aseptically to each well in the 6-well plate. Drug solutions are made in medium with the solvent (DMSO). DMSO in media served as control. Each well was added with 0.7 ml of medium DMSO drug solutions. The plate was then incubated at 37 ^{0} C for 72 hours in CO_2 incubator. After 72 hours of incubation, the plate was taken and the viable cells using Haemocytometer. A separate count was maintained for viable and non-viable cells. The percentage inhibition of growth was calculated by comparing the percentage viability in the well with test compound with that of the control.[26]

Calculations:

$$Cells\ per\ ml = Average\ count\ per\ square \times dilution\ factor$$

$$Total\ viable\ cells = Cells\ per\ ml \times Original\ volume\ of\ cell\ suspension$$

$$Cell\ viability\ \% = Total\ viable\ cells(unstained) \div Totalcells$$

$$\% \, Cytotoxicity = \frac{(T_{dead} - C_{dead})}{T_{total}} \times 100$$

Where,

T_{dead} is the number of dead cells in the test

C_{dead} is that in the control group

T_{total} is the total number of dead and live cells in the test compound.

DNA binding activity: This activity is based on the binding capacity of drug with the DNA. Now, the amount of DNA detected in HPLC is reduced and can be estimated by the reduction in peak area. With varying concentration of drug, Herring sperm DNA,(Himedia, Mumbai, India) sodium aqueous solution was prepared with the final concentration being 0.1 mg/ml. Test/standard (Cisplatin) drug solutions were made in water with concentration ranging from (10µl to 100µl). DMSO was used to solubilize the drugs.100µl of DNA solution was taken in an eppendorff tube and to it 100µl of test/standard solution was added and the mixture was incubated at 37^0C for 30 minutes. 40µl of the mixture was then injected into HPLC with 90:10(methanol: water) mobile phase and detected at 254 nm wave length. The peak area of the test/standard is compared with that of the blank to get the percentage binding. Percentage of DNA bound at different drug concentration is calculated and plotted.[27]

Anti mitotic activity (using germinating Bengal gram seeds): Bengal gram seeds (Cicer *auratinum*) (locally available in the market) of a good quality were taken and soaked overnight with water to hasten the germination process. The next day the seeds were distributed in a group of ten each in petridishes, (HI media, Mumbai, India) on moistened filter paper. Drug solutions were prepared 1%DMSO, Dimethyl sulfoxide (DMSO) (E.Merk, Mumbai, India) at concentrations ranging from 10µl to 100µl and added to the filter paper in the petridishes. One petridish served as DMSO control, and one served as cisplatin (positive) control. The seeds were allowed to germinate for 7 days and care was taken to moisten the filter paper with control and drug solutions every 24 hours. The length of the radicals was measured in cm at the end of 7th day and percentage mean values of the solvent control treated and percentage inhibition growth is calculated the values are plotted to get IC_{50} value.[28]

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