School of Sciences[1], Indiana University Kokomo, Kokomo, USA; Drug Discovery and Development Research Group[2], College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, Canada

Cytotoxic benzylidene hydrazides of terephthalic acid and related compounds

M. Hossain[1,*], S. C. Hall[1], P. J. Wiggington[1], S. M. Roth[1], S. Das[2], U. Das[2], P. K. Roayapalley[2], J. R. Dimmock[2]

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**Corresponding author: Mohammad Hossain, School of Sciences, Indiana University Kokomo, Kokomo, IN 46904-9003, USA*
mohoss@iu.edu

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The present investigation involved the synthesis of a number of novel benzylidene hydrazides as candidate cytotoxic agents. The preparation of these compounds from terephthalic acid and isophthalic acid proceeded satisfactorily. However, the reaction of phthalic acid hydrazide with various aryl aldehydes was unsuccessful in general. Some of the unexpected products were identified. The shapes and also the distances between the centers of the aryl rings designated B and C of three representative compounds **1b**, **2b** and **3b** were determined. The compounds designated **1a-e**, **2a-e** and **3b** were screened against human HCT116 and HT29 colon cancer cells as well as human CRL1790 non-malignant colon cells which revealed the tumor-selective toxicity displayed by these compounds.

1. Introduction

A major problem in current cancer chemotherapy is multidrug resistance to established antineoplastics. In this study, a series of compounds have been designed and synthesized which are structurally dissimilar from contemporary anticancer medication. Hence neoplastic cells may be sensitive to these molecules.

The compounds are designed to have different groups which could react with cellular constituents. Thus, reaction of the carbon atom of an azomethine group could act as an alkylating agent towards cellular thiols. The presence of a dimethylaminomethyl group vicinal to a phenolic hydroxy group may lead to deamination to an orthoquinone methide which is likely chemically reactive. Furthermore, hydroxy and secondary amino groups permit hydrogen bonding while the oxygen atom of the carbonyl group acts as a hydrogen receptor. In addition, the aryl rings may form van der Waals bonds with aromatic rings present in cellular compounds. These concepts are portrayed in Fig. 1 for the representative compound **1b**.

The decision was made to mount two benzylidenehydrazides potential cytotoxic groups on phenyl ring A with the aim of increasing potency. In order to develop structure-activity relationships, the elimination of the dimethylaminomethyl group giving rise to **1a** was suggested while the addition of a dimethylaminomethyl group to **1b** would give **1c**. The mitochondrial membrane potential of some cancer cells is in excess of –200 mV (Bonnet et al. 2007; Fantin et al. 2006) while for non-malignant cells it is often in the region of –140 mV (Bagkos et al. 2014a, 2014b). Thus, positively charged compounds may preferentially accumulate in the mitochondria in cancer cells leading to greater toxicity to neoplasms than normal cells. Hence the formation of the quaternary ammonium analogs of **1b**,**c** namely **1d**,**e** was suggested.

Another objective of this study was to investigate whether there is a correlation between cytotoxic potencies and the relative location of the two benzylidene hydrazide groups attached to ring A. Hence the formation and bioevaluation of **2a-e** and **3a-e** was planned. However as will be presented later, only **3b** in series **3** was isolated. The structures of **1a-e**, **2a-e** and **3b** are shown in Fig. 2.

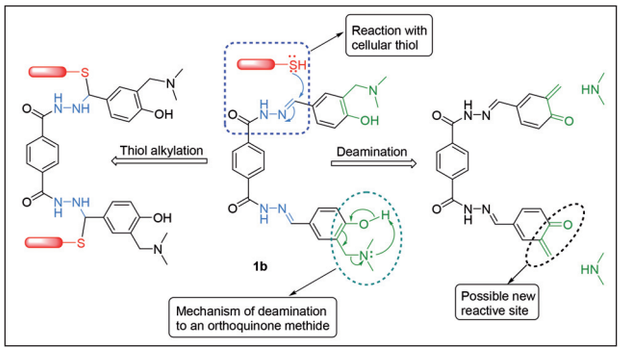


Fig. 1: Possible modes of action of a representative compound **1b** with cellular constituents.

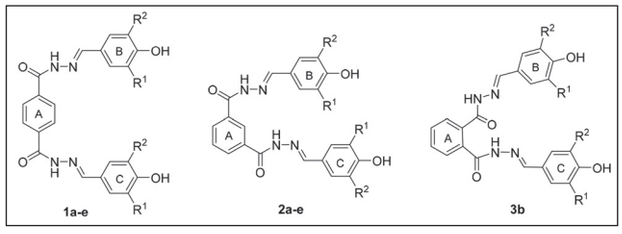
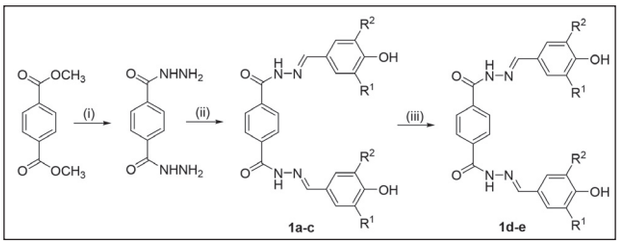


Fig. 2: Structures of three series of hydrazides **1**-**3**. **a**: $R^1 = R^2 = H$; **b:** $R^1 = H$, $R^2 = CH_2N(CH_3)_2$; **c:** $R^1 = R^2 = CH_2N(CH_3)_2$; **d:** $R^1 = H$, $R^2 = CH_2N^+(CH_3)_3I^-$; **e:** $R^1 = R^2 = CH_2N^+(CH_3)_3I^-$.

2. Investigations and results

Compounds **1a**-**e** were synthesized according to Scheme 1. A similar synthetic chemical route was followed for the preparation of **2a**-**e** from dimethylisophthalate. Dimethylphthalate reacted with hydrazine to give the corresponding hydrazide. However, reaction of this compound and 4- hydroxybenzaldehyde led to the isolation of **4a** and **4b** as indicated in Scheme 2. Nevertheless, reaction of 3-dimethylaminomethyl-4-hydroxybenzaldehyde with phthalohydrazide led to the formation of the desired compound **3b**. Models of **1b**, **2b** and **3b** were made using Spartan (Spartan 2021) and the distances between the centers of the aryl rings B and C in these compounds were obtained.

Our principal interest was to find compounds effective against colon cancer (Addala et al. 2017; Hossain et al. 2020). The benzylidenehydrazides **1a**-**e**, **2a**-**e** and **3b** were evaluated against human HCT116 and HT29 colon cancer cells. In addition, these molecules were assessed for cytotoxicity against human CRL1790 non-malignant colon cells. These biodata are presented in Table 1.

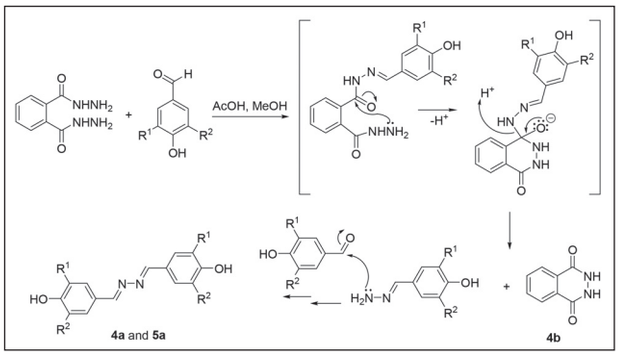


Scheme 1: Synthesis of series **1**. The reagents are as follows: (i) NH_2NH_2. H_2O, Ethanol, reflux at 85 °C; (ii) Appropriate 4-hydroxybenzaldehyde, AcOH, Ethanol, reflux at 85 °C; (iii) CH_3I, DMSO. The substituents in aryl rings A and C are indicated in Fig. 2.

3. Discussion

The synthesis of **1a**-**e** and **2a**-**e** proceeded in a straightforward manner. However, in an attempt to prepare **3a**, bis(4-hydroxybenzylidene)hydrazine (**4a)** and phthalhydrazide **4b** were obtained. In a subsequent experiment, phthalohydrazide reacted with 3,5-bis(dimethylaminomethyl)-4-hydroxybenzaldehyde and yielded **5a** and **4b**. A possible way that **4a**, **4b** and **5a** were formed is given in Scheme 2.

The evaluation of **1a**-**e**, **2a**-**e**, **3b**, **4a**,**b** against HCT116 and HT29 colon cancer cells is presented in Table 1. Examination of the biodata reveals that in general these compounds are potent cytotoxins towards HCT116 and HT29 cells and are much more toxic than 5-flurouracil (5-FU) which is used clinically to treat colon cancers. Of particular note are **1b** and **1c** which have 1422 and 141 times the potency of 5-FU towards HT29 and HCT116 cells, respectively. No less than 77% of the compounds have submicromolar IC_{50} values towards HCT116 cells and 69% towards HT29 neoplasms. While somewhat tangential to the present study, the average potency of **4a**, but not **4b**, towards HCT116 and HT29 cells is impressive possessing approximately 13 times the average potency of 5-FU.



Scheme 2: A possible mechanism for the formation of compound **4a** ($R^1 = R^2 = H$), **4b** and **5a** ($R^1 = R^2 = CH_2N(CH_3)_2$).

The next phase of the investigation was to determine whether mounting the benzylidene hydrazono group at positions 1 and 4 (series **1**) or at the 1 and 3 locations (series **2**) mounted on ring A (see Fig. 1), led to compounds with the greatest potencies. Hence the IC_{50} values of **1a** and **2a** were compared, then **1b** and **2b** and so on. The biodata using HCT116 cells revealed that **1a** and **2a** are equipotent, **2b>1b**, **1c>2c**, **2d>1d** and **2e>1e**, suggesting greater potencies are found with the analogs in series **2**. A similar comparison was made using HT29 cells in which case **1a>2a**, **1b>2b**, and **2e>1e**. However, **1c** and **2c** as well as **1d** and **2d** are equipotent which prevents firm conclusions from being made.

The average IC_{50} values for **1a**-**e** against HCT116 and HT29 cells are 1.25 and 0.45 µM, respectively. The average IC_{50} figures for **2a**-**e** against HCT116 and HT29 cells are 0.26 and 2.97 µM, respectively. Thus, the relative potencies depend on the cell line in view.

A further investigation was to find if the compounds demonstrate tumor-specific toxicity, i.e., whether the compounds are more toxic to neoplasms than to non-malignant cells. Hence **1a**-**e**, **2a**-**e**, **3b**, **4a**,**b** as well as 5-FU were evaluated against human CRL1790 non-malignant colon cells. These data are presented in Table 1. The higher the selectivity index (SI) value, the greater the tolerance of the cells to the compounds. Of particular note are the high SI figures of **2a** and **3b** using comparisons with HCT16 cells and **1b** for comparisons with HT29 cells.

Table 1: Cytotoxic evaluation of 1a-e, 2a-e, 3b, 4a,b against HCT116, HT29 and CRL1790 cells

Compound	HCT116		HT29		Average		CRL1790	PSE[c]
	IC_{50} (µM)[a]	SI[b]	IC_{50}(µM)[a]	SI[b]	IC_{50}(µM)[a]	SI[b]	IC_{50}(µM)[a]	
1a	0.17 ± 0.08	27.4	0.04 ± 0.01	116	0.11	71.8	4.65 ± 1.09	65,273
1b	4.09 ± 0.86	3.43	0.01 ± 0.00	1402	2.05	703	14.0 ± 2.96	34,293
1c	0.03 ± 0.01	94.3	0.42 ± 0.28	6.74	0.23	50.5	2.83 ± 0.70	21,957
1d	1.65 ± 0.20	3.09	0.20 ± 0.01	25.5	0.93	14.3	5.10 ± 0.24	1,538
1e	0.32 ± 0.22	48.4	1.56 ± 0.37	9.93	0.94	29.2	15.5 ± 1.28	3,106
2a	0.23 ± 0.09	288	14.1 ± 2.13	4.72	7.15	146	66.3 ± 2.43	2,042
2b	0.27 ± 0.17	98.4	0.36 ± 0.18	73.8	0.32	86.1	26.6 ± 1.90	26,906
2c	0.21 ± 0.13	120	0.15 ± 0.02	169	0.18	145	25.3 ± 1.89	80,556
2d	0.54 ± 0.21	29.6	0.19 ± 0.08	84.3	0.37	57.0	16.0 ± 0.16	15,405
2e	0.06 ± 0.02	64.8	0.09 ± 0.03	43.2	0.08	54.0	3.89 ± 0.42	67,500
3b	0.30 ± 0.08	182	16.6 ± 1.27	3.29	8.45	92.5	54.5 ± 1.83	1,095
4a	0.98 ± 0.09	4.39	0.45 ± 0.03	9.56	0.72	6.97	4.30 ± 1.48	968
4b	7.90 ± 1.68	4.82	17.0 ± 0.84	2.24	12.5	3.53	38.1 ± 1.11	28.35
5 FU	4.22 ± 0.43	6.59	14.2 ± 0.60	1.95	9.22	4.27	27.8 ± 1.17	46.31

[a] The IC_{50} values are the concentrations of compounds required to inhibit the growth of the cells by 50%.
[b] The letters SI refers to the selectivity index. The SI figures are the ratios of the IC_{50} value of the compounds towards non-malignant CRL1790 cells and the IC_{50} figure of a compound against a specific neoplastic cell line.
[c] The letters PSE refer to the potency-selectivity expression. These figures are the products of the reciprocal of the average IC_{50} values against HCT116 and HT29 cells and the average SI value multiplied by 100.

The selectivity index (SI) figures were obtained by dividing the IC_{50} values against CRL1790 cells by the IC_{50} figures towards a particular neoplastic cell line. These data are presented in Table 1. All of the SI values are greater than 1 and thus the compounds outlined in this study demonstrate tumor-specific toxicity. The SI values for 5-FU are less than 10 while 69% of the compounds described in this report have figures greater than 5-FU towards HCT116 cells and 54% against HT29 cells. The average SI values for **1a**-**e** are 35.3 and 312 against HCT116 and HT29 cells, respectively, while using these two cell lines, the average SI figures for **2a**-**e** are 120 and 75.0, respectively. Thus, the relative magnitude of the SI values is cell line specific. This result may be influenced by differences in the shapes of different compounds. Hence molecular modeling of three representative compounds **1b**, **2b** and **3b** was undertaken and the result is shown in Fig. 3 (Spartan, 2021). Compound **1b** adopts an eclipsed conformation, **2b** is essentially in the staggered form while **3b** is intermediate between the staggered and eclipsed structural isomers **1b** and **2b**. For example, the distances *q* between the centers of the aryl rings B and C in **1b**, **2b** and **3b** are 4.85, 15.33 and 9.56 Å, respectively, which likely influences the relative cytotoxic potencies.

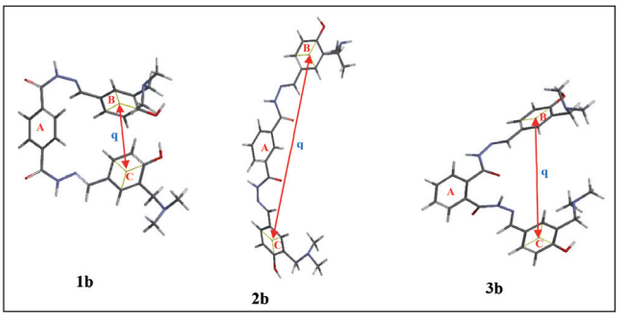


Fig. 3: Shapes of three representative compounds **1b**, **2b** and **3b** determined by molecular modeling.

In order to identify those compounds which display the most favorable features in terms of both potency and tumor-selective toxicity, the potency-selectivity expression (PSE) values were obtained for **1a**-**e**, **2a**-**e**, **3b**, **4a**,**b** as well as 5-FU. The PSE figures are the products of the reciprocals of the average IC_{50} values against the neoplastic cell lines and the average SI values times 100. These data are presented in Table 1. With the exception of **4b**, all of the compounds have substantially higher PSE figures than 5-FU. For example, the PSE value of **2c** is 1740 times greater than the figure for 5-FU. The compounds with the highest values (PSE figures in parentheses) are **2c** (80,556), **2e** (67,500) and **1a** (65,273) which may be identified as lead molecules. A comparison was made between the PSE figures in series **1** and **2** which have the same substituents in the aryl rings B and C. While the PSE figures for **1a**,**b** are greater than **2a**,**b** respectively, **2c**-**e** have higher PSE figures than **1c**-**e**, respectively, which indicates the need to expand both series considerably in order to draw meaningful structure-activity relationships. In addition, both **1b** and **2b** have substantially higher PSE values than **3b**.

Finally in view of the excellent potency and selectivity of these compounds in general, an estimate of their drug-like properties was undertaken. For good bioavailability, the molecular weights of the compounds should not exceed 500 and the logP values should not be greater than 5. In addition, the numbers of hydrogen bond acceptor atoms should not be greater than 10 while the number of hydrogen bonding donor atoms should not exceed 5 (Lipinski et al. 2001). The number of rotatable bonds should be less than 10 while the polar surface area should be lower than 140 $Å^2$ (Veber et al. 2002). The oral bioavailability score should be more than 0.50 (SwissADME, 2021). The compounds with PSE values in excess of 15,000 were examined for drug-like properties and the results are presented in Table 2. There are no violations in terms of the logP figures, the numbers of hydrogen bond acceptor atoms, hydrogen bond donor atoms and PSA values but molecular weights and rotatable bonds are outside of the recommended limits in most cases. In future, the formation of compounds where flexible groups such as the dimethylaminomethyl group are incorporated into rings may address this problem.

In conclusion, a cluster of compounds designed as candidate cytotoxic agents has been prepared and evaluated against HCT116 and HT29 colon cancer cells. The potency displayed by most of these compounds is highly impressive. These compounds are much less toxic to CRL1790 non-malignant cells giving rise to huge selectivity indices in many cases. These observations lead inexorably to the decision to develop these compounds by analog formation, assessment against a wide range of neoplasms, and some non-malignant cell lines, as well as mode of action studies on representative compounds.

4. Experimental

4.1. Chemistry

4.1.1. General

Melting points were determined using an electrochemical digital melting point apparatus, model number 1A9100 instrument and are uncorrected. ^1H and ^{13}C NMR spectra were determined using a Bruker Avance III 500 MHz NMR spectrometer in DMSO-d_6, or $CDCl_3$. Chemical shifts are reported in d (ppm) units relative to the internal standard tetramethylsilane (TMS). High resolution mass spectra (HRMS) were obtained using a QSTAR XL Hybrid LC/MS/MS system. Purity of all final compounds was established by Agilent 1200 HPLC with a diode-array detector. All compounds were found to be >95% pure by HPLC analysis. All chemicals and solvents used were of reagent grade without being purified or dried before use.

4.1.2. Terephthalohydrazide/isophthalohydrazide/phthalohydrazide

Each hydrazide was synthesized from the corresponding dimethylester following published procedures (Zahmatkesh 2015).

4.1.3. Substituted benzaldehydes

4-Hydroxybenzaldehyde was purchased from Sigma-Aldrich and used as it is whereas 3-((dimethylamino)methyl)-4-hydroxybenzaldehyde and 3,5-bis((dimethylamino) methyl)-4-hydroxybenzaldehyde were synthesized by following a published procedure (Karki et al. 2016) which was modified as described below.

3-((Dimethylamino)methyl)-4-hydroxybenzaldehyde: To a solution of 4-hydroxybenzaldehyde (1.000 g, 8.2 mmol) in CH_3CN (20 mL) was added *N*,*N*,*N*',*N*'-tetramethyldiaminomethane (1.2 mL, 9.0 mmol). The reaction mixture was heated

Table 2: Drug-like properties of selected compounds

Compound	MW (g/mol)	log P	HBA	HBD	RB	PSA ($Å^2$)	Oral bioavailability score	Violations
1a	402.4	2.57	6	4	8	123.38	0.55	0
1b	516.59	2.71	8	4	12	129.86	0.55	2
1c	630.78	2.88	10	4	16	136.34	0.17	3
2b	516.59	2.64	8	4	12	129.86	0.55	2
2c	630.78	2.77	10	4	16	136.34	0.17	3
2d	800.47	-0.93	6	4	12	123.38	0.55	2
2e	1198.54	-6.70	6	4	16	123.38	0.17	3
Ideal compound	≤500	≤5	≤10	≤5	<10	<140$Å^2$	> 0.50	0

[a] The abbreviations in some of the headings of each column refer to molecular weight (MW), logarithm of the partition coefficient (log P), hydrogen bond acceptor atoms (HBA), hydrogen bond donor atoms (HBD), rotatable bonds (RB) and polar surface area (PSA).

to 50 °C for 16 h. Volatiles were removed by rotary evaporation to yield a crude product which was purified by FCC using two solvent systems (EtOAc:hexane 50:50, DCM:MeOH:NH_4OH 95:5:1). Each fraction was collected in 100 mL portion. Starting material was eluted with EtOAc:hexane solvent system in F1-F2, pure product was eluted with DCM:MeOH:NH_4OH solvent system in F3-F5. F3-F5 were combined and dried to yield the target benzaldehyde (97% yield after recovery of starting material). Compound appeared as a light orange oil which solidified under refrigeration. Melting point = 63.1-65.9 °C. ^{1}H NMR ($CDCl_3$): 11.12 (s, br, 1H, Ar-OH), 9.77 (s, 1H, CHO), 7.67 (d, 1H, Ar-H, 8.4 Hz), 7.52 (s, 1H, Ar-H), 6.87 (d, 1H, Ar-H, 8.3 Hz), 3.70 (s, 2H, C-CH_2-N), 2.33 (s, 6H, N-CH_3) ppm.

3,5-bis((Dimethylamino)methyl)-4-hydroxybenzaldehyde: To a solution of 4-hydroxybenzaldehyde (1.000 g, 8.2 mmol) in CH_3CN (20 mL) was added *N,N,N',N'*-tetramethyldiaminomethane (2.4 mL, 17.2 mmol). The reaction mixture was refluxed at 85 °C for 24 h. The volatiles were removed by rotary evaporation to yield the target benzaldehyde (99 % yield). The compound appeared as an orange oil which solidified under refrigeration. Melting point = 80.1-83.0 °C. ^{1}H NMR ($CDCl_3$): 9.76 (s, 1H, CHO), 7.55 (s, 2H, Ar-H), 3.56 (s, 4H, C-CH_2-N), 2.28 (s, 12H, N-CH_3) ppm.

*4.1.4. N^1,N^4-bis(4-Hydroxybenzylidene)terephthalohydrazide (**1a**)*

To a solution of terephthalohydrazide (200 mg, 1 mmol) in 15 mL ethanol were added a solution of 4-hydroxybenzaldehyde (2.2 mmol) in ethanol (5 mL) and 2-3 drops of acetic acid. The mixture was then refluxed at 85 °C for 24 h. After about half of the solvent was evaporated using a rotary evaporator and the mixture was cooled to room temperature, then placed in an ice-water bath. The precipitate formed was collected by vacuum filtration, washed with ice-cold ethanol (about 2 mL) and finally dried in the oven at 50 ºC to give compound **1a**. Compound **1a** appeared as white solid powder, yield 65%, melting point = 347.2-350.1 °C, ^{1}H NMR (DMSO-d_6): δ 11.78 (s, 2H, CONH), 9.94 (s, br, 2H, Ar-OH), 8.38 (s, 2H, N=CH), 8.03 (s, 4H, Ar-H), 7.58 (d, 4H, Ar-H, 8.0 Hz), 6.85 (d, 4H, Ar-H, 8.1 Hz) ppm. ^{13}C NMR (DMSO-d_6): δ 162.6, 160.0, 149.1, 136.6, 129.4, 128.1, 125.6, 116.2 ppm. HRMS (ESI): m/z 403.1413 ([M+1]$^+$), calcd 403.1401 for $C_{22}H_{19}N_4O_4$.

*4.1.5. N^1,N^4-bis(3-((Dimethylamino)methyl)-4-hydroxybenzylidene) terephthalohydrazide (**1b**)*

Compound **1b** was prepared from terephthalohydrazide (200 mg, 1 mmol) and 3-((dimethylamino)methyl)-4-hydroxybenzaldehyde (2.2 mmol) using the same procedure for preparing **1a**. The title compound **1b** appeared as off-white solid powder, yield 68%, melting point = 173.3-174.9 °C, ^{1}H NMR (DMSO-d_6): δ 11.78 (s, 2H, CONH), 8.36 (s, 2H, N=CH), 8.03 (s, 4H, Ar-H), 7.53 (s, 2H, Ar-H), 7.46 (d, 2H, Ar-H, 8.3 Hz), 6.81 (d, 2H, Ar-H, 8.3 Hz), 3.63 (s, 4H, $ArCH_2N$), 2.26 (s, 12H, $N(CH_3)_2$) ppm. ^{13}C NMR (DMSO-d_6): δ 161.5, 158.9, 148.0, 135.6, 127.6, 127.1, 127.0, 124.4, 122.9, 115.1, 59.4, 43.7 ppm. HRMS (ESI): m/z 517.2546 ([M+1]$^+$), calcd 517.2558 for $C_{28}H_{33}N_6O_4$.

*4.1.6. N^1,N^4-bis(3,5-bis((Dimethylamino)methyl)-4-hydroxybenzylidene) terephthalohydrazide (**1c**)*

Compound **1c** was prepared from terephthalohydrazide (200 mg, 1 mmol) and 3,5-bis((dimethylamino)methyl)-4-hydroxybenzaldehyde (2.2 mmol) using the same procedure for preparing **1a**. The title compound **1c** was obtained by evaporating ethanol using a rotary evaporator and recrystallized using CH_2Cl_2/CH_3CN (1:1) to yield the pure compound. Compound **1c** appeared as yellow solid powder, yield 57%, melting point = > 400 °C, ^{1}H NMR (DMSO-d_6): δ 11.79 (s, 2H, CONH), 8.34 (s, 2H, N=CH), 8.02 (s, 4H, Ar-H), 7.46 (s, 4H, Ar-H), 3.59 (s, 8H, $ArCH_2N$, peak overlapping with the water peak in DMSO-d6), 2.25 (s, 24H, $N(CH_3)_2$) ppm. HRMS (ESI): m/z 631.3700 ([M+1]$^+$), calcd 631.3715 for $C_{34}H_{47}N_8O_4$.

*4.1.7. 1,1'-(((Terephthaloylbis(hydrazin-2-yl-1-ylidene))bis(methaneylylidene))bis(6-hydroxy-3,1-phenylene))bis(N,N,N-trimethylmethanaminium) iodide (**1d**)*

To a solution of **1b** (200 mg, 0.39 mmol) in DMF (1.5 mL) iodomethane (3.1 mmol) was added. The mixture was stirred at room temperature for 24 h. After that excess iodomethane was evaporated using a rotary evaporator and the precipitate formed was collected by vacuum filtration, washed several times with acetonitrile to yield compound **1d**. The compound **1d** was further re-crystallized using minimum amount of boiling water. Yield 73%. melting point = > 400 °C, ^{1}H NMR (DMSO-d_6): δ 10.89 (s, 2H, CONH), 8.44 (s, 2H, N=CH), 8.04 (s, 4H, Ar-H), 7.82 (s, 2H, Ar-H), 7.75 (d, 2H, Ar-H, 8.3 Hz), 7.08 (d, 2H, Ar-H, 8.3 Hz), 4.54 (s, 4H, $ArCH_2N$), 3.09 (s, 18H, $N^+(CH_3)_3$) ppm. ^{13}C NMR (DMSO-d_6): δ 167.5, 164.5, 153.0, 141.4, 139.0, 138.9, 136.5, 133.0, 130.9, 121.9, 120.7, 68.0, 57.4 ppm. HRMS (ESI): m/z 273.1483 (M^{2+}), calcd 273.1472 for $C_{30}H_{38}N_6O_4$.

*4.1.8. 1,1',1'',1'''-(((Terephthaloylbis(hydrazin-2-yl-1-ylidene))bis(methaneylylidene))bis(2-hydroxybenzene-5,1,3-triyl))tetrakis(N,N,N-trimethylmethanaminium) iodide (**1e**)*

To a solution of **1c** (100 mg, 0.16 mmol) in DMSO (1.5 mL) iodomethane (3.2 mmol) was added. The mixture was stirred at room temperature for 24 h and excess iodomethane was evaporated using a rotary evaporator. After that acetonitrile was added dropwise to the reaction mixture until a precipitate formed. The mixture was cooled in an ice-water bath and the precipitate was collected by vacuum filtration, washed several times with acetonitrile to yield compound **1e** which was re-crystallized using minimum amount of boiling water to give pure **1e**. Yield 65%, melting point = > 400 °C, ^{1}H NMR (DMSO-d_6): δ 11.96 (s, 2H, CONH), 8.49 (s, 2H, N=CH), 8.05 (s, 4H, Ar-H), 7.97 (s, 4H, Ar-H), 4.70 (s, 8H, $ArCH_2N$), 3.11 (s, 36H, $N^+(CH_3)_3$) ppm. ^{13}C NMR (DMSO-d_6): δ 162.2, 158.9, 147.0, 136.0, 135.9, 127.7, 126.1, 118.0, 62.6, 52.0 ppm.

*4.1.9. N^1,N^4-bis(4-Hydroxybenzylidene)isophthalohydrazide (**2a**)*

Compound **2a** was prepared from isophthalohydrazide (200 mg, 1 mmol) and 4-hydroxybenzaldehyde (2.2 mmol) using the same procedure for preparing **1a**. Compound **2a** appeared as white solid powder, yield 92%, melting point = 311.3-315.1 °C, ^{1}H NMR (DMSO-d_6): δ 11.83 (s, 2H, CONH), 9.96 (s, br, 2H, Ar-OH), 8.44 (s, 1H, Ar-H), 8.39 (s, 2H, N=CH), 8.09 (d, 2H, Ar-H, 7.7 Hz), 7.66 (dd, 1H, Ar-H, 7.7, 7.8 Hz), 7.58 (d, 4H, Ar-H, 8.2 Hz), 6.85 (d, 4H, Ar-H, 8.2 Hz) ppm. ^{13}C NMR (DMSO-d_6): δ 162.9, 160.0, 149.0, 134.4, 131.0, 129.4, 129.2, 127.3, 125.7, 116.2 ppm. HRMS (ESI): m/z 403.1413 ([M+1]$^+$), calcd 403.1401 for $C_{22}H_{19}N_4O_4$.

*4.1.10. N^1,N^4-bis(3-((Dimethylamino)methyl)-4-hydroxybenzylidene) isophthalohydrazide (**2b**)*

Compound **2b** was prepared from isophthalohydrazide (200 mg, 1 mmol) and 3-((dimethylamino)methyl)-4-hydroxybenzaldehyde (2.2 mmol) using the same procedure for preparing **1b**. Compound **2b** appeared as yellow solid powder, yield 73%, melting point = >400 °C, ^{1}H NMR (DMSO-d_6): δ 11.83 (s, 2H, CONH), 8.43 (s, 1H, Ar-H), 8.36 (s, 2H, N=CH), 8.08 (d, 2H, Ar-H, 7.7 Hz), 7.67 (dd, 1H, Ar-H, 7.7, 7.8 Hz), 7.54 (s, 2H, Ar-H), 7.46 (d, 2H, Ar-H, 8.3 Hz), 6.81 (d, 2H, Ar-H, 8.3 Hz), 3.63 (s, 4H, $ArCH_2N$), 2.26 (s, 12H, $N(CH_3)_2$) ppm. ^{13}C NMR (DMSO-d_6): δ 162.8, 159.9, 149.0, 134.4, 131.0, 129.2, 128.7, 128.1, 127.3, 125.5, 124.0, 116.2, 60.5, 44.7 ppm. HRMS (ESI): m/z 517.2546 ([M+1]$^+$), calcd 517.2558 for $C_{28}H_{33}N_6O_4$.

*4.1.11. N^1,N^4-bis(3,5-bis((Dimethylamino)methyl)-4-hydroxybenzylidene) isophthalohydrazide (**2c**)*

Compound **2c** was prepared from isophthalohydrazide (200 mg, 1 mmol) and 3,5-bis((dimethylamino)methyl)-4-hydroxybenzaldehyde (2.2 mmol) using the same procedure for preparing **1c**. Compound **2c** appeared as yellow solid powder, yield 66%, melting point = >400 °C, ^{1}H NMR ($CDCl_3$): δ 11.90 (s, 2H, CONH), 8.29 (s, 1H, Ar-H), 8.21 (s, 2H, N=CH), 7.72 (br, 2H, Ar-H), 7.28 (s, 4H, Ar-H), 7.20 (dd, 1H, Ar-H, 7.2, 7.0 Hz), 3.50 (s, 8H, $ArCH_2N$), 2.25 (s, 24H, $N(CH_3)_2$) ppm. ^{13}C NMR ($CDCl_3$): δ 165.1, 159.6, 149.7, 133.7, 131.2, 128.7, 128.6, 124.4, 123.3, 59.9, 44.7 ppm. HRMS (ESI): m/z 631.3710 ([M+1]$^+$), calcd 631.3715 for $C_{34}H_{47}N_8O_4$.

*4.1.12. 1,1'-(((Isophthaloylbis(hydrazin-2-yl-1-ylidene))bis(methaneylylidene))bis(6-hydroxy-3,1-phenylene))bis(N,N,N-trimethylmethanaminium) iodide (**2d**)*

Compound **2d** was prepared from **2b** (200 mg, 0.39 mmol) using the same procedure for preparing **1d**. The compound **2d** was re-crystallized using minimum amount of boiling water. Yield 80%, melting point = > 400 °C, ^{1}H NMR (DMSO-d_6): δ 11.90 (s, 2H, CONH), 8.44-8.42 (1H, Ar-H and 2H, N=CH), 8.10 (d, 2H, Ar-H, 7.7 Hz), 7.82 (s, 2H, Ar-H), 7.75 (d, 2H, Ar-H, 8.5 Hz), 7.68 (dd, 1H, Ar-H, 7.7, 7.8 Hz), 7.08 (d, 2H, Ar-H, 8.5 Hz), 4.54 (s, 4H, $ArCH_2N$), 3.09 (s, 18H, $N^+(CH_3)_3$) ppm. ^{13}C NMR (DMSO-d_6): δ 162.9, 159.7, 148.2, 134.3, 134.2, 131.7, 131.1, 129.3, 127.5, 126.1, 117.2, 115.9, 63.2, 52.7 ppm. HRMS (ESI): m/z 273.1478 (M^{2+}), calcd 273.1472 for $C_{30}H_{38}N_6O_4$.

*4.1.13. 1,1',1'',1'''-(((Isophthaloylbis(hydrazin-2-yl-1-ylidene))bis(methaneylylidene))bis(2-hydroxybenzene-5,1,3-triyl))tetrakis(N,N,N-trimethylmethanaminium) iodide (**2e**)*

Compound **2e** was prepared from **2c** (57 mg, 0.10 mmol) in DMSO (0.30 mL) and iodomethane (3.3 mmol) using the same procedure for preparing **1e**. The compound **2e** was re-crystallized using CH_3OH/CH_2Cl_2 (3:7) mixture. Yield 90%, melting point = > 400 °C, ^{1}H NMR (DMSO-d_6): δ 12.01 (s, 2H, CONH), 10.51 (s, br, 2H, Ar-OH), 8.48 (s, 2H, N=CH), 8.42 (s, 1H, Ar-H), 8.12 (d, 2H, Ar-H, 7.6 Hz), 7.98 (s, 4H, Ar-H), 7.72 (dd, 1H, Ar-H, 7.4, 7.4 Hz), 4.71 (s, 8H, $ArCH_2N$), 3.11 (s, 36H, $N^+(CH_3)_3$) ppm. ^{13}C NMR (DMSO-d_6): δ 163.0, 159.2, 147.3, 136.5, 134.3, 131.2, 129.3, 127.6, 127.0, 118.6, 63.1, 52.6 ppm.

*4.1.14. N^1,N^4-bis(3-((Dimethylamino)methyl)-4-hydroxybenzylidene) phthalohydrazide (**3b**)*

Compound **3b** was prepared from phthalohydrazide (200 mg, 1 mmol) and 3-((dimethylamino)methyl)-4-hydroxybenzaldehyde (2.2 mmol) using the same procedure for preparing **1b**. Compound **3b** appeared as white solid powder, yield 79%, melting point = 191.3-193.0 °C, ^{1}H NMR (DMSO-d_6): δ 8.54 (s, 2H, N=CH), 8.07 (m, 2H, Ar-H), 7.87 (m, 2H, Ar-H), 7.62 (s, 2H, Ar-H), 7.59 (d, 2H, Ar-H, 8.8 Hz), 6.82 (d, 2H, Ar-H, 8.3 Hz), 3.63 (s, 4H, $ArCH_2N$), 2.25 (s, 12H, $N(CH_3)_2$) ppm. ^{13}C NMR (DMSO-d_6): δ 160.8, 160.7, 133.0, 132.0, 129.7, 129.3, 127.7, 125.6, 125.3, 123.8, 116.2, 60.5, 44.7 ppm. HRMS (ESI): m/z 517.2576 ([M+1]$^+$), calcd 517.2558 for $C_{28}H_{33}N_6O_4$.

*4.1.15. 4,4'-((1E,1'E)-Hydrazine-1,2-diylidenebis(methaneylylidene)) diphenol (4a) and 2,3-Dihydrophthalazine-1,4-dione (**4b**)*

To a solution of phthalohydrazide (200 mg, 1 mmol) in 20 mL methanol were added 4-hydroxybenzaldehyde (2.1 mmol) and 2-3 drops of acetic acid. The mixture was then refluxed at 65 °C for 5 h. After that about half of the solvent was evaporated using rotary evaporator and the mixture was cooled in an ice-water bath for 10 min. The precipitate formed was collected by vacuum filtration, washed with ice-cold methanol (about 1 mL) and finally dried in the oven at 50 ºC to give compound **4b**. The filtrate was collected, and the volume was reduced in a rotary evaporator to yield a yellow solid which was isolated by vacuum filtration and identified as compound **4a**.
Compound **4a** appeared as a yellow solid powder, yield 40%, melting point = 276.1-277.8 °C, ^{1}H NMR (DMSO-d_6): δ 10.08 (s, br, 2H, Ar-OH), 8.55 (s, 2H, N=CH), 7.69 (d, 4H, Ar-H, 8.1 Hz), 6.86 (d, 4H, Ar-H, 8.1 Hz) ppm. ^{13}C NMR (DMSO-d_6): δ 160.8, 160.7, 130.6, 125.6, 116.2 ppm. HRMS (ESI): m/z 241.0978 ([M+1]$^+$), calcd 241.0972 for $C_{14}H_{13}N_2O_2$. HRMS (ESI): m/z 241.0978 ([M+1]$^+$), calcd 241.0972 for $C_{14}H_{13}N_2O_2$.
Compound **4b** appeared as a white solid powder, yield 43%, melting point = > 400 °C, ^{1}H NMR (DMSO-d_6): δ 11.54 (s, 2H, CONH), 8.06-8.08 (m, 2H, Ar-H), 7.86-7.88 (m, 2H, Ar-H) ppm. HRMS (ESI): m/z 163.0508 ([M+1]$^+$), calcd 163.0502 for $C_8H_7N_2O_2$.

*4.1.16. 4,4'-((1E,1'E)-Hydrazine-1,2-diylidenebis(methaneylylidene)) bis(2,6-bis((dimethylamino)methyl)phenol) (**5a**)*

To a solution of phthalohydrazide (100 mg, 0.52 mmol) in 3 mL ethanol were added a solution of 3,5-bis((dimethylamino)methyl)-4-hydroxybenzaldehyde (255 mg, 1.1 mmol) in ethanol (5 mL) and 2-3 drops of acetic acid. The mixture was then refluxed at 85 °C for 24 hours. After about half of the solvent was evaporated using rotary evaporator and the mixture was cooled in an ice-water bath for 10 min. The precipitate formed was collected by vacuum filtration, washed with ice-cold ethanol (about 1 mL) and finally dried in the oven at 50 ºC to give compound **4b** (75 mg, yield 90%). The filtrate was collected, and the solvent was evaporated in a rotary evaporator to yield an oily residue. The residue was suspended in 2 mL of $CHCl_3$ and the undissolved material was removed by filtration. The clear chloroform solution was collected and evaporated to yield compound **5a** (290 mg, yield 62%). Compound **5a** appeared as oily liquid. ^{1}H NMR ($CDCl_3$): δ 11.77 (s, br, 2H, Ar-OH), 8.50 (s, 2H, N=CH), 7.55 (s, 4H, Ar-H), 3.69 (s, 8H, $ArCH_2N$), 2.36 (s, 24H, $N(CH_3)_2$) ppm. ^{13}C NMR ($CDCl_3$): δ 161.0, 160.3, 130.0, 124.9, 122.6, 59.3, 44.2 ppm.

4.2. Molecular modeling

The data in Fig. 3 was generated using a Spartan program (Spartan 2021). The calculated minimum energy of **1b**, **2b** and **3b** are 722.3, 651.4 and 634.2 kJ/mol, respectively.

4.3. Cytotoxicity assay

Evaluation of **1a**-**e**, **2a**-**e**, **3b**, **4a**,**b** and 5-FU against HCT116, HT29 and CRL 1790 was undertaken by a literature method (Vichai and Kirtikara 2006). In brief, HCT116, HT29 and CRL1790 cells were obtained from the ATCC. The HCT116 and HT29 cells were cultured in McCoy's 5A medium while the CRL1790 cells were cultured in Minimum Essential Medium. The assay medium was supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin was added to the culture medium. These bioassays were carried out for 48 h in triplicate on three different occasions.

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