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Design, synthesis, in silico and in vitro studies of novel 4-methylthiazole-5-carboxylic acid derivatives as potent anti-cancer agents

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ABSTRACT

Since inhibitors of mucin onco proteins are potential targets for breast cancer therapy, a series of novel 4-methylthiazole-5-carboxylic acid (**1**) derivatives **3a–k** were synthesized by the reaction of **1** with $SOCl_2$ followed by different bases/alcohols in the presence of triethylamine. Once synthesized and characterized, their binding modes with MUC1 were studied by molecular docking analysis using Aruglab 4.0.1 and QSAR properties were determined using HyperChem. All synthesized compounds were screened for in vitro anti-breast cancer activity against MDA-MB-231 breast adenocarcinoma cell lines by Trypan-blue cell viability assay and MTT methods. Compounds **1**, **3b**, **3d**, **3e**, **3i** and **3f** showed good anti-breast cancer activity. Since **1** and **3d** exhibited high potent activity against MDA-MB-231 cell lines, they show could be effective mucin onco protein inhibitors.

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It is almost counter-intuitive to believe that the complexity of a disease can be made even more complicated by the results of scientific research. Breast cancer is the most common malignancy in women and it accounts for nearly fifty per cent of cancer deaths in women. Like all chronic diseases breast cancer also poses a series of threats and difficulties, which may further lead to the development of mental problems in the patients. The incidence of breast cancer has been increasing steadily from one out of twenty in 1960 to one out of eight in women now.[1–4] Mammography is the most effective tool for screening and early detection of breast cancer in women.[5] Estrogens play crucial roles in breast cancer development and growth, and estrogen-stimulated growth in tumor cells requires estrogen receptors (ERs).[5–8] About two-thirds of human breast tumors reveal higher levels of ERs than normal breast tissues.[9] Inhibitions of proliferative pathways were considered an effective strategy to fight cancer and nowadays much attention has been paid to the discovery and development of new and more selective anti-cancer drugs.[9,10] A number of drug probables are preceded to clinical trials and few of them are in clinical use. It is a challenging target for synthetic chemists because of the complex structures of drug problems and handling of toxic starting materials and reagents. Under these circumstances, we aimed at the structural modification of 4-methylthiazole-5-carboxylic acid (**1**) to synthesize novel, safe and effective derivatives that can represent a promising pathway in search of new anti-cancer agents.

The present study is to predict and evaluate the efficacy of molecules against MUC1 onco protein, a member of mucin family. Mucins are the predominantly glycoproteins and they act as physical barriers and protect the apical borders of epithelial cells in adverse conditions. Mucins are largely unrecognized as effectors of carcinogenesis and intimately involved in breast malignancy. It also has been reported that mucins are predominantly over expressed in various human malignancies in addition to breast malignancy and their role in signaling cell growth and survival.

All of the mucin family proteins contain the tandem repeats of proline, threonine and serine residues which are called PTS domains and these domains are involved in the glycosylation process. MUC1 is the heavily glycosylated high molecular weight membrane protein comprising more than 50% of carbohydrate moiety

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and can be expressed by various epithelial cells. It is overexpressed in the entire cell membranes of carcinoma cells and allows them to interact with restricted receptors.[11] They contain large α sub unit and smaller β unit. The N-terminal region of α sub unit is fully exposed to cell surface and contains variable number of repeats containing PTS domains. Glycosylation of these repeats is altered in human carcinomas which in turn play a role in the immunosurveillance of cancer.[12,13] This N-terminal region is anchored to cell membrane through C-terminal region and blocks the cell–cell and cell–extracellular matrix interactions and when it is released the C-terminal region acts as a putative receptor which is engaged in signaling path ways related to tumor progression.[14]

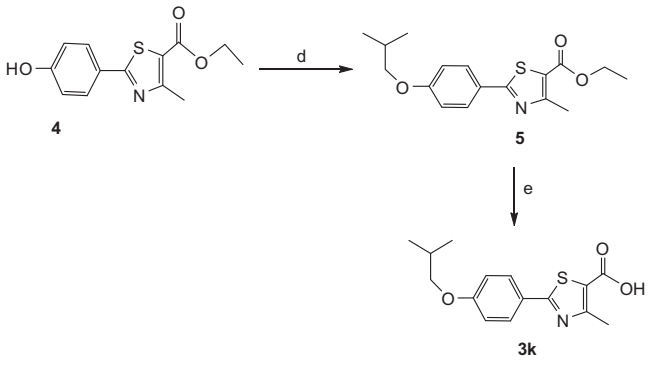
Hence, mucins are approved as therapeutic targets and adverse prognosis markers. In addition, Food and Drug Administration (FDA) approved MUC1 as a serum biomarker for breast cancer and targeting MUC1 is the ideal choice of controlling breast cancer.[15] Inhibitors of mucins function can become the promising agents to control and manage the breast cancerous condition.[14]

The derivatives (**3a**–**h**) of 4-methylthiazole-5-carboxylic acid (**1**) were prepared by the reaction of 4-methylthiazole-5-carboxylic acid (**1**) with thionyl chloride in the presence of catalytic amount of *N*,*N*-dimethyl formamide to form corresponding acid chloride **2** and its further reaction with substituted benzyl amines, aniline, 6-fluoro-3-(piperidin-4-yl)benzo[*d*]isoxazole and bioactive amines like cytosine, 5-fluoro-cytosine and alcohols in the presence of triehyl amine in toluene afforded the corresponding derivatives **3a**, **3b**, **3c**, **3d**, **3f**, **3g**, **3i**, **3j**, and **3e**, **3h** (Scheme 1 & Table 1). Substituted benzyl amines, 6-fluoro-3-(piperidin-4-yl)benzo[*d*] isoxazole[16] and aniline directly reacted with **2** in the presence of triethylamine as a base in toluene. But **2** did not react with cytosine and 5-fluoro-cytosine in the presence of triehylamine; hence they were prepared through silylated cytosine[17] and 5-fluoro-cytosine[17] in the presence of hexamethyldisilazane in toluene using catalytic amount of methane sulphonic acid and triethyl amine, to get **3i** and **3j**. Reaction of **4** with isobutyl bromide in the presence of K_2CO_3 and *N*-methyl-2-pyrrolidone produced **5** and it is further hydrolyzed to afford **3k** (Scheme 2).[18]

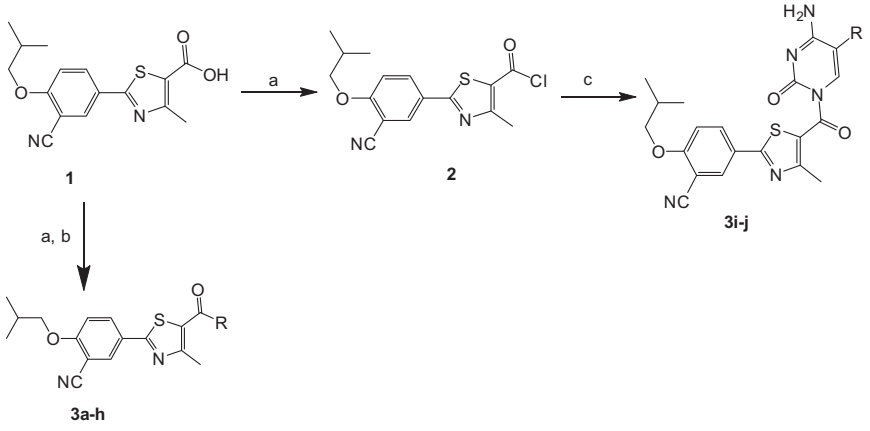
Characteristic IR stretching absorptions were observed in the regions 1245–1262 (O═C—N), 1530–1558 (N—H_{aliph}), 1685–1692 (C═O) for aliphatic and 1648–1655 for aromatic and 1661–1671 for piperazines, 2228–2232 (CN) cm^{-1}, respectively.[19,20] In the ^1H NMR spectra of compounds **3a**–**h**, NH proton chemical shift appeared in the region of 8.91–8.99 ppm[21] but this signal is not observed for **3e**, **3g**, **3h**, **3i** and **3j** indicating the amide group formation. In ^{13}C NMR spectra of compounds **3a**–**k**, chemical shifts were observed in the expected regions.[22] The chemical shift at 116.9–117.9 ppm is assigned to CN. ^{13}C chemical shift in the region 169.5–172.9 ppm is attributed to C═O of the amide group (see Schemes 1 and 2, Table 1).

The models were built for ligand molecules and molecular dynamics simulations were observed for a period of 10 ps. The total energy graphs of dynamics simulations showed that the confirmations were stabilized in the 10 ps dynamics run. The stabilized conformations of the ligands were saved and a QSAR study was carried out and their molecular descriptors were studied in HyperChem software tools.[23–25] All the ligand molecules were filtered with Lipinski filters. The drug likeness of the molecules was predicted from their molecular descriptors and Lipinski data.[23–25] Among all the molecules **3g** is showing a molecular weight of 518 Daltans which should be below 500 Daltans for any molecule to behave as a drug (Table 2). Hence **3g** may be antigenic to the host system. The remaining molecules are showing their descriptors in optimal range indicating their potential to behave as drugs.

The CASTp predicted binding site of MUC1 contain the amino acid residues Pro 1061, Tyr 1066, Gln 1070, Arg 1071, Ser 1074, Leu 1089, Ser 1090, Asn 1091 and Ile 1092. The molecular docking between the binding domain of mucin and stabilized conformations of the molecules showed that the lowest docking energy of −8.882 Kcal/mol was found with **3d** with the formation of two hydrogen bonds and highest docking energy of −4.387 Kcal/mol was found with **3j** with no hydrogen bond formation. Compounds **3c** & **3g** are not showing any docking energy, but forming single hydrogen bond which indicates the existence of weak interaction (Table 3). These docking results could explain that (except **3c** & **3g**)



Scheme 2. Synthetic route for the preparation of 2-(4-isobutoxyphenyl)-4-methylthiazole-5-carboxylic acid. Reagents and conditions: (d) iso butyl bromide, K_2CO_3, *N*-methyl-2-pyrrolidone, (e) 5% NaOH, acetone, 5% HCl.



Scheme 1. Protocol for the synthesis of 4-methylthiazole-5-carboxylic acid derivatives. Reagents and conditions: (a) $SOCl_2$, toluene, DMF, 90–95 °C. (b) R-H, TEA, toluene, 70–75 °C. (c) TEA, *N*-(trimethylsilyl)-2-((trimethylsilyl)oxy)-1,2-dihydropyrimidin-4-amine, 55–60 °C, H_2O.

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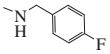
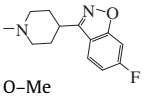
Table 1
Protocol for the synthesis of 4-methylthiazole-5-carboxylic acid derivatives

Entry	R	Time	Yield (%)	Mp (°C)
3a		2 h	85	142–149
3b		2.5 h	84	167–169
3c		3 h	80	172–174
3d		1.5 h	88	139–141
3e		20 min	95	148–150
3f		4 h	92	297–299
3g		3.5 h	85	164–166
3h	O–Me	10 min	98	160–162
3i	H	5 h	89	238–240
3j	F	5 h	91	282–284

all other molecules are having inhibitory activity against mucin protein. Hence, they may be considered as anti-inflammatory agents against mucin protein. The molecular interaction of all molecules with CASTp predicted domain of MUC1 protein is shown in Figure 1.

The anti-proliferative activity of newly synthesized 4-methylthiazole-5-carboxylic acid derivatives (**3a**–**k**) against MDA-MB-231 breast cancer cells was examined by Trypan blue and MTT assays. Compounds **1**, **3b**, **3d**, **3e**, **3f** and **3i** exhibited more potent inhibition against MDA-MB-231 cell lines when compared to others. The anti-proliferative activities of test compounds against MDA-MB-231 cells are shown in Figures 2 and 3 and compared the data with the standard Celecoxib.

Table 3
Docking energies and interacting atoms of the lead molecules with amino acid residues of binding domain of mucin protein

Molecule	Docking Energy (Kcal/mol)	No. of Hydrogen bonds	Hydrogen bond information Length(A°) Atom of molecule & Residue involved in H- bond formation
1	-8.307	5	2.966 A° (O &Arg 1071)
			2.659 A° (N &Gln 1070)
			2.636 A° (N & Tyr 1066)
			2.904 A° (O & Tyr 1066)
			2.473 A° (N & Ile 1092)
3a	0	4	2.361 A° (O &Arg 1071)
			2.164 A° (O &Gln 1070)
			2.746 A° (O & Tyr 1066)
			2.757 A° (N &Ser 1074)
3b	-7.190	1	2.466 A° (N &Leu 1089)
3c	0	1	2.058 A° (N &Leu 1089)
3d	-8.082	2	2.313 A° (N &Leu 1089)
			2.766 A° (O & Tyr 1066)
3e	-7.525	2	2.691 A° (N &Gln 1070)
			2.618 A° (N & Ile 1092)
3f	-6.850	3	2.899 A° (O &Gln 1070)
			2.193 A° (O &Gln 1070)
			2.426 A° (N &Leu 1089)
3g	0	1	2.908 A° (O & Tyr 1066)
3h	-5.336	3	2.942 A° (O &Arg 1071)
			2.886 A° (O &Gln 1070)
			2.953 A° (O & Tyr 1066)
3i	-7.704	4	2.999 A° (O &Arg 1071)
			2.888 A° (O &Arg 1071)
			2.504 A° (O &Arg 1071)
			2.883 A° (N &Leu 1089)
3j	-4.387	0	--------
3k	-5.826	2	2.752 A° (N &Ser 1074)
			2.901 A° (O &Ser 1074)

Table 2
Molecular descriptors of test molecules (**1** and **3a**–**k**)

Property	1	3a	3b	3c	3d	3e	3f	3g	3h	3i	3j	3k
Molecular Weight (Daltans)	316.00	382.00	423.00	423.00	423.00	372.00	391.00	518.00	330.00	470.00	427.00	319.00
Hydrogen Bond Donors	1	0	1	1	1	0	3	0	0	3	2	0
Hydrogen Bond Acceptors	3	2	4	4	4	4	6	5	4	6	7	4
LogP	1.35	2.2	2.90	2.86	2.86	0.00	4.569	2.70	1.63	1.59	0.94	3.08
Molar Refractivity (A^{03})	90.01	115.87	117.737	117.737	117.737	66.93	66.61	146.99	89.689	121.819	114.27	88.155
Surface area (A^{02})	503.53	385.68	631.82	653.36	656.77	689.11	723.26	705.31	553.12	640.14	557.51	572.09
Volume (A^{03})	997.85	1102.02	1308.30	1316.24	1313.43	1152.38	1317.57	1535.25	1051.09	1388.47	1239.47	1037.02
Hydration energy (K.cal/mol)	-12.15	-4.34	-3.99	-4.59	-6.02	-6.96	-18.90	-7.80	-7.97	-15.26	-9.93	-3.06
Polarizability (A^{03})	36.20	46.66	48.89	48.89	48.89	40.47	45.99	58.51	38.03	50.55	46.73	36.68
Gradient energy (K.cal/molA^{o})	0.096	0.087	0.099	0.097	0.099	0.093	0.099	0.099	0.099	0.099	0.099	0.089
Total energy (K.cal/mol)	97.779	217.771	119.001	134.196	125.194	85.192	114.316	166.611	101.002	167.109	123.368	99.653

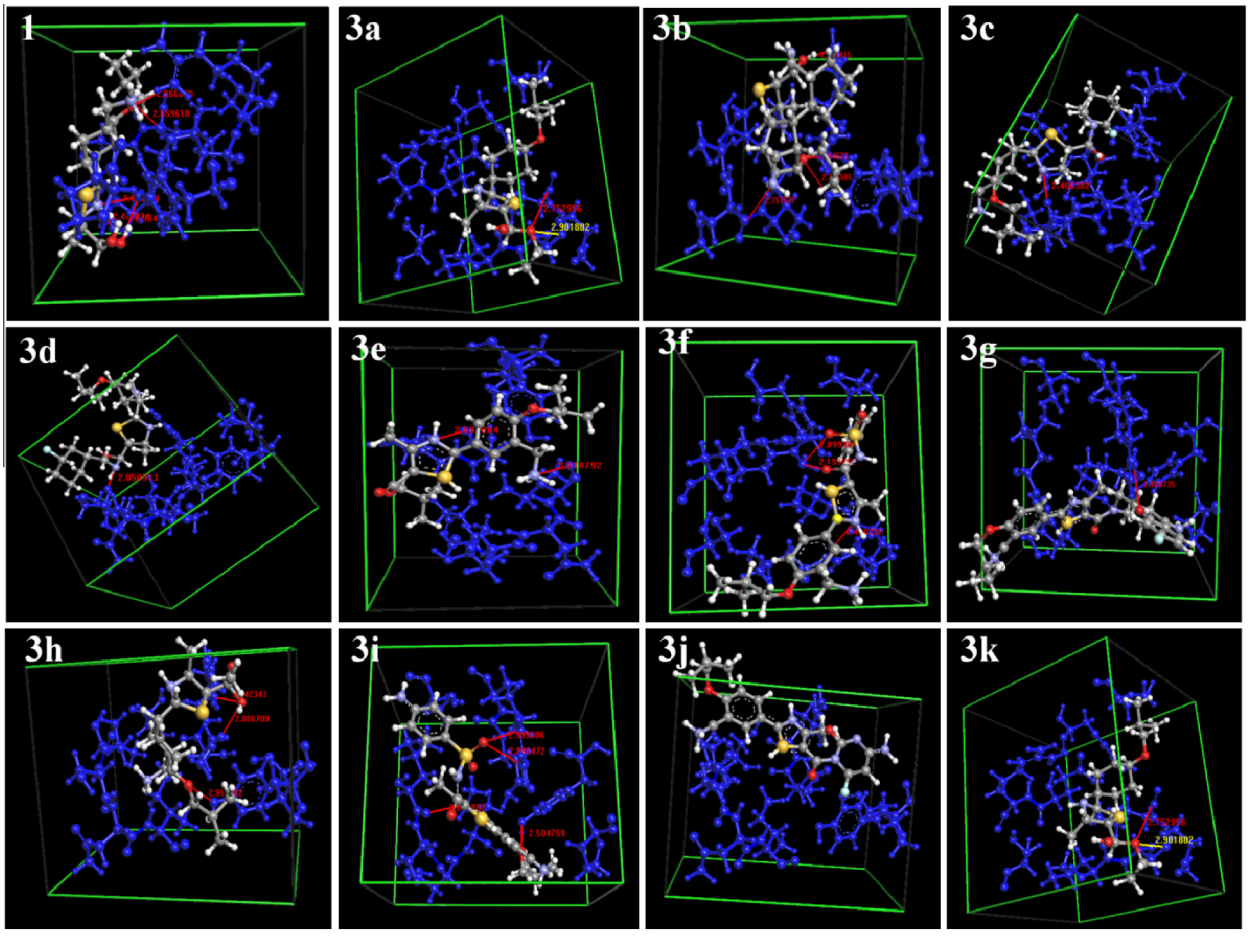


Figure 1. Image showing the molecular interaction of compounds **1**, **3a**, **3b**, **3c**, **3d**, **3e**, **3f**, **3g**, **3h**, **3i**, **3j** and **3k** with mucin. Compounds are in CPK colors and protein in indigo color. Red colored lines indicate the hydrogen bonds between the drug molecules and mucin protein.

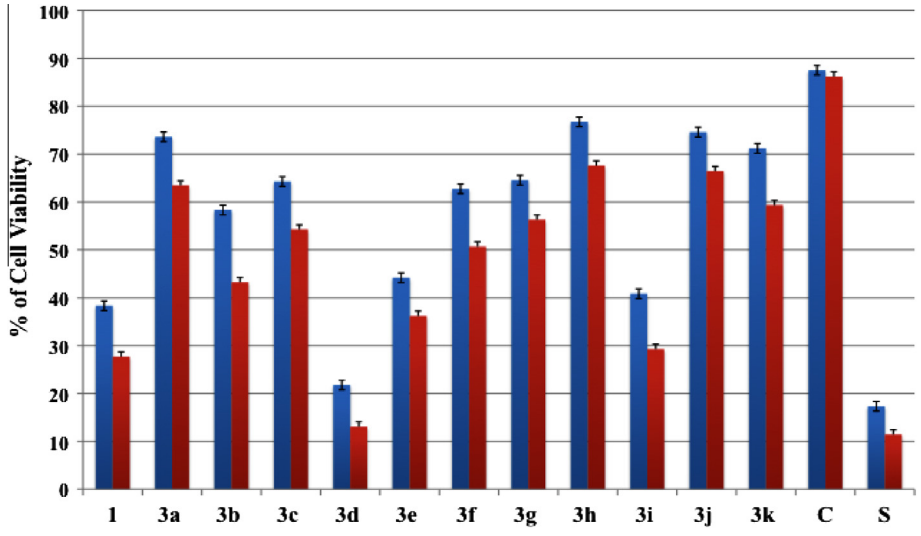


Figure 2. Cytotoxicity of test compounds against MDA-MB-231 cells by Trypan blue assay. Cytotoxicity of test compounds against MDA-MB-231 cells by Trypan blue assay at 10 μg/mL (Blue) and 100 μg/mL (Red) concentrations, respectively. C = control (DMSO), S = standard (Celecoxib), results mentioned as mean ± standard deviation for % of cell viability. *P*-Value <0.001.

In conclusion, the molecular descriptors from QSAR study, docking energies and in vitro assays on MDA-MB-231 adeno carcinoma breast cancer cell lines indicate that the molecules **1**, **3a**, **3b**, **3c**, **3d**, **3e**, **3j** and **3k** can be used as anti-cancer agents against mucin onco protein. The effectiveness from in vitro assays can form the basis to proceed with the molecules to the next phase of clinical trials to test their in vivo efficacy. The compounds **1**, **3b**, **3d**, **3e**, **3f** and **3i** exhibited higher anti-proliferative activity when

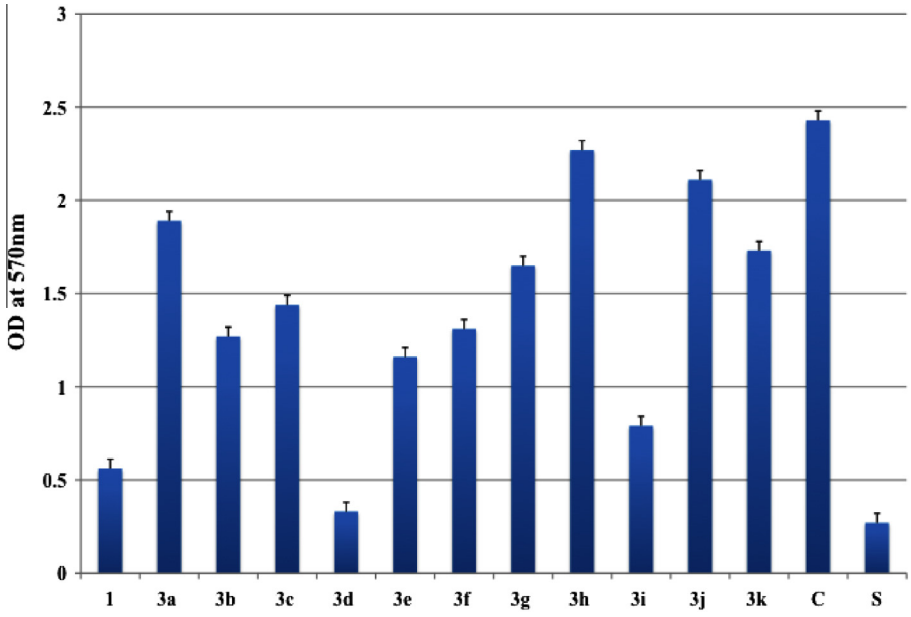


Figure 3. Anti-proliferative activity of test compounds against MDA-MB-231 cells by MTT Assay. Anti-proliferative activity of test compounds against MDA-MB-231 cells by MTT Assay. C = control (DMSO), S = standard (Celecoxib), results mentioned as mean ± standard deviation for OD values at 570 nm, *P*-Value <0.001.

compared with other derivatives. These results pave the way for future design and development of adeno carcinoma breast cancer compounds and also more potent drugs for cancer. The compounds **1** and **3d** can be considered as lead molecules which are to be screened in vivo to assess their efficacy.

General procedure for the synthesis of title compounds: A solution of 2-(3-cyano-4-isobutoxyphenyl)-4-methylthiazole-5-carboxylic acid (**1**) (1.5 mmol), thionyl chloride (1.5 mmol) and catalytic amount of dimethyl formamide in toluene (10 mL) was stirred at 90–95 °C for 3 h until clear solution was formed and formation of acid chloride was determined by TLC. The reaction mixture was cooled to 70 °C then slowly added phenylmethanamine (1.5 mmol) was added slowly in the presence of Et_3N and stirred for 2 h at 75 °C. Progress of reaction was monitored by TLC (methylene dichloride/methanol, 9:1 v/v). The solid separated was filtered off, washed with toluene (10 mL) followed by washed with ice-cold water (10 mL) and then recrystallized from 2-propanol to afford pure product. This general synthetic protocol was followed for the preparation of **3a**–**h** by reacting 4-methylthiazole-5-carboxylic acid chloride (**2**) with various amines and alcohols in the presence of Et_3N in toluene.

Molecular dynamics and QSAR study of ligand molecules: The models were constructed in HyperChem software tool and optimization was done.[26,27] A molecular dynamics study was carried out in AMBER 99 force field for a period of 10 pico seconds (ps) at 300 K and the stabilized confirmations and their energy values were saved. QSAR study was carried out to know the molecular descriptors of the molecules and their reactivity against mucin protein.

Preparation of MUC1 protein: The X-ray crystallographic structure of MUC1 (PDB ID: 2ACM) was retrieved from Protein Data Bank (http://www.rcsb.org/pdb/home/home.do).[28,29] Except A-chain all other chains and water molecules were removed, hydrogen atoms were added and energy minimization was done to get the stable confirmation of the protein.

Prediction of binding site on MUC1: The binding site of MUC1 protein was predicted from CASTp server[30,31] (Computed Atlas of Surface Topography of proteins) which predicts the binding regions based on the pocket algorithm of the alpha shape theory. This algorithm facilitates the accurate computation of surface area and volume of numerous binding cavities from high resolution structures where small molecules can be accommodated.

Docking analysis: Individual dockings were carried out between the binding site of mucin and all the molecules using Arguslab 4.0.1 free software developed by Mark A. Thompson, Planaria Software LLC, Seattle, WA (http://www.arguslab.com/downloads.htm).[32,33] After docking process, the docking poses with best energies were saved and analyzed for the reactivity of all ligand molecules with binding site of mucin.

Pharmacology: Cell lines and culturing. The MDA-MB-231 human breast adenocarcinoma cells were routinely maintained as monolayer in DMEM medium supplemented with 2 mM/L glutamine (Himedia), 10% fetal bovine serum (FBS, Himedia) and 10 μg/mL ciprofloxacin in humidified incubator (Binder) containing 5% CO_2, 95% air at 37 °C.

Trypan blue assay: The MDA-MB-231 cells were seeded at 2×10^5 cells per well in 6-well tissue culture plates in DMEM media supplemented with 10% FBS, incubated for 24 h at 37 °C in CO_2 incubator for cell viability assay by Trypan blue dye exclusion.[34] Later the medium was replaced with fresh medium containing various concentrations of synthesized compounds and 0.1% of DMSO (served as solvent control), incubated for 24–48 h at 37 °C. Celecoxib, a potent inhibitor of MDA MB 231 cell lines was used as Standard control to evaluate the potency of the test compounds.[35] To assess the cell viability after incubation aliquots from both floating and trypsinized adherent cells were mixed with a 0.4% trypan blue solution in 1:1 (v/v) and loaded on to a haemocytometer. The cells were observed and counted under light microscope, live cells are clear and the dead cells are blue in color. The concentration of the compound that inhibited cell growth by 50% (IC_{50}) was determined from cell survival plots. Data are expressed as mean ± SE ($n = 3$) percentage of viable cells per sample was calculated by;

$$\text{Viable cells}(\%) = [(\text{Total cells} - \text{Dead cells})/\text{Total cells}] \times 100\%.$$

MTT assay: Cell proliferation was assayed by MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay as described by Mosmann.[36] The exponentially growing MDA-MB-231 cells were collected and resuspended in fresh culture

medium with 10% FBS. Before the treatment with synthesized compounds and the standard (Celecoxib), cells were washed with PBS and fresh medium was added and incubated in 96-well plates in the presence or absence of various concentrations of synthesized compounds for 24 h in a final volume of 100 μL. At the end of the treatment, 20 μL of MTT (5 mg/mL in PBS) was added to each well and incubated for an additional 4 h at 37 °C. The purple-blue formazan precipitate was dissolved in 100 μL of DMSO and the optical density was measured at 570 nm on Bio-Rad, micro titer plate reader. An increase in number of living cells results in an increase in the total metabolic activity in the sample. This increase directly correlates to the amount of purple formazan crystals formed. Data are expressed as mean ± SD, (n = 3).

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Supplementary data

Supplementary data (spectral data for all the title compounds and Tables 4 and 5, and ^{1}H and ^{13}CNMR spectra of the title compounds) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.07.058.

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