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RESEARCH ARTICLE

Substituted chloroacetamides as potential cancer stem cell inhibitors: Synthesis and biological evaluation

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Abstract

Cancer kills, irrespective of geographical and cultural origin. Novel modalities for treating cancer are desperately needed. Cancer stem cells (CSCs), main culprits behind chemoresistance and tumor relapse, are one of the few logical choices. Herein, we report the synthesis and biological evaluation of small molecules with chloroacetamide war-head. These molecules were screened for viability against various breast, prostate, and oral cancer cell lines using MTT and soft-agar assays. Further, promising hits were screened in sphere-forming assay with the aim of discovering potential anti-CSC agents. Our optimism yielded four hits inhibiting self-renewal of cancer cells with stem-like characters in vitro. Finally, the hits were evaluated for in vitro toxicity against human peripheral blood mononuclear cells and mouse embryonic fibroblast cell line. Overall, these preliminary investigations yielded three hits exhibiting promising anti-CSC potential with little or no toxicity against normal cells.

KEYWORDS

breast cancer, cancer stem cells, chloroacetamides, CSCs, oral cancer, prostate cancer, soft-agar assay, sphere-forming assay

1 | INTRODUCTION

Cancer, one of the most dreadful diseases in the history of mankind, is characterized by uncontrolled, abnormal cell growth, and metastasis. Despite the availability of efficacious drugs in the anticancer armamentarium and other treatment modalities such as surgery and radiation therapy, cancer is mostly incurable; metastasis being the most notable reason (Anderson et al., 2019). Recent statistics on cancer is horrifying. In the United States alone, 1.7 million new cases were registered in 2018 and by year 2030, more than 20 million cases will be prevailing worldwide, as speculated (Cancer Statistics, 2019). It is a growing cause of concern in both the developing and the developed nations affecting millions of people.

Apart from the adverse effects associated with the treatment which drain the patient mentally, physically, and financially, the chances of relapse in majority of the cases are quite high. The recently proposed cancer stem cell (CSC) hypothesis challenged the conventional clonal hypothesis of cancer development and paved a way toward preventing tumor relapse and metastasis (Yu, Pestell, Lisanti, & Pestell, 2012). The identification of highly chemoresistant, cancer-initiating CSCs responsible for the growth, metastasis, and relapse of the disease has rekindled the spirit to battle the disease (Hanahan & Weinberg, 2011). Compared to the phenotypical stem cells, the CSCs exhibit unique characteristics (self-renewal, differentiation, and tumorigenicity), which enable them to divide asymmetrically producing an identical daughter cell and a more differentiated cell, which later forms the bulk of the tumor (Ratajczak, Bujko, Mack, Kucia, & Ratajczak, 2018; Yu et al., 2012). Being less susceptible to chemo- and radiotherapies compared to the bulk tumor, CSCs are responsible for the treatment failure and cancer recurrence. Thus, based on the characteristic nature of CSCs, targeting and exterminating CSCs represents a budding approach for significantly improving clinical outcome or for possibly achieving complete eradication of cancer.

The research on CSCs has matured to an extent that it gives us fairly complete picture of the association between CSCs and the deadly disease including its relapse (Soundararajan, Paranjape, Maity, Aparicio, & Mani, 2018). We now have far better idea about the pathways and molecular targets associated with the CSCs. This information can help us in differentiating CSCs from the normal stem cells and normal cells of the body (Ratajczak et al., 2018). Despite the identification and targeting of many pathways that control cancer growth, we are still far from complete eradication of the disease. Even though several regulatory signaling pathways are known, the major challenge lies in identifying the underlying targets unique to CSCs making up the druggable hotspot. Significantly large number of research groups (including the authors') worldwide has been working on the development of anti-CSC therapeutics (Kaur, Sharma, Dogra, & Singh, 2018; Kharkar, 2017; Luo & Wicha, 2019; Saeg & Anbalagan, 2018; Skoda et al., 2018; Yadav et al., 2018). The most advanced anti-CSC therapeutics, napabucasin (BBI-608), an orally administered small-molecule investigational agent proposed to be an inhibitor of cancer stemness pathways, is currently in Phase III clinical trials in combination with 5-fluorouracil (NCT02753127) for the treatment of previously treated metastatic colorectal cancer (mCRC; Canstem 303c Trial, 2019). The search for novel, effective anti-CSC therapeutic is on, for the radical change it is expected to bring about in the cancer therapy arena.

In the author's lab, while working on a series of 4-aryl-3-chloro-2-azetidinones (**1**, 1) as potential anti-CSC agents, we tested an intermediate of the title compounds represented by **2**, in a battery of assays comprising of MTT, soft-agar, and sphere formation, against a couple of breast and prostate cancer cell lines. To our surprise, intermediate **2** exhibited quite potent, submicromolar cellular activity against the cell lines tested. Further, we searched DrugBank (Wishart et al., 2018) for molecules similar to **2** (substructure search with default settings) for understanding their potential macromolecular targets. The hits are listed in Table 1. We reasoned that the electrophilic "war-head," that is, the chloroacetamide moiety, could potentially modify certain biological macromolecules covalently as long as they selectively bound to them. Further, it was hypothesized that if these chloroacetamides were to behave as general toxins (owing to the presence of war-head), they should exhibit potent cell-killing activity irrespective of the nature of the aryl/arylalkyl group attached to the amide N. On the other hand, if these molecules were to bind selectively (even if covalently) to their putative molecular target(s), then the nature of the N-aryl/arylalkyl group would affect the biological activity due to underlying ligand–receptor binding kinetics. Also, the aryl/arylalkyl group may potentially influence the cell permeability with implications on the activity. Given the tiny nature of these molecules and differential activity despite the presence of a "war-head," we were pretty convinced that these molecules were hitting some target systematically (as inferred from the structure–activity relationship). These targets could be kinases, ion channels, or enzymes, critical for the survival of the cancer, and/or CSC. Intrigued by the biological results of **2** and the literature reports, a full-fledged design, synthesis, and biological evaluation of a focused library based on chloroacetamide core structure was undertaken.

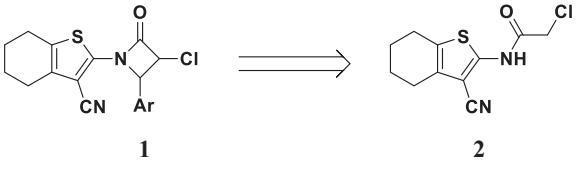


CHART 1 Serendipitous design strategy of the 2-chloroacetamide series

Around the same time, Roberts et al. (2017) published an interesting study featuring chemoproteomic screening of cysteine-reactive fragment-based covalent ligands library belonging to acrylamide and 2-chloroacetamide series in search of antipancreatic cancer therapeutics. The authors started off with the sole aim of impairing pancreatic cancer cells along with identification of druggable hotspots. The study resulted in successful identification of a covalent ligand with pancreatic cancer cell killing in vitro and inhibiting tumor growth in vivo. The ligand covalently modified the catalytic cysteine of the ubiquitin-like modifier activating enzyme 5 (UBA5), which was involved in activating the ubiquitin-like protein UFM1 to UFMylate proteins. In a similar study, anticancer natural product withaferin A and its cysteine-reactive covalent ligand selectively targeted breast cancer signaling, proliferation, and in vivo tumor growth (Grossman et al., 2017). The authors screened a library of cysteine-reactive covalent ligands against 231MFP breast cancer cells in order to discover compounds with phenotypes similar to withaferin A. The screening process identified few hits, the best one being a synthetically easily tractable chloroacetamide derivative.

These investigations, along with our in-house efforts and literature, motivated us to further evaluate the medicinal chemistry-intuitive, designed compounds in the ongoing anti-CSC therapeutics programme to supplement our efforts to develop a novel anti-CSC therapeutic for breast and prostate cancers, two major types of cancers occurring globally. In this study, we report a focused library of 14 structurally-diverse small molecules containing chloroacetamide war-head.

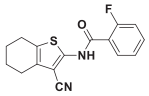
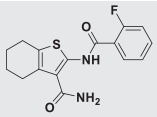
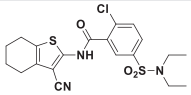
2 | MATERIALS AND METHODS

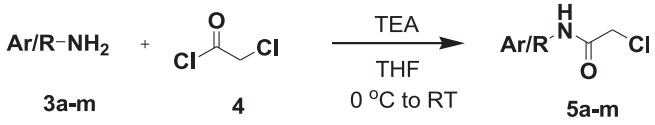
2.1 | Chemistry

2.1.1 | General

All common reagents or materials were obtained from commercial sources and used without further purification. All solvents were dried prior to use with appropriate drying agents. Column chromatography was performed using silica gel 60 (#230-400). Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel plates with QF-254 indicator and visualized under short UV (λ254 nm). Proton nuclear magnetic resonance (^{1}H-NMR) spectra were obtained on Bruker NMR 400 instrument (Billerica, MA), and chemical shifts were given in ppm from tetramethylsilane (TMS) as internal standard. The purity of the compounds (≥95%) were determined using HPLC conducted on Agilent 1220 Infinity system (Santa Clara, CA), using a C18Kromasil® column (25 cm × 4.6 mm, 5 μl, 100 Å pore size). Mass

TABLE 1 Hits from substructure search based on query **2** from DrugBank

Sr. No.	DrugBank ID	Structure	Status	Molecular target
1	DB07217		Experimental drug	MAP kinase 10
2	DB07398		Experimental drug	Serine/threonine-protein kinase PknG
3	DB06790		Experimental drug	UDP-*N*-acetylmuramoyl-tripeptide-D-alanyl-D-alanine ligase



SCHEME 1 Synthesis of substituted chloroacetamides (**5a-m**)

spectral data were collected on Shimadzu 8040 LC-MS/MS system (Kyoto, Japan). The FT-IR spectra were recorded on Perkin Elmer RX1 instrument (Waltham, MA).

2.1.2 | General procedure for the syntheses of chloroacetamide derivatives (2, 5a-m)

A 100-ml round-bottomed flask was charged with anhydrous THF (10 ml) and substituted amine **3a-m** (1 mmol; Scheme 1) was stirred and cooled in ice-bath till complete dissolution of the solid material. Triethylamine (TEA; 1.5–3 mmol, 1.5–3 equiv.) was added to the flask, followed by dropwise addition of cold solution of chloroacetyl chloride **4** (1.5 mmol, 1.5 equiv.) in anhydrous THF (10 ml) while maintaining the temperature below 5°C. The resulting solution was allowed to stir at RT overnight. On completion of reaction, as monitored by TLC, the solvent was evaporated under reduced pressure and the residue was washed with ice-cold water and the precipitated solid was filtered off at the suction pump. The crude product was then quickly purified by syringe column chromatography using 20–30% ethylacetate/hexane as eluent.

2.2 | Biology

2.2.1 | Cell lines and reagents

The cells were obtained from National Centre for Cell Science (NCCS; Pune, India). Cells used were in culture for no more than 3 months. Briefly, the cells were maintained in complete media with 10% fetal bovine serum (Gibco Life Technologies, Grand Island, NY). Unless otherwise noted, cells were seeded at 40% confluency for all assays.

50X B27 Supplement (Life Technologies, Invitrogen, Catalogue No.: 17502-044), fibroblast growth factor (FGF; Sigma-Aldrich, Catalogue No.: F029125), epidermal growth factor (EGF; Sigma-Aldrich, Catalogue No.: E9644), insulin (Sigma, Catalogue No.: 19278), Dulbecco's Modified Eagle Medium/F12 (DMEM-F12; HiMedia, Catalogue No.:AL139-6), minimum essential media (HiMedia, Catalogue No.: AL255G), Rosewell Park Memorial Institute medium 1640 (RPMI 1640; HiMedia, Catalogue No.: AL223A), Dulbecco's phosphate buffered saline (HiMedia, Catalogue No.: TL1006), Trypan Blue (TCI 93), prostate epithelial media (LONZA, Catalogue No.: CC-3166), MEGM (LONZA, Catalogue No.: CC-3051), Heparin (Sigma, Catalogue No.: H3393), Penstrep (HiMedia, Catalogue No.: A002).

2.2.2 | MTT assay

Briefly, the cells were plated in 96-well plate as per predetermined plating efficiency (Table S2). The plates were then incubated for 24 hr in 5% CO_2 atmosphere at 37°C. Appropriate concentrations of the test compounds were then added to the plate and further incubation was carried out for 48 hr (in 5% CO_2 atmosphere at 37°C). The assay plate was then centrifuged twice at 3,000 rpm for 3 min and supernatant was then discarded. A total of 100 μl of MTT solution (0.5 mg/ml) was then added to each well of the plate and it was further incubated for 4 hr (in 5% CO_2 atmosphere at 37°C). Following 4 hr incubation, the plate was then centrifuged twice, and supernatant was aspirated off very carefully. Then, 200 μl of DMSO was then added to each well to solubilize MTT crystals and mixed well by shaking the plate. The XY graph of log % viability was then plotted against log drug concentration. The IC_{50} (test compound concentration inhibiting 50% of cell population) was then calculated by regression analysis.

2.2.3 | Soft-agar assay

Briefly, a mixture of 50 μl of 2× medium (taken appropriately as per cell line) and 50 μl of 1.2% Bacto™ Agar were plated onto each well of 96-well microtiter assay plate. A 10 μl of cells (of specific plating efficiency prestandardized for respective cell line, Table S3) were mixed with 20 μl of 2× medium and 30 μl of 0.8% of Bacto™ Agar and 1.6 μl of test compound (of appropriate concentration) in a vial and transferred to the

solidified pre-layers of the assay plates. The cells were then allowed to grow and form colonies at 37°C and 5% CO_2 for 1 week. An intermittent feeding with 50 μl of appropriate 2× medium was performed after 3 days of experimental set up. Then l6 μl of alamarBlue® (1.5 mg/ml) was then added to all the wells to quantify the developed colonies. The plates were incubated for 24 hr at 37°C. Absorbance was then measured at 630 nm. The XY graph of log % viability was then plotted against log drug concentration. The IC_{50} (test compound concentration inhibiting 50% of cell population) was then calculated by regression analysis.

2.2.4 | Sphere assay

Serum-free media under low attachment condition induces sphere formation. Only CSCs (prepared as single-cell suspension) can survive this condition and grow in the form of spheres. Depending upon the cell line used the serum-free media was prepared as under: mammosphere media preparation (for 100 ml): methyl cellulose (1 g) added to plain media (mammosphere epithelial basal medium; 100 ml) and dissolved under magnetic stirring. After complete dissolution, FGF-80E, EGF-40 μL, Penstrep-1 ml, Heparin-400 μl were added.

Prostosphere media preparation (for 100 ml): methyl cellulose (1 g) added to plain media (prostate epithelial basal medium; 100 ml) and dissolved under magnetic stirring. After complete dissolution, Insulin-40 μl, B27-2 ml, EGF-80 μl, Penstrep-1 ml was added.

Briefly, the cells were trypsinized and made into single-cell suspension by passing through cell strainers (100 μl and 40 μl, respectively). The cells were diluted at a concentration of 2,000 cells/100 μl and suspended in either mammosphere (for breast cell lines) or prostosphere (for prostate cell lines) medium. Then 100 μl of this suspension was added into each well of 96-well suspension plates and incubated at 37°C, 5% CO_2 for 24 hr. Appropriate concentrations of the test compounds (2 μl) were added into the respective wells with 100 μl of stem cell culture medium. Plates were incubated at 37°C, 5% CO_2 for 72 hr. After incubation 2.5 μl of the respective test compound concentration and 50 μl of stem cell culture medium were added into each well and plates were further incubated at 37°C, 5% CO_2 for 72 hr. Then 3 μl of the respective test compound concentration was added with 50 μl of stem cell culture medium again after incubation and plates were incubated again for 72 hr at 37°C, 5% CO_2. The number of primary spheres formed for each concentration was counted. Live spheres appear to be bright and transparent under phase contrast microscopy (Figure 1). The spheres were counted/scored manually at the end of experimental period and % viability for each concentration was calculated by using following formula

$$\%\text{viability} = \frac{\text{Number of spheres formed per well} \times 100}{\text{Number of spheres formed in Control well}}$$

A comparative graph of the number of spheres formed was then plotted against the concentration and the growth curve was compared with the positive control.

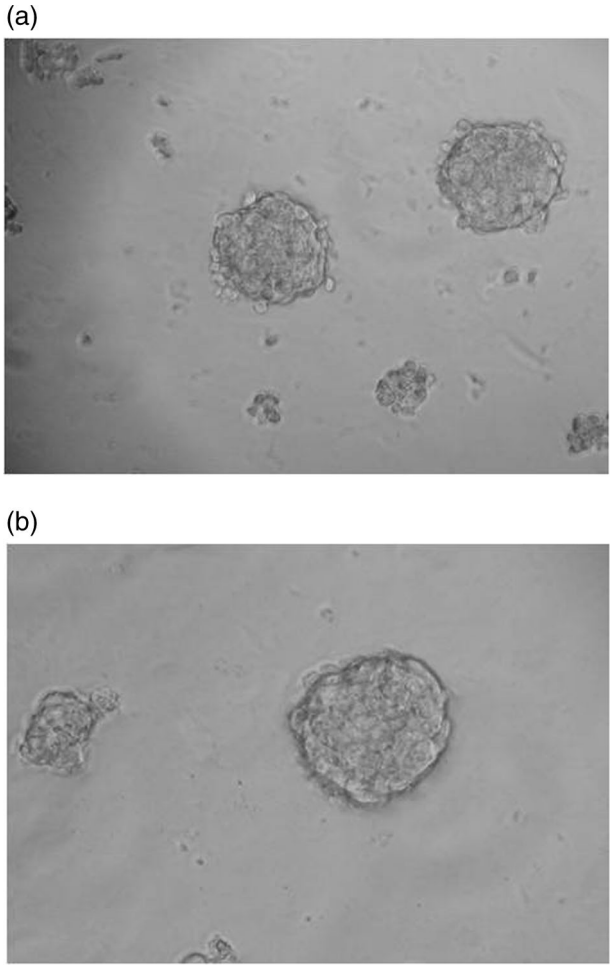


FIGURE 1 Images of (a) mammospheres (MDA-MB-231) (b) prostatospheres (PC-3) under 20× magnification

2.2.5 | hPBMC assay

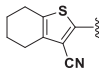
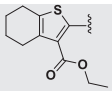
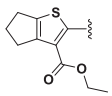
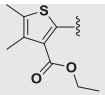
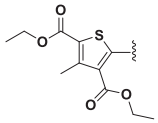
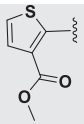
This assay was performed using freshly isolated lymphocytes from blood of a healthy donor. Briefly, diluted defibrinated fresh blood was overlaid gradually on HiSep™ LSM1077 and centrifuged at low speed for 30 min. The lymphocyte layer (the buffy coat) was carefully removed in a new collection tube. The buffy coat was given another wash, by the diluent buffer, to reduce the platelet contamination. The supernatant was discarded, and the pellet was resuspended in diluent buffer. The viability was checked by hemocytometer. Viable cells with at least 95% purity were considered for the assay. The MTT assay was performed with these cells as described above with plating efficiency of 0.7 million/ml.

3 | RESULTS AND DISCUSSION

3.1 | Chemistry

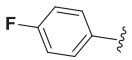
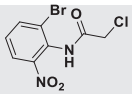
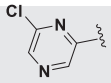
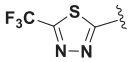
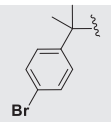
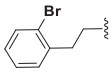
The title compounds were synthesized by reacting various aliphatic and (hetero)aromatic amines with chloroacetyl chloride in presence of

TABLE 2 Median effect of chloroacetamide derivatives on viability of human breast, prostate and oral cancer cell lines[a]

Compound	Structure	IC_{50} values (μM)[b] in cell lines[c]								
		MDA-MB-231	MCF7	T-47D	DU145	LNCaP	PC-3	KB	KB-3-1	KB-Ch^R-8-5
Cisplatin	–	61.94 ± 0.15	26.61 ± 0.03	77.62 ± 0.07	24.15 ± 0.07	33.49 ± 0.02	22.91 ± 0.14	35.48 ± 0.15	24.54 ± 0.07	28.18 ± 0.04
Sunitinib	–	3.15 ± 0.09	3.05 ± 0.06	3.61 ± 0.05	1.93 ± 0.07	2.82 ± 0.04	2.11 ± 0.07	2.51 ± 0.02	1.73 ± 0.06	1.86 ± 0.1
2		2 ± 0.05	2.19 ± 0.06	0.33 ± 0.01	1.78 ± 0.06	nt	1.81 ± 0.09	nt		
5a		5.48 ± 0.06	7.87 ± 0.05	nt	2.07 ± 0.11		3.57 ± 0.03			
5b		4.35 ± 0.21	4.8 ± 0.13		5.23 ± 0.06		3.86 ± 0.07			
5c		7.87 ± 0.08	11.27 ± 0.1		9.18 ± 0.16		4.75 ± 0.06			
5d		10.14 ± 0.1	14.76 ± 0.12		7.36 ± 0.04		2.72 ± 0.09			
5e		0.314 ± 0.05	7.67 ± 0.09	>100	1.73 ± 0.06	24.66 ± 0.03	4.04 ± 0.09	30.90 ± 0.14	4.16 ± 0.1	16.98 ± 0.07

(Continues)

TABLE 2 (Continued)

Compound	Structure	IC_{50} values (μM)[b] in cell lines[c]								
		MDA-MB-231	MCF7	T-47D	DU145	LNCaP	PC-3	KB	KB-3-1	KB-Ch^R-8-5
5f		9.77 ± 0.11	18.41 ± 0.1	69.18 ± 0.2	1.12 ± 0.15	14.35 ± 0.05	6.68 ± 0.07	>100	4.46 ± 0.09	67.6 ± 0.25
5g		19.72 ± 0.06	18.75 ± 0.04	nt	8.71 ± 0.06	2.15 ± 0.02	nt			
5h		>100	>100	66.22 ± 0.13	24.6 ± 0.25	41.4 ± 0.06	>100	>100	61.65 ± 0.13	>100
5i		0.304 ± 0.08	3.6 ± 0.03	4.62 ± 0.06	2.72 ± 0.07	2.88 ± 0.03	2.29 ± 0.09	7.58 ± 0.1	3.89 ± 0.27	7.94 ± 0.12
5j		3.37 ± 0.16	>100	>100	44.05 ± 0.21	12.19 ± 0.05	4.09 ± 0.04	>100	18.92 ± 0.11	>100
5k		0.439 ± 0.12	>100	9.79 ± 0.07	5.90 ± 0.11	22.18 ± 0.06	23.07 ± 0.14	>100	7.58 ± 0.21	89.12 ± 0.08
5l		12.36 ± 0.04	8.69 ± 0.03	nt	4.74 ± 0.07	2.35 ± 0.03	nt			
5m		0.729 ± 0.11	13.77 ± 0.09	13.61 ± 0.16	1.06 ± 0.05	9.2 ± 0.02	6.14 ± 0.05	27.54 ± 0.07	4.89 ± 0.13	7.94 ± 0.16

Abbreviation: nt, not tested.

[a]Determined by MTT assay.

[b]Compounds tested in triplicate.

[c]Cancer cell line origin: Breast cancer cell lines: MDA-MB-231, MCF7, T-47D; prostate cancer cell lines: DU 145, PC-3, LNCaP, and oral cancer cell lines: KB, KB-3-1, and KB-Ch^R-8-5.

TABLE 3 Median effect of chloroacetamide derivatives on viability in human breast, prostate, and oral cancer cell lines in soft-agar assay[a]

	IC_{50} values (μM)[b] in cell lines[c]					
Compound	**MDA-MB-231**	**DU 145**	**PC-3**	**KB**	**KB-3-1**	**KB-Ch^R-8-5**
Cisplatin	35.56 ± 0.15	33.11 ± 0.16	25.82 ± 0.24	33.73 ± 0.08	41.4 ± 0.02	21.57 ± 0.11
Sunitinib	2.8 ± 0.07	1.13 ± 0.04	2.64 ± 0.25	3.71 ± 0.09	4.13 ± 0.01	1.74 ± 0.05
2	1.83 ± 0.05	0.27 ± 0.04	4.2 ± 0.12	nt		
5a	1.46 ± 0.08	4.14 ± 0.03	nt			
5b	2.22 ± 0.13	2.39 ± 0.2				
5c	2.18 ± 0.04	4.5 ± 0.13				
5d	2 ± 0.12	2.71 ± 0.06				
5e	3.09 ± 0.05	1 ± 0.08	3.09 ± 0.08	3.98 ± 0.03	14.93 ± 0.03	1.59 ± 0.12
5f	nt	1.91 ± 0.09	1.97 ± 0.2	2.63 ± 0.06	22.38 ± 0.11	2.07 ± 0.11
5h		30.48 ± 0.18	28.84 ± 0.22	>100	>100	>100
5i	3.52 ± 0.17	3.01 ± 0.05	1.41 ± 0.16	11.53 ± 0.05	4.71 ± 0.01	2.82 ± 0.21
5j	nt	3.42 ± 0.07	2.75 ± 0.15	11.43 ± 0.07	>100	8.93 ± 0.06
5k		2.53 ± 0.07	2.09 ± 0.14	9.68 ± 0.07	>100	3.18 ± 0.12
5m	3.28 ± 0.05	1.16 ± 0.06	2.11 ± 0.29	4.46 ± 0.05	7.64 ± 0.02	3.24 ± 0.13

Abbreviation: nt, not tested.

[a]Determined by soft-agar assay.

[b]Compounds tested in triplicate.

[c]Cancer cell line origin: Breast cancer cell lines: MDA-MB-231; prostate cancer cell lines: DU145, PC-3, and oral cancer cell lines: KB, KB-3-1, and KB-Ch^R-8-5.

a base in anhydrous THF to yield **2**, **5a-m** (70–90% yield) as per previously described procedure in Section 2.1.2. The purified and structurally characterized molecules were taken up for biological screening in a battery of assays.

3.2 | Biology

3.2.1 | MTT assay

Initially, the title compounds were screened in cell viability (MTT) assay against breast (MDA-MB-231, MCF7, and T-47D), prostate (DU145, LNCaP, and PC-3), and oral (KB, KB-3-1, and KB-Ch^R-8-5) cancer cell lines. The optimal plating densities of each cell line are given in Table S2. Cisplatin and sunitinib were used as positive controls and the vehicle DMSO as a negative control. Cisplatin represented conventional chemotherapy modality to which CSCs are resistant, while sunitinib was used as a representative anti-CSC drug (Yuan et al., 2016). The choice of cell line for biological evaluation was governed by several factors, particularly, their aggressiveness, tumorigenic potential, invasive behavior, in addition to others. MDA-MB-231 is a perfect model for chemotherapy while MCF7 is a suitable model for hormone therapy. T-47D is quite similar to MCF7 except that it is quite susceptible to progesterone (Yu, Kim, Yoo, & Kang, 2017). In addition, T-47D cells form tightly cohesive mass structures in 3D cultures owing to extensive cell–cell adhesion (Holliday & Speirs, 2011). In case of prostate cancer cell lines, PC-3 is highly metastatic and is characteristic of prostatic small cell carcinoma, an extremely aggressive prostate cancer unresponsive to hormonal therapy (Tai et al., 2011).The oral cancer cell lines, KB, KB-3-1, and KB-Ch^R-8-5, were used to further assess the anticancer potential of the title compounds, since oral cancer is among the top 10 most commonly affecting cancer types. These cells represent highly drug-(colchicine, vinblastine, actinomycin D or adriamycin)-resistant phenotypes owing to abundance of multidrug-resistant efflux transporters such as P-gp, an ideal scenario for anti-CSC activity evaluation (Richert, Akiyama, Shen, Gottesman, & Pastan, 1985; Shen et al., 1986). Very recently, Fang et al. (Fang et al., 2019) have established KB cell line overexpressing P-gp for understanding structural requirements of flavonoids undergoing P-gp mediated efflux.

Table 2 lists the IC_{50} (μM) values for the title compounds **5a-m** against the breast, prostate and oral cancer cell lines. Quite a few compounds—**5e**, **5i**, **5k**, and **5m** (MDA-MB-231) and **2** (T-47D) exhibited submicromolar potency (better than sunitinib). Other relatively potent compounds included **2** (MCF7), **5f** (DU145), **5m**, **5g**, **5l**, and **2**. None of the tested compounds were more potent than sunitinib against the oral cancer cell lines. Moving down Table 2 starting from the original lead structure **2**, replacement of 3-CN (**2**) with 3-COOEt (**5a**) on the thiazole core decreased potency. Further contraction of the cyclohexane ring (**5a**) with cyclopentyl ring (**5b**) led to slight gain in potency in breast cancer cell lines while the potency at prostate cancer cell lines decreased marginally. Further optimization of potency yielded **5e** (MDA-MB-231, IC_{50} = 0.314 ± 0.05 μM). Careful observation of the results indicated the requirement of limited bulk on the amine. Extended aromatic substituent (e.g., **5l**), presence of a polar group on the aromatic ring of amine (e.g., **5h**) had detrimental

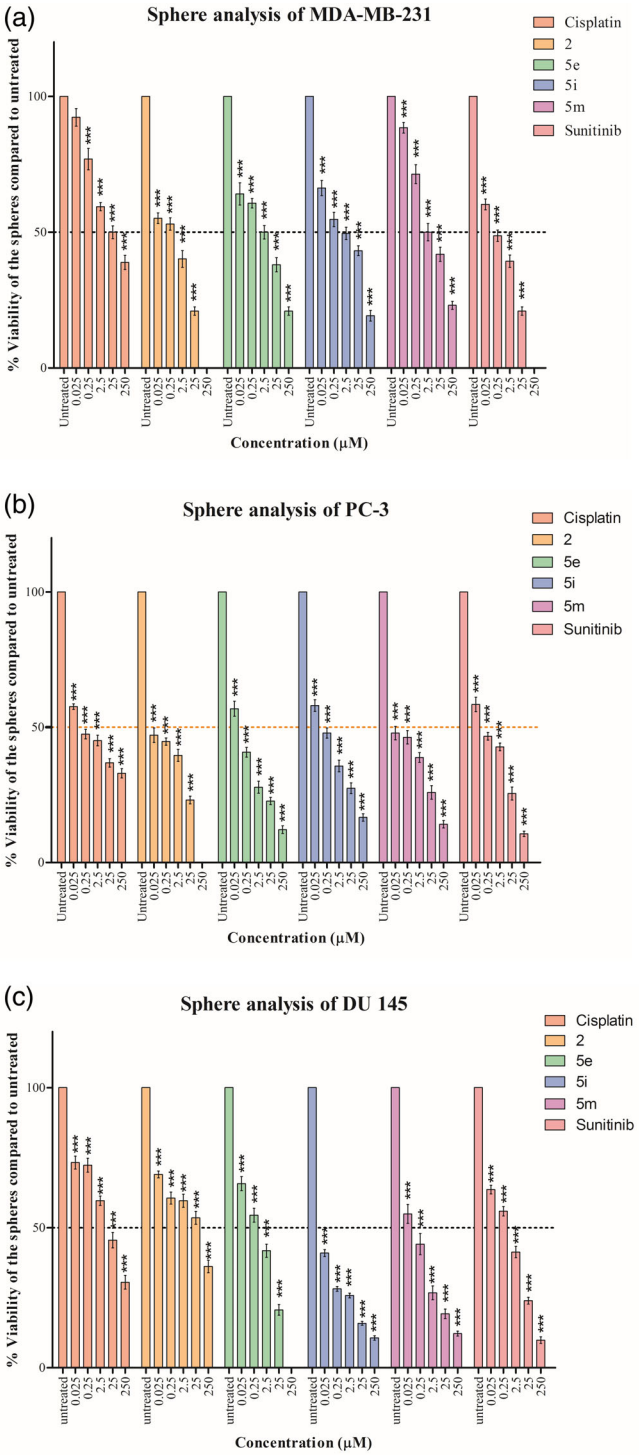


FIGURE 2 Results from sphere-forming assay of the hits. (a) % Viability of mammospheres (MDA-MB-231); (b) % viability of prostatospheres (PC-3); (c) % viability of prostatospheres (DU 145) compared to the untreated. (The data are expressed as mean ± *SEM* [*n* = 6]. Asterisk above columns indicate statistically significant difference compared to control. ***p < .001)

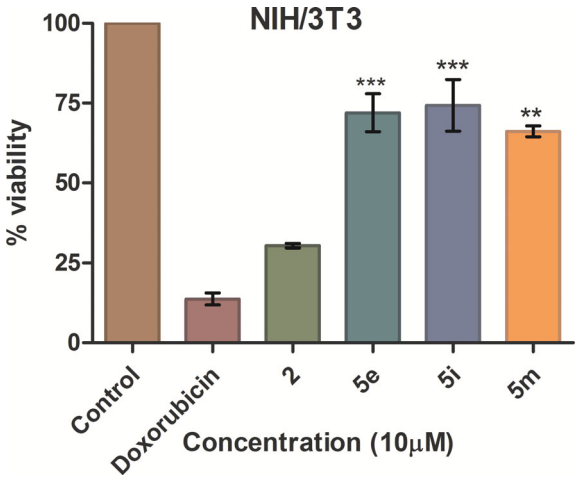


FIGURE 3 % Cell viability of the hits against NIH/3T3 cell line (data are mean ± *SD*, *n* = 3). (Asterisk above columns indicate statistically significant difference compared to control. **p < .01, ***p < .001)

TABLE 4 Median effect of the hits on lymphocyte viability (hPBMC assay)

Sr. No.	Compound code	IC_{50} (μM)
1	Cisplatin	98 ± 1.3
2	Sunitinib (+ve control)	2.8 ± 0.07
3	**2**	0.33 ± 0.02
3	**5e**	>100
4	**5i**	>100
5	**5m**	>100

Abbreviation: hPBMC, human peripheral blood mononuclear cell.

effect on potency in some (MDA-MB-231) cell line(s), while beneficial in others (LNCaP). Overall, our concern that these molecules would otherwise be general toxins was invalid since there were definitive trends in the activity profiles of the title compounds. Some of these activity patterns could be due to the impaired cellular permeability owing to unfavorable molecular properties in less potent compounds. Overall, presence of a halogen on the aromatic ring alone or with a heteroaromatic ring bearing amine contributed positively to impaired cell viability.

3.2.2 | Soft-agar assay

Majority of the title compounds (except **5g** and **5l**) exhibiting relatively potent activity in MTT assay were further taken up for evaluation in soft-agar assay (SAA). Also known as anchorage-independent assay, SAA is widely used to understand the effect of test compounds on the ability of cancer cell's proliferation and migration to form colonies in semisolid matrix mimicking in vivo condition, that is, tumor suppression in vitro. It is indeed the test of a cell to undergo "unlimited" division. If a compound inhibits colony formation in a dose-dependent manner, it can be concluded that it is an inhibitor of tumorigenicity in vitro, an important characteristic of CSCs. Previously, Horibata, Vo, Subramanian, Thompson, and Coonrod (2015) have used SAA for

identifying inhibitors of tumorigenicity in breast cancer cells. In a very recent report, Seyfrid et al. (2019) have described SAA as one of the inexpensive and convenient assays for evaluating the stemness properties of brain tumor stem cells.

In the present investigation, representative cell lines from breast (MDA-MB-231), prostate (DU 145, PC-3), and oral (KB, KB-3-1, KB-ChR-8-5) cancer types were used. The IC_{50} (μM) values were calculated based on the capacity of the molecules to inhibit 50% colony formation compared to the control (Table 3). Since the number of formed colonies varies with cell type, the cell plating density was optimized for each cell type used (Table S3).

Compounds **5a** (MDA-MB-231), **2** (DU 145), **5i** (PC-3 and KB-3-1), **5f** (KB), and **5e** (KB-Ch^R-8-5) were the most potent against the indicated cell line. Several of the tested compounds were more potent than sunitinib (Table 3). The results were encouraging to warrant further evaluation of the pan-active compounds **2**, **5e**, **5i** and **5 m** (Table 3) in sphere formation or sphere assay to understand the potential effects of these hits on CSCs. These hits were selected on the basis of combined activity data from MTT and SAA.

3.2.3 | Sphere-formation assay

Sphere-formation assay (SFA) is widely utilized for identification of CSCs in vitro based on the very characteristic of CSCs to grow in serum-free gel matrix. Under the nonadherent conditions, only CSCs will survive and proliferate to form spheres (Pastrana, Silva-Vargas, & Doetsch, 2011). Very recently, Bahmad et al. (2018), used SFA for assessing the presence and self-renewal potential of prostate CSCs in different models as an alternative to various in vivo methods. In this study, we evaluated selected hits from MTT and SAA against breast (MDA-MB-231) and prostate (DU 145 and PC-3) CSCs at five different concentrations (0.025, 0.25, 2.5, 25, 250 μM; Figure 2a–c). Surprisingly, all the hits (**2**, **5e**, **5i**, and **5 m**) exhibited moderate to high activity at 0.025–2.5 μM inhibiting 50% or more number of spheres in all cell lines tested. In totality, the tested hits were more active against PC-3 compared to the other two cell lines. Hits **2** and **5m** were even better at 25 nM concentration than sunitinib, the positive control (Figure 2b). Overall, **2** and **5m** turned out to be the clear winners in SFA against breast (MDA-MB-231) and prostate (PC-3 and DU 145) CSCs, respectively.

3.2.4 | hPBMC assay

Given the nature of the hits, we intended to evaluate the in vitro toxicity of the hits on normal cells. Two cell lines—human peripheral blood mononuclear cells (hPBMCs) and mouse embryonic fibroblasts (NIH/3T3) were used. The hPBMC assay is used by the scientific community as a cost-effective tool for demonstrating the toxicity, in particular immunotoxicity, on humans (Pourahmad & Salimi, 2015). Similarly, NIH/3T3 cell line was another choice representing the mesenchyme-derived cells forming the most basic unit of the connective tissues. Previously, Sahinturk, Kacar, Vejselova, and Kutlu (2018) have used NIH/3T3 cell line for evaluating cytotoxicity of acrylamide. All the hits (**2**, **5e**, **5i**, and **5m**) along with doxorubicin, representing cytotoxic drugs as positive control and vehicle as negative control, were evaluated in MTT assay using hPBMCs and NIH/3T3 cell line. The hits **5e**, **5i**, and **5m** were found to be significantly less toxic (~fourfold) compared to doxorubicin at 10 μM dose against NIH/3T3 cell line (Figure 3). In a contrasting scenario, **2**, the original hit, which was one of the best hits in cell viability assays toxic at the dose level tested (10 μM). Similar trend was observed during screening of the hits against hPBMCs. The molecules were screened (highest concentration tested 100 μM) and IC_{50} values were calculated. While **5e**, **5i**, and **5m** showed no activity (IC_{50} > 100 μM) toward hPBMCs, hit **2** was found to be relatively toxic (IC_{50} = 0.33 μM; Table 4) compared to the positive control sunitinib (IC_{50} = 2.8 μM). In summary, the hits **5e**, **5i**, and **5m** were relatively nontoxic to the normal cells.

4 | CONCLUSION

Design and development of anti-CSC therapeutics is likely to offer a promise for successful clinical outcome of cancer treatment modalities. These agents are expected to act in conjunction with the standard chemotherapy regimen(s), depleting CSCs, the root cause of relapse. Given the complex nature of cancer as a heterogeneous disease, anti-CSC agents must to act on CSC-specific pathways to be efficacious. The basic research establishing the utility of these approaches in clearing CSCs is crucial for validation of the concept. This is only possible with the availability of unique agents acting via distinct mechanism(s) of action. In this study, our hypothesis to use a war-head to direct activity against CSCs led to interesting results. The chloroacetamide derivatives incorporating structurally varied amines exhibited definitive potential as anti-CSC therapeutics against breast and prostate cancers. The relatively lesser incidence of toxicity against normal cell at the doses tested ensured us that these hits were not general toxins. Rather, there was a distinct structure–activity pattern. These preliminary results are likely to serve as a foundation for more elaborate, distinctive, definitive, and confirmatory studies. The possible mode of action of these compounds may involve covalent modification of their target(s). Moving forward, extensive experimental investigations are likely to prove the utility of this promising, potential anti-CSC therapeutics.

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CONFLICT OF INTEREST

Authors declare no conflict of interests from a financial or commercial standpoint.

AUTHOR CONTRIBUTIONS

P.K. conceived the project. K.P. synthesized, characterized, purified, and partly done biological characterization. M.A. and S.S carried out majority of the biological evaluation. P.K. and S.S. monitored the progress of the project and contributed to manuscript compilation and critical reading.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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