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Synthesis and Evaluation of the Diarylthiourea Analogs as Novel Anti-Cancer Agents

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FULL TITLE: Synthesis and evaluation of the diarylthiourea analogs as novel anti-cancer agents

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ABSTRACT:

Ten *p*-nitrodiarylthiourea analogs were designed, synthesized and evaluated in breast (MCF-7, T-47D, MDA-MB-453) and prostate (DU-145, PC-3, LNCaP) cancer cell lines for their anticancer activities. Majority of the compounds were able to inhibit the growth of these six cancer cell lines at low micromolar concentrations. Compound **7** was found to be the most potent anticancer agent in this series with GI_{50} values of 3.16 μM for MCF-7, 2.53 μM for T-47D, 4.77 μM for MDA-MB-453 breast cancer lines and 3.54 μM for LNCaP prostate cancer cell line. These GI_{50} values were comparable to the original parent compound, SHetA2.

GRAPHICAL ABSTRACT:
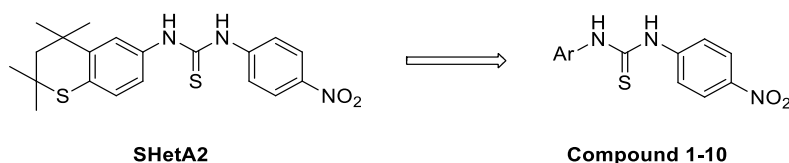
ABBREVIATIONS:

SHetA2: *N*-(2,3-dihydro-2,2,4,4-tetramethyl-6-benzothiopyranyl), *N'*-(4-nitrophenyl)thiourea; ATRA: all trans retinoic acid; NOAEL: no observed adverse effect level; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; MTS: MTT coupled with phenazine methosulfate (soluble form of MTT) ; ER: estrogen receptor; AR: androgen receptor

Previously, we synthesized *N*-(2,3-dihydro-2,2,4,4-tetramethyl-6-benzothiopyranyl), *N'*-(4-nitrophenyl)thiourea (SHetA2, NSC 726189)[1] which has been shown to be a novel potential cancer prevention agent and is now in preclinical development for cancer prevention and treatment through the National Cancer Institute (NCI) Rapid Access to Intervention Development (RAID) program. The studies of SHetA2 were reviewed extensively.[2,3] SHetA2 was evolved from a lead optimization

process from all *trans*-retinoid acid (ATRA, Tretinoin®, Mean GI_{50} 51 μM, Figure 1),[2,3,4] which is used for the treatment of acute promyelocytic leukemia. However, the exploitation of retinoic acid's full potential as chemopreventive and/or therapeutic drugs, particularly against solid tumors, has been hampered mainly by their local and systemic toxicity and side effects, which are often associated with their ability to activate nuclear retinoid receptors.[2] Thus, efforts have been made to develop novel compounds that retain retinoid anticancer activity with minimal retinoid toxicity and side effects. SHetA2 is such a compound, a more effective anticancer agent than ATRA and appears to function without activating retinoid receptors.[1] As a result, it has been shown to lack retinoid toxicities when tested in animal models.[3] SHetA2 exerts its selective anticancer activity through regulating apoptosis, cell growth, differentiation and angiogenesis.[5,6] SHetA2 has been shown to induce proteasomal degradation of cyclin D1,[7] generate mitochondrial swelling and endoplasmic reticulum stress, promote the formation of reactive oxygen species, and induce apoptosis in cancer cells while sparing normal cells.[6] More importantly, it was reported recently in a dog study, that no toxicity of SHetA2 was observed in any tested dose groups. The lowest observed adverse effect level (NOAEL) for ShetA2 was not established and was considered to be above 1,500 mg/kg/day.[8]

However, SHetA2 may not be an ideal drug candidate due to the following two limitations: its high lipophilicity and cumbersome six step synthesis 1) Its high lipophilicity (LogP 7.09, higher than the upper limit of "Lipinski rule of 5" and the Log P of most marketed drugs),[9,10] might contribute to its extremely low (<1%) systemic bioavailability for all doses tested in rat and high plasma protein binding (99.3-99.5% at low micromolar concentrations).[6, 11] In addition, this high lipophilicity might potentially cause nonselectivity, liver toxicity and drug-drug interactions.[12] 2) Its synthesis involves 6 steps with low overall yield of 3%, which hinders its large scale supply and new analog synthesis.[13] To address these issues, we have modified the SHetA2 structure based on the previous structure-activity relationship.[1, 2] We hypothesized that the nitrophenyl group and the thiourea linker in SHetA2 structure are important for its anticancer activity. Thus, our modification strategy was to keep these two moieties intact while replacing the thiochromane ring with another ring structure. In this way, we anticipate to reduce both the lipophilicity of the designed compounds and simplify the six-step synthesis to one step using readily available and inexpensive starting materials.

Here, we report the design, synthesis and biological activity of ten diarylthiourea analogs. These compounds were synthesized according to our previously published procedure.[1] The newly

synthesized compounds were evaluated for their ability to inhibit the growth of breast cancer cell lines-MCF-7, T-47D, and MDA-MB-453 and prostate cancer cell lines-DU-145, LNCaP and PC-3. The potency of compounds **5** and **7** in this series were shown to be comparable to the parent compound, SHetA2.

General procedure for the preparation of compounds **1-10** with the overall yield of 19%-87% from amines and 4-nitrophenyl isothiocyanate is shown in Scheme 1. To a solution of amines (1.0 mmol) in dry THF (4.5 mL) at 0°C under nitrogen, 4-nitrophenyl isothiocyanate (1.02 mmol) in dry THF (5 mL) was added dropwise over 3 min. After the addition, the reaction mixture was allowed to warm to room temperature and then was stirred overnight. The solvent was evaporated, and residue was recrystallized or purified by flash column chromatography to give an analytically pure sample of the corresponding thiourea product. The chemical structures of compounds **1-10** were confirmed by ^{1}H, C[13] NMR and LC-MS. The Log P, calculated Log P and the overall yield is summarized in table 1. Further reaction details and characterizations are provided in the Supplementary data.

Cell Culture: MCF-7, T-47D, MDA-MB-453, DU-145, PC-3 and LNCaP cells were obtained from ATCC and cultured and maintained according to ATCC protocols. Briefly, MCF-7 and MDA-MB-453 cells were maintained in Dulbeco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. T-47D, DU-145, LNCaP and PC-3 cells were maintained in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were maintained in an incubator at 37°C, 95% relative humidity and 5% CO_2 atmosphere. Sub-culturing of all cells was done every three days. 10 mM stock solutions of compound **1-10** were made in DMSO and stored at -20°C. Working solutions (100µM) were made in cell culture media by serial dilution just prior to addition of the compounds in 96 well plates.

Cell viability assay: To test the effects of the diarylthiourea analogs on breast cancer cells, two thousand cells (MCF-7, MDA-MB-453, or T-47D) were plated in 96 well flat bottom plates. Twenty-four hours later, cells were treated with varying concentration (0.5 µM, 1.0 µM, 5.0 µM, 10.0 µM, or 20.0µM) of either a diarylthiourea analog (**1-10**), ShetA2, all trans retinoic acid (ATRA), or mock treated with the vehicle. Cytotoxicity was monitored 48 hours later by adding

20μL of MTT (Sigma). Cell viability was determined after a 2-hour incubation by dissolving the tetrazolium crystals with DMSO and absorbances were measured at 595nm with plate reader (Biorad, Hercules, CA). Means and standard deviations represent at least two independent experiments done in six replicates.

The analogs were also tested on prostate cancer cells, where five thousand DU-145, PC-3 and LNCaP cells were plated in 96-well flat bottom plates for 24 hours prior to treating them with varying concentrations (0.5 µM, 1.0 µM, 5.0 µM, 10.0 µM, or 20.0 µM) of either a diarylthiourea analog, ShetA2, all trans retinoic acid (ATRA), or mock treated with the vehicle. Following 48-hours incubation, 20µL of MTS reagent (CellTiter 96® Aqueous One Solution Reagent; Promega, Madison, WI) was added to each well according to the manufacturer's instructions. Cell viability was determined after a 2-hour incubation by measuring the absorbance at 490nm using a Spectra Max M5 plate-reader (Molecular Devices, Sunnyvale, CA). Means and standard deviations represent at least two independent experiments done in triplicates. Cell proliferation assays were performed in a similar manner, except cell growth was monitored for 2, 3 and 4 days after addition of the drug with either MTT or MTS (breast cancer cell lines and prostate cancer cell lines, respectively).

Growth inhibition 50 (GI_{50}) is defined as the concentration at which 50% of cell-growth is inhibited. The GI_{50} was calculated for each analog as follows. For each concentration value c (c = 0.5 µM, 1.0 µM, 5.0 µM, 10.0 µM, or 20.0 µM), the absorbance of the test well after a 48-hour period of exposure was computed and averaged over all replicates to yield a mean absorbance T(c). An exponential equation ($\mathbf{T(c) = b_1 \exp(b_2 c) + b_3}$) was used to model the absorbance as a

function of concentration c, using non-linear least squares regression, weighted by the inverse variance of the observations. For some analogs, the exponential model degenerated to a linear model; weighted linear least squares regression was used instead for these analogs. GI_{50} was computed as the concentration c for which (T(c) - T0)/(C - T0) = 0.5, using the exponential or linear model to interpolate, where T0 represents absorbance at time zero and C represents vehicle control absorbance, averaged over all replicates in the experiment. The reported means and standard deviations of GI_{50} represent at least two independent experiments with either six replicates (breast cancer cells) or triplicates (prostate cancer cells).

The preclinical studies of SHetA2 on cell culture and animal models showed that it is a promising agent for cancer prevention and treatment without significant toxicity and side effects.[6-8] However, SHetA2 is too lipophilic, with a Log P value of 7.09, which can hinder its oral bioavailability as an anticancer drug, since optimal Log P for most oral drugs is between 1-3. We have designed the SHetA2 analogs with Log P value between 2.99 and 6.36 (Table 1) so that all our compounds are less lipophilic than the lead compound SHetA2 with the premise that these compounds would have better pharmacokinetic profiles. In addition, as previously reported, the six step synthesis of SHetA2 suffers from a low overall yield of 3% and this procedure involves use of reagents that are toxic and have unpleasant odor.[8] Tallent's group reported a modified procedure for the synthesis of key intermediates to the SHetA2 with improved yield, but started with much more expensive chemicals and used column chromatography after every synthesis step.[13] We have developed a simple, one step synthesis procedure of the target compounds with cheap and commercially available chemicals with overall yield of 19%-87% (Table 1). By far in published literature, only certain diarylurea multi-target kinase inhibitors have chemical structures related to our compounds.[14,15] Sorafenib is among one of these compounds that have been approved for the treatment of advanced renal cell cancer by the FDA. In addition, the wide diversity of cellular and molecular targets that can be regulated by judiciously modifying *N, N'*-diarylureas and ureas, suggests this part of the molecule may serve as privileged scaffolds for anticancer agents.[14,15]

The synthesized diarylthiourea analogs **1-10** were tested on three breast cancer cell lines — MCF7, T-47D and MDA-MB-453 cells (Table 2). While majority of the compounds showed some effect on inhibiting growth of all three breast cancer cell lines, diarylthiourea analogs **5** and **7** showed the most significant effects. The GI_{50} values of compounds **5** and **7** were comparable to the parent SHetA2 (2.94-6.27 µM and 2.53-4.77 µM vs. 3.27-4.13 µM, respectively), with the T-47D cells displaying the greatest sensitivity to these compounds when compared to MCF7 and MDA-MB-453. Since compound **7** appeared to be the most effective in inhibiting the growth of all three breast cancer cell lines, its ability to inhibit cell growth over a longer period of time was evaluated (Figure 2 A-C). Results in figure 2 indicate that compound **7** continued to inhibit cell growth 4 days after treatment and appeared to effectively inhibit the growth of all three breast cancer cell lines.

The diarylthiourea analogs of SHetA2 were also tested on three prostate cancer cell lines— DU-145, PC-3 and LNCaP. Overall, LNCaP cells were more susceptible to the growth inhibitory effects of these agents (Table 2). Compound **7** showed the most potent growth inhibitory effect in

LNCaP cells as evidenced by a low GI_{50} value of 3.54 µM. This value was comparable to the GI_{50} value (2.25 µM) of SHetA2 in the same cell-line (Table 2). Being the most potent of the diarylthiourea analogs, compound **7** was tested for its ability to inhibit cell growth over a longer period of time (Figure 2D). This experiment was carried out in LNCaP cells that were most vulnerable to the growth inhibitory effects of compound **7**. Cell growth was significantly inhibited with 5 µM compound **7** staring at day 2 and remained so until day 4. Similar trend in growth inhibition was observed at higher concentrations (10 and 20 µM) as well (Figure 2D). Moreover, while SHetA2 inhibited the growth of all three prostate cancer cell-lines uniformly, compound **5** and **7** selectively inhibited the growth of only one of the prostate cancer cell lines, the LNCaP cells.

Here we report a simple, one step synthesis procedure to obtain a variety of diarylthiourea derivatives of SHetA2 and their biological evaluation in human breast and prostate cancer cells. The simple syntheses involved a coupling reaction of 4-nitropheny isothiocyanate in dry THF with commercially available amines.. Most of them caused growth inhibition of these cancer cells. Among them, two compounds showed potency comparable to the lead compound, SHetA2. The study also validated our previous hypothesis that the 4-nitrophenyl group and the thiourea linker are important for the anticancer activity of SHetA2 because all the compounds tested have these moieties. Modification of other functional groups in SHetA2 resulted in active anticancer agents. Therefore, this study has provided a new approach to the design and synthesis of the next generation of novel anticancer agents, perhaps with novel mechanisms of action.

We tested the new diarylthiourea analogs along with SHetA2 on three breast cancer and three prostate cancer cell lines and our results (Table 2) show that diarylthiourea analog **5** and **7** are effective in inhibiting cancer cell growth of four cancer cell lines— MCF7, T-47D, MDA-MB-453 (breast cancer cells) and LNCaP (prostate cancer cell). SHetA2 was previously evaluated in the panel of National Cancer Institute (NCI) human tumor cell lines by the Developmental Therapeutics Program (DTP) and results showed that SHetA2 inhibited growth of most cancer cells in the micromolar range. Specifically, reported GI_{50} values of 4.5 µM for MCF-7, 4.8 µM for T-47D, 5.0 µM for MDA-MB-453, 4.9 µM for DU145 and 5.0 µM for PC-3,[6,16] is consistent with our results shown in Table 2. Our newly synthesized compounds (**5** and **7**) showed equivalent potency in comparison to the lead compound SHetA2 (Table 2). Noteworthy are the effects of compounds **5** and **7** on the three prostate cancer cell lines. Both these agents have shown to be more selective in

inhibiting the growth of LNCaP cells in comparison to PC-3 and DU-145 cell-lines. As seen in Table 2, compound **7** is about six times more potent in inhibiting growth of LNCaP cells than PC-3 and DU145 cells and compound **5** is at least twice as potent in LNCaP cells than in PC-3 and DU-145 cells. This observation is significant since SHetA2 does not show any such selectivity (Table 2). One explanation for this selectivity could be due to differential androgen receptor signaling patterns in the three cell lines. Both PC-3 and DU-145 cell-lines do not express androgen receptors (AR) and are not androgen dependent for their growth, while LNCaP cells express AR (with a point mutation in the ligand binding domain) and are considered androgen-dependent cells[17]. Therefore, we hypothesize that these new analogs, compounds **5** and **7**, may employ a different mechanism to induce growth inhibition in prostate cancer cell lines as compared to SHetA2. Whether this mechanism involves the regulation of androgen receptors (AR) requires further investigation.

However, this differential response was not as significant in the breast cancer models. MCF7 and T-47D cells are both estrogen receptor (ER) positive (presence of ERα) and are considered hormone-dependent, whereas MDA-MB-453 is ER negative (absence of ERα). While the $G1_{50}$ for the ER positive cells were lower in comparison to the ER negative breast cancer cells, the difference is not statistically significant (Table 2). The differences observed in the breast and prostate cancer cells might be attributed to different mechanisms of action in the two cancer types. Although MDA-MD-453 is considered ER negative breast cancer cells, similar to MCF7 and T-47D, these cells do express ERβ.

In conclusion, this is the first report that demonstrates the growth inhibitory properties of SHetA2 and a new generation of diarylthiourea analogs on both breast and prostate cancer cells. While compounds **1-10** are not more potent than the SHetA2, we report for the first time the effects of *p*-nitrodiarylthiourea compounds on the inhibition of both breast and prostate cancer cells, along with a more simplified synthetic scheme. Data from this study indicate that these novel potential anticancer agents are promising lead compounds for further evaluation. While the mechanism of action and pharmacokinetic profile of these compounds remain to be elucidated in future studies, results presented in this study provide a strong foundation for further preclinical studies of these compounds as potential therapeutic agents for both breast and prostate cancers.

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Supplementary Material: The synthesis procedures and spectrum data of each compound are provided as a separate electronic file. It can be transformed into PDF format.

FIGURE LEGENDS

Scheme 1: General procedure for the synthesis of the compound **1-10**
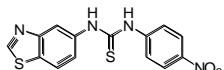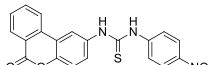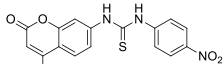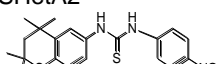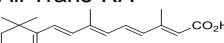
Figure 1: Evolution of SHetA2
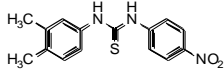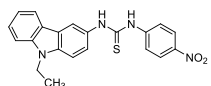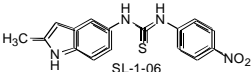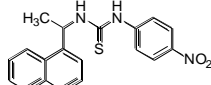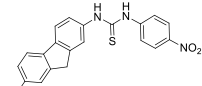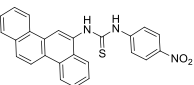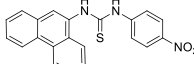
Figure 2: Breast cancer (A) MDA-MB-453, (B) MCF7, (C) T-47D, and prostate cancer (D) LNCaP cells were plated in 96-plates and treated with varying concentrations of SL-01-18 for 2, 3 and 4 days. Cell growth was analyzed with MTT (A-C) or MTS (D). Means and standard deviations represent at least two independent experiments done in six replicates.

Table 1. Some SHetA2 derivatives (1-10) produced according to Scheme 1

Compounds	Name	Log P[a]	CLog P[a]	Total Yield (%)
1	SL-01-03	3.83	3.51	83
2	SL-01-05	ND	5.01	19
3	SL-01-06	3.84	3.05	23
4	SL-01-08	ND	2.83	24
5	SL-01-09	4.11	4.52	87
6	SL-01-15	5.34	5.39	35
7	SL-01-18	6.36	6.08	42
8	SL-01-19	5.24	4.91	70
9	SL-01-20	3.59	3.54	60
10	SL-01-21	2.88	2.66	73
Parent	SHetA2	7.09	5.74	3

[a]Log P and Clog P were calculated with ChemDraw.

Table 2. Potency (GI$_{50}$ values) in μM

Name and Chemical Structure	Breast cancer cell lines (μM) Mean±SD			Prostate cancer cell lines (μM) Mean±SD		
	MCF-7	T-47D	MDA-MB-453	DU-145	PC-3	LNCaP
1	16.96 ±3.35[1]	8.14±1.79[2]	13.48±1.96[1]	35.15±15.42[1]	26.77±13.25[1]	10.58±1.95[1]
2	12.81±2.46[1]	9.44±0.12[1]	6.88±0.83[1]	5.87±0.92[2]	19.59±0.68[1]	5.86±0.41[2]
3	ND*	21.62±0.85[1]	ND*	ND*	ND*	40.47±28.82[1]
4	ND*	15.28±3.27[1]	ND*	ND*	ND*	23.53±7.11[1]
5	6.21±1.95[1]	2.94 ±0.91[2]	6.27±0.39[1]	13.69±4.15[1]	13.90±1.09[2]	6.05±0.18[2]
6	7.81±1.19[2]	3.96 ±0.58[2]	6.20±2.42[2]	17.48±2.20[1]	16.50±1.17[1]	11.32±3.90[1]
7	3.16±1.79[2]	2.53±0.39[2]	4.77±2.21[2]	21.78±3.98[1]	19.96±3.32[1]	3.54±0.17[2]
8	13.30±2.73[1]	11.55±3.87[1]	14.49±4.58[1]	6.11±1.30[2]	19.27±0.41[1]	7.29±2.35[2]
9	13.08±1.74[1]	8.68±2.34[1]	27.59±7.22[1]	24.10±5.40[1]	22.56±3.98[1]	12.75±0.60[1]
10	9.89±1.62[2]	ND*	ND*	ND*	ND*	ND*
SHetA2	4.13±0.57[2]	3.99±0.06[2]	3.27±0.33[2]	3.47±0.42[2]	4.80±1.53[2]	2.25±1.04[2]
All Trans-RA	12.39±0.26[1]	0.82±0.03[2]	9.51±0.61[1]	11.64±0.54[1]	12.64±0.38[1]	10.33±0.75[1]

[1] Based on linear model
[2] Based on exponential model
* ND=Not determined or GI50 >>50µM