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Antiproliferative activity of 3,5-disubstituted tetrahydro-2*H*-1,3,5-thiadiazine thione (THTT) derivatives and evaluation as potential prodrug

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Abstract: Four series of tetrahydro-2*H*-1,3,5-thiadiazine thione derivatives were screened for their *in vitro* antiproliferative activities against two human cancerous PC3 and HeLa cell lines. The cytotoxicity of all the compounds (series A-D) was also determined on mammalian mouse fibroblast 3T3 cells. Most of the compounds showed significant anticancer potential against both cancer cell lines within the range of IC_{50} = 6.4-29.9 and 2.4-23.8 μM respectively when compared with standard doxorubicin (IC_{50} = 0.3 μM). All compounds demonstrated a notable selectivity for Hela cells and found either non-toxic or relatively less toxic for 3T3 cell lines model. The structure-activity relationship indicated that antiproliferative activity mainly influenced by the nature and position of substituents at thidiazine nucleus. In general, the presence of aryl groups for example 3,4-$(OMe)_2$.Bzl and CH(Ph)Me at N-3 position resulted in a significant activity. Under enzymatic hydrolysis, complete conversion (100%) of ester derivative of thiadiazine thione (10a) into its acidic counterpart (7c) was achieved during 20 min which indicated that these types of THTT ester derivatives can be a possible lead for future investigations as prodrug anticancer probes.

Keywords: Tetrahydro-1,3,5-thiadiazine thiones, anticancer, antiproliferative, ester, prodrugs.

INTRODUCTION

Cancer is the next principal cause of fatal outcomes in humans following cardiovascular diseases (Sung *et al*., 2021). The present existing modes of chemotherapy suffer harsh side effects and resistance to drugs, which have led severe medical problems (Akin and Guner, 2019). Thus, there is an extreme need to explore more selective, successful, and nontoxic chemotherapeutic agents. In this context, prodrugs are becoming an integral part of the drug discovery paradigm for chemotherapy (Zhao *et al*., 2020). Their importance is supported by i) less toxicity, ii) better selectivity, and iii) improved ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties (Rashin *et al*., 2020). Prodrugs are bio-reversible and inactive derivatives of drug molecules. Prodrugs require an enzymatic or chemical conversion (Shah *et al*., 2017) to release an active parent drug *in vivo*, which can then induce its related pharmacological effects (Zawilska *et al*., 2013).

In an approach of prodrugs designing, hydrophilic groups such as carboxyl group on the parent drug are chemically modified into alkyl or aryl esters (Hamada, 2017). These esters undergo biotransformation into parent active species by ubiquitous carboxylesterases which are benefitted by substrate specificities and varied tissue distribution (Satoh and Hosokawa, 2006). Thus, by modifying the length, size and shape of alkyl/aryl esters, the modularity in lipophilicity, membrane permeability, and selectivity in pharmaceutical targets can be achieved. Currently, a substantial number of ester prodrugs have been advanced into clinical use for the treatment of various types of illnesses, for example, capecitabine 1 (fig. 1) as an anticancer drug (Koukourakis *et al*., 2008), temocapril 2 (fig. 1) as an ACE inhibitor (Yamamoto *et al*., 2005) and oseltamivir as an anti-influenza (Shi *et al*., 2006).

As prodrugs, thiadiazine derivatives have been well-investigated for their diverse biological activities including antiepileptic (Semreen *et al*., 2010), antifungal (Wang *et al*., 2019), antiprotozoal (compound 3, fig. 1) (Bermello *et al*., 2011), antileishmanial (compound 4, fig. 1) (Monzote *et al*., 2005), antibacterial (Sim and Teo, 2018), antitubercular (Kumar and Rao, 2008) and anticancer (compound 5, fig. 1) (Pérez *et al*., 2000). Considering thiadiazine anticancer activity, it is recognized that thiadiazines exert *in vivo* anticancer effect through their metabolites isothiocyanates and dithiocarbamic acid species by disrupting cell cycle (Radwan *et al*., 2012).

Inspired by the above-mentioned importance of thiadiazine derivatives and the increasing popularity of prodrugs in present drug discovery, we became interested in developing new thiadiazine derivatives with diverse

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structural modifications at positions N-3 and N-5. These also include carboxylate ester derivatives in search of potential prodrug candidates as anticancer agents.

MATERIALS AND METHODS

General experimental details

^{1}H NMR (one-dimensional; 1D) spectra were obtained by using 300, 400 and 500 MHz Bruker instruments. Mass was measured by electrospray ionization (ESI) and electron ionization (EI) on QSTAR XL MS/MS System and JEOL (JMS-600H), respectively. Analytical HPLC Shimadzu LC-20 system was used for monitoring reaction profiles. All synthesized and impure thiadiazines derivatives were purified by silica gel column chromatography or biotage automated flash chromatography system. TLC was performed using silica gel 60 HF_{254} precoated plates. All reagents, chemicals and anhydrous solvents were obtained from commercial sources and were utilized without any additional treatment.

General procedure for the synthesis of Series-A (3,5-disubstituted tetrahydro-2H-1,3,5-thiadiazine-2-thiones, 7a–f)

Carbon disulfide (60 mmol) was added portion-wise to a stirred mixture of the corresponding alkyl-, or aralkylamine (6a-f) (10 mmol) and potassium hydroxide (10 mmol) in 10 mL absolute ethanol (EtOH). The reaction mixture was continuously stirred for 3 hours at room temperature (25 ºC). Afterward, formalin (22 mmol) was slowly added to the reaction mixture containing dithiocarbamate and kept for a further 1 hour of stirring. Then the resulted solution was added in 15 minutes to a mixture of 10 mmol various alkyl amines and 20 mL phosphate buffer (pH 7.8). The mixture was allowed to stir for 1 hour before it was cooled at 0ºC and pH was lowered up to 2 with 8% HCl. After 30 minutes of stirring, resulted precipitates were filtered and recrystallized from EtOH to give THTT (7a-f) as a pure white solid. Spectroscopic analysis of the THTT 7a-f (series A) was found in good agreement with previously reported data (Arshad *et al*., 2018).

General procedure for the synthesis of Series-B (3,5-disubstituted tetrahydro-2H-1,3,5-thiadiazine-2-thiones, 8a–d)

For the preparation of THTT products (8a-d), the same procedure was used as for series A, except that initially glycine (10 mmol) was used instead of alkyl-, or aralkylamine. Spectroscopic analysis of the THTT 8a–d (series B) was found in good agreement with previously reported data (Arshad *et al*., 2018).

General procedure for the synthesis of Series-C (3,5-disubstituted tetrahydro-2H-1,3,5-thiadiazine-6-thiones, 9a–f)

For the preparation of THTT products (9a-f), the same procedure was used as for series A, except that in later stage glycine (10 mmol) was used instead of various alkyl amines. The resulting THTT (9a-f) as pure white solid were subjected for characterization. Spectroscopic analysis of the THTT 9a-f (series C) was found in good agreement with previously reported data (Arshad *et al*., 2018).

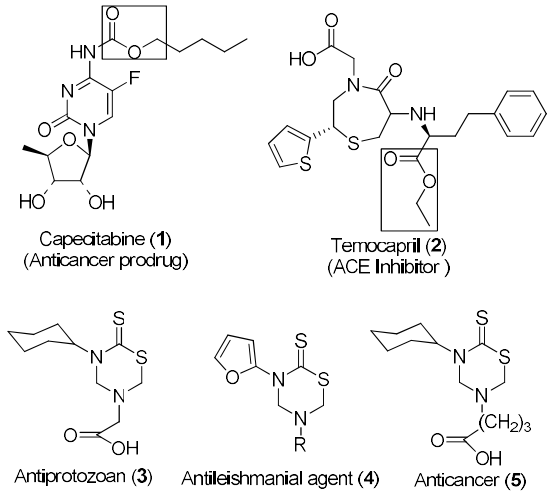
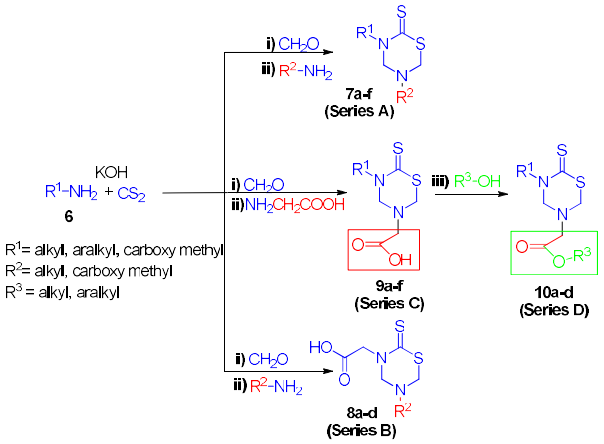


Fig. 1: Prodrugs and biologically active THTT derivatives.



Scheme 1: Synthetic routes to tetrahydro thiadiazine thiones series A (7a-f), series B (8a-d), series C (9a-f) and series D (10a-d). Reagents and conditions: i) 1º amines 6a-g (10 mmol), KOH (1 equiv.), EtOH (10 mL), CS_2 (6 equiv.), rt, 3 hours; then CH_2O (2.2 equiv.), rt, 1 hour; ii) 1º amines (1 equiv.), phosphate buffer (pH 7.8, 20 mL), rt, 1 hour; iii) THTT 9c-d (1 mmol), $SOCl_2$ (1.5 equiv.), R^3OH (1.5 mL), 0-4 ºC, 2 hours.

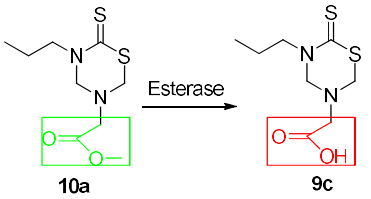
General procedure for the synthesis of Series-D (ester derivatives of 3,5-disubstituted tetrahydro-2H-1,3,5-thiadiazine-6-thiones, 10a-d)

Thionyl chloride (1.5 mmol) was added slowly (10 minutes) to an ice-cooled solution of 1 mmol THTT derivatives (9c-d) and 1.5 mL of corresponding alcohol. The reaction mixture was kept for 2 hours stirring then the alcohol was evaporated by rotary evaporator and 25 mL

water was added to the mixture. Afterward, the product was extracted with EtOAc (ethyl acetate) and washed with saturated sodium bicarbonate (10 mL). Ethyl acetate was dried over magnesium sulfate and subsequent evaporation afforded the crude ester derivatives of THTT. Flash column chromatography of crude products delivered the gummy THTT ester derivatives (10a-f) as pure compounds. Spectroscopic analysis of the THTT 10a-f (series D) was found in good agreement with previously reported data (Arshad *et al*., 2018).

Materials for Biological Assay Protocol

The Fetal bovine serum (Cat No. S181H-100, Lot No. S11302S181H) from Biowest, France. PC3 cell lines (ATCC No. CRL-1435, Lot No. 58501591) and 3T3 cell lines (ATCC No. CRL-1658, Lot No. 59049195).



Scheme 2: THTT ester derivative (10a) evaluation as prodrug

Antiproliferative Assay Protocol

The antiproliferative activities of THTT compounds (series A-D) were evaluated in 96-well flat bottomed microplates by using the standard 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) colorimetric assay (Mosmann, 1983; Dimas *et al*., 1998). In this method, Dulbecco's Modified Eagle Medium (DMEM) supported with 5% of fetal bovine serum (FBS), 100 IU/mL of penicillin, and 100 µg/mL of streptomycin was used for PC3 cells (prostate cancer)/HeLa cells (cervical cancer)/3T3 cells (mouse fibroblast) culture at 37 ºC in 5% CO_2 incubator. Before dilution with a specific medium, the exponentially growing cells were harvested and counted with a haemocytometer. Cell cultures of PC3 with the concentration of 1×10^5 cells/mL, HeLa of 6×10^4 and 3T3 of 5×10^4 cells/mL were developed and put (100 µL/well) separately into 96-well plates. The medium was removed after incubation and fresh medium (200 µL) was introduced with various concentrations of THTT derivatives (1-30 µM) and kept for 48 hours. Subsequently, each well was added with MTT (200 µL, 0.5 mg/mL) and kept for further 4 hours incubation before adding 100 µL of DMSO in each well. The reduction proportion of MTT to formazan within cells was measured by the absorbance at 570 ηm through a microplate reader. IC_{50} values demonstrated as concentration causing 50% growth inhibition for PC3/HeLa/3T3 cell lines. The following formula was used to calculate percent growth inhibition:

$$\%\ inhibition = 100 - \left(\frac{mean\ OD\ of\ A - mean\ OD\ of\ B}{mean\ OD\ of\ C - mean\ OD\ of\ B} \times 100\right)$$

Where A, B and C stand for the tested compound; negative control and positive control, respectively.

Experimental detail of enzymatic hydrolysis (prodrug evaluation)

1.0 mL of substrate solution (1.0 mM solution in 1% phosphate buffer of pH 7.5 and 10% acetone) was placed in a 5.0 mL glass vessel. Then 0.5 mL (3 units total) of the porcine liver esterase (PLE) stock solution was added (the stock solution was prepared by 4.0 mg of the PLE in 10 mL of 1% phosphate buffer of pH 7.5). The sample was placed in a shaking incubator at 37 ºC for 20 minutes and monitored by analytical HPLC on a C18 reversed-phase (RP) analytical column (Athena CN, 150 × 4.6mm, particle size 5 µm) at 40 ºC using a mobile phase-A (water/methanol 95:5 (v/v) + 0.1% formic acid) and B (MeOH + 0.1% formic acid) at a flow rate of 2.0 mL/min. The binary gradient starting with 5% B solution to 95% B in 15 minutes.

STATISTICAL ANALYSIS

All biological experiments were performed in triplicate and results for cell viability are expressed as mean ± SD. Absorbance was measured using a microplate reader (Spectra Max plus, Molecular Devices, CA, USA). IC_{50} values were calculated by using EZ-Fit Enzyme Kinetics by Perrella Scientific Inc. (Massachusetts, USA).

RESULTS

The title compounds 7a-f, 8a-d and 9a-f (series A, B and C) were synthesized in good to excellent yields (65-86%) according to the reaction pathways illustrated in scheme 1 at 10 mmol scales by following the literature (Aboul-Fadl *et al*., 2002; Arshad *et al*., 2018). Subsequently, tetrahydro thiadiazine-6-thiones 9c-d of series C were converted into ester analogs 10a-d (series D) at 1 mmol scale in good isolated yields by utilizing 2.0 equivalence of thionyl chloride in corresponding alcohol under 2 hours ice-cooled environment (0–4 ºC) adopting the literature protocol (Arshad *et al*., 2018). The characterization of all synthesized compounds was confirmed by EI and ^1H NMR and found in good agreement with the literature (Arshad *et al*., 2018).

Antiproliferative Activity

All four series (A-D) with NMR pure compounds were examined for their *in vitro* anticancer potential against two human cancer cell lines i.e. PC3 (prostate carcinoma) and HeLa (human cervical carcinoma) by employing MTT Assay (Mosmann, 1983; Dimas *et al*,. 1998). The

Table 1: Antiproliferative activity of various THTT scaffolds 7-10 (series A–D) in % growth inhibition zone[a]

S No.	Compound	% Growth inhibition zone		
		PC3[b]	HeLa[b]	3T3
Series A				
1.	7a	69.40	99.90	68.43
2.	7b	86.80	98.60	69.50
3.	7c	86.14	96.40	75.81
4.	7d	80.20	76.20	72.01
5.	7e	79.90	93.80	77.74
6.	7f	22.15	48.70	51.20
Series B				
7.	8a	15.20	54.90	22.70
8.	8b	11.60	21.60	26.40
9.	8c	27.70	26.07	5.90
10.	8d	15.30	13.70	3.81
Series C				
11.	9a	53.16	64.59	31.94
12.	9b	34.51	56.80	23.73
13.	9c	58.01	62.26	56.59
14.	9d	46.60	80.30	44.61
15.	9e	94.30	100	86.80
16.	9f	81.9 0	100	72.89
Series D				
17.	10a	49.60	11.02	36.93
18.	10b	36.01	71.12	44.03
19.	10c	43.04	62.70	48.53
20.	10d	41.30	45.99	51.90
21.	Doxorubicin	83.80	99.08	–
22.	Cycloheximide	–	–	65.95

[a] Results represented in % inhibition; testing incubation period of 48 hours at 37±1 ºC. [b] PC3 = Prostate cancer cell lines; HeLa = Cervical cancer cell lines; 3T3 = Mammalian mouse fibroblast cell lines. [c] % inhibition of std. drugs doxorubicin (30 µg/mL) = 83.80 for PC3; 99.08 for HeLa cells and cycloheximide (50 µg/mL) = 65.95 for 3T3 cell lines.

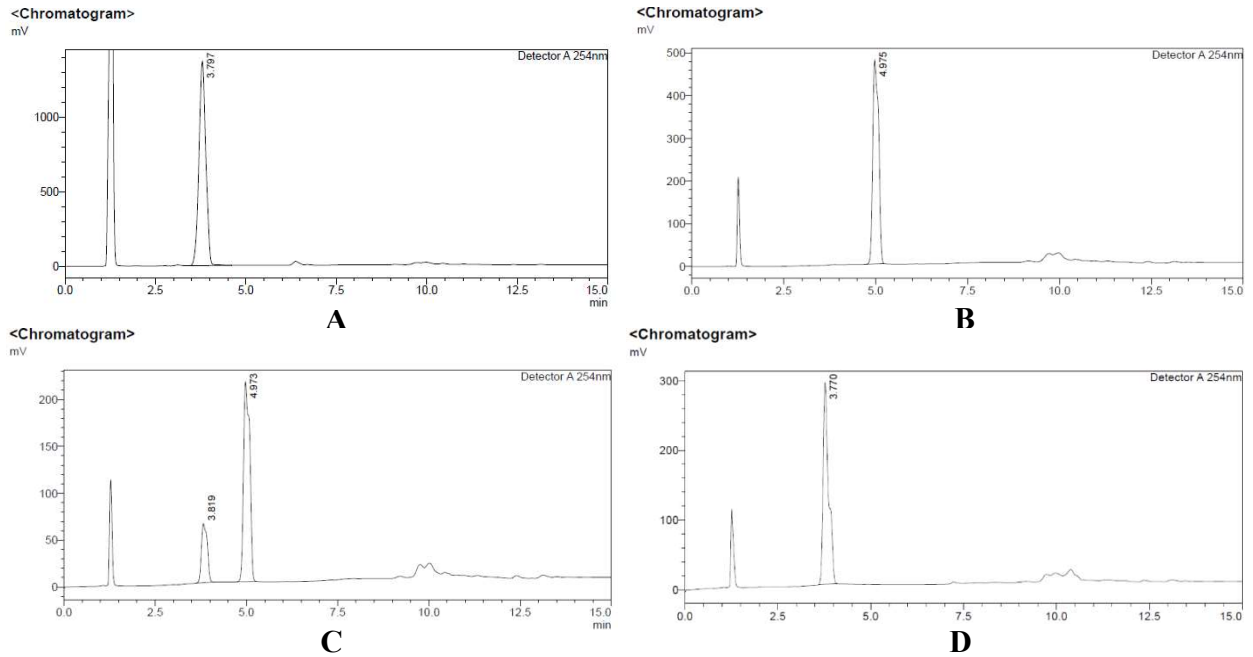
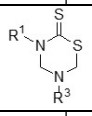
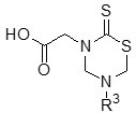
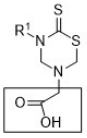
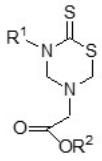


Fig. 2: HPLC chromatogram of enzymatic hydrolysis. A) THTT ester derivative 10a in buffer and acetone; B) THTT acidic analog 7c in buffer and acetone; C) After 2 minutes; esterase hydrolysis of THTT ester derivative (10a); D) After 20 minutes; complete esterase hydrolysis of THTT (10a) into its acidic counterpart (7c).

Table 2: Antiproliferative activity of various THTT scaffolds 9-12 (series A-D)[a]

Entry	Compound	R^1	R^2	R^3	$IC_{50} \pm SEM^b$ (μM)		
					PC3[c]	HeLa[c]	3T3
			Series A 				
1.	7a	CH(Ph)Me	–	Et	11.4 ± 0.9	4.0 ± 0.2	10.8 ± 1.4
2.	7b	CH(Ph)Me	–	*n*-Pr	9.1 ± 0.2	3.9 ± 0.3	15.8 ± 3.3
3.	7c	CH(Ph)Me	–	*i*-Pr	6.4 ± 2.7	3.8 ± 0.6	18.7 ± 0.3
4.	7d	CH(Ph)Me	–	*n*-Bu	8.2 ± 0.2	3.2 ± 0.1	33.1 ± 4.5
5.	7e	CH(Ph)Me	–	$(CH_2)_2OH$	8.9 ± 1.1	3.7 ± 0.5	15.6 ± 2.2
6.	7f	Et	–	Et	NA	NA	41.9 ± 5.0
			Series B 				
7.	8a	–	–	Et	NA	21.8±3.2	NA
8.	8b	–	–	*n*-Pr	NA	NA	NA
9.	8c	–	–	$(CH_2)_2OH$	NA	NA	NA
10.	8d	–	–	*n*-Bu	NA	NA	NA
			Series C 				
11.	9a	Et	–	–	23.4 ± 3.1	23.8± 1.6	NA
12.	9b	*i*-Pr	–	–	NA[d]	14.0± 0.2	NA
13.	9c	*n*-Pr	–	–	14.4 ± 0.6	20.4± 1.5	NA
14.	9d	*n*-Bu	–	–	NA	11.0± 3.2	NA
15.	9e	3,4-$(OMe)_2$.Bzl	–	–	7.9 ± 0.7	8.4 ± 0.8	22.7 ± 2.5
16.	9f	CH(Ph)Me	–	–	8.7 ± 0.2	2.4 ± 0.3	28.4 ± 2.3
			Series D 				
17.	10a	*n*-Pr	Me	–	29.9±0.3	NA	NA
18.	10b	*n*-Pr	Et	–	NA	17.9± 5.1	NA
19.	10c	*n*-Pr	Bzl	–	NA	14.8± 4.3	NA
20.	10d	*n*-Bu	Et	–	NA	NA	42.8 ± 2.5
21.[e]	Doxorubicin	–	–	–	0.3 ± 0.02	0.3± 0.02	–
22.[e]	Cycloheximide	–	–	–	–	–	0.61±0.17

[a] Antiproliferation results represented in IC_{50} as the mean of triplicate; testing incubation period of 48 hours at 37±1 ºC. [b] SEM = ± standard error of the mean. [c] PC3 = Prostate cancer cell lines; HeLa = Cervical cancer cell lines; 3T3 = Mammalian mouse fibroblast cell lines. [d] NA = Not active. [e] IC_{50} values of std. drugs doxorubicin = 0.30 μM for PC3/HeLa cell lines and cycloheximide = 0.61 μM for 3T3 cell lines.

cytotoxicity of series A–D was also examined against 3T3 cell lines model. The antiproliferative activity of allsynthesized analogs was initially screened at the concentration of 30 μg/mL for PC3, HeLa and 50 μg/mL for 3T3 cell lines, the results were calculated as a percentage of inhibition as shown in table 1. Subsequently, the IC_{50} values for PC3 and HeLa cell lines were determined for all active compounds and compared with the standard drug doxorubicin as listed in table 2. Most of the compounds showed significant anticancer potential against both cancer cell lines within the range of IC_{50} = 6.4–29.9 and 2.4–23.8 μM respectively when compared with standard doxorubicin (IC_{50} = 0.3 μM). Cytotoxic evaluation of series A-D against 3T3 cell lines in IC_{50} values as compared with standard drug cycloheximide (IC_{50} = 0.61 μM) was also performed and

found non- or less toxic.

Esterase Catalyzed Hydrolysis (prodrug evaluation)
Hydrolysis of 3,5-disubstituted tetrahydro-2*H*-1,3,5-thiadiazine-6-thione (THTT) ester derivative (10a) was carried out by using porcine liver esterase (PLE) under phosphate buffer (pH 7.0–7.5) in a shaking incubator at 37 ºC for 20 minutes duration. The reaction successfully hydrolyzed the ester derivative into its acidic counterpart (9c), whereas acid analogue of THTT (9c) appeared stable when subjected for the same hydrolytic conditions.

DISCUSSION

As part of our interest for the investigations of biologically active molecules, herein, we report the antiproliferative activity of four (04) series of thiadiazine thiones analogs. This includes disubstituted tetrahydro-2*H*-1,3,5-thiadiazine-2-thiones with N-3 and N-5 alkyl groups 7a-f (series A), carboxylic group attached at -N-CS-S- fragment of the THTT ring skeleton 8a-d (series B), thiadiazine-6-thiones with N-3 carboxylic and N-5 alkyl groups 9a-f (series C) and N-3 carboxylic esters and N-5 alkyl groups 10a-d (series D).

The obtained biological screening results showed that antiproliferative activity primarily depends upon the position and nature of the substituents at the thidiazine nucleus. The structure activity relationship indicated the importance of aryl and alkyl groups at both nitrogen atoms for better antiproliferation (series A). Generally, the presence of aryl groups such as 3,4-$(OMe)_2$.Bzl and CH(Ph)Me at -N-CS-S- of the THTT ring skeleton resulted in significant antiproliferation activity against both cancerous cell lines (PC3 and HeLa) as can be noticed for compounds 7a-e, 9e and 9f (table 1). Gratifyingly, all the compounds of series A and series C showed higher activity against HeLa cells, note that compound 9f from series C was found the most active in the series (IC_{50} = 2.4±0.3 μM). In the same way, 7c from series A was found as the most active molecule against PC3 cells (IC_{50} = 6.4±2.7 μM). On the other hand, changing the position of alkyl and carboxylic groups at N-3 and N-5 positions (series B) resulted in the loss of the activity against both cancerous cell lines. For instance, on comparing the compounds from series B and series C, both have the same functional groups but the positions are altered, the former found inactive while later showed significant antiproliferation.

For an anticancer drug, it is critical to harm cancer cells, while be tolerated very well by normal tissues. To assess this requirement, cytotoxicity of all the compounds was also evaluated on 3T3 (mammalian mouse fibroblast) at the concentration of 50 μM initially and the results as the percentage growth inhibition are listed in table 1 (see result section). The IC_{50} values were also determined and compared with standard cycloheximide as shown in table 2 (result section). Interestingly, all the synthesized compounds found either non-toxic and/or relatively less toxic for 3T3 cell lines model. It is important to note that all the esters (series D) are generally found with reduced cytotoxic effects not only for 3T3 cells as well as for both cancerous cell lines, in comparison to their acidic counterparts. This behavior may be attributed to their prodrug criterion of being inactive and non-toxic (normal cells).

To check the potential of bio-reversibility and prodrug phenomenon of thiadiazine esters to release its parent acid form, we performed enzymatic hydrolysis of THTT ester 10a. Enzymatic hydrolysis of ester derivative (10a) was carried out by using porcine liver esterase (PLE) under phosphate buffer (pH 7.0–7.5) at 37 ºC for 20 minutes stirring. Acetone was used as a co-solvent to overcome solubility concerns and the reaction was monitored by analytical HPLC. Analysis of reaction mixture after two minutes by HPLC (fig. 2, Chromatogram C) gave two major peaks representing the ester 10a at retention time 4.9 minutes (fig. 2, Chromatogram B) and hydrolyzed product at retention time 3.8 minutes, presumably THTT acid 7c. On behalf of the HPLC analysis of the reference compound 7c (fig. 2, A), it was easily concluded that the hydrolyzed product at retention time 3.8 minutes was actually the THTT acid (7c). After twenty minutes of hydrolysis, HPLC monitoring resulted in only one peak which exactly matched the acid (7c) at retention time 3.8 minutes (fig. 2, D), thus it showed the complete conversion of THTT ester (10a) into its acidic counterpart (7c). This bio-reversibility is in compliance with prodrug criterion of being inactive, non-toxic and bio-transformable into parent active species.

CONCLUSION

In this report, various derivatives of thidiazine skeleton through several structural modifications at N-3 and N-5 positions were evaluated for their *in vitro* antiproliferative activity against two cancerous cell lines; HeLa (human cervical carcinoma) and PC3 (prostate cancer). The cytotoxicity of these THTT analogs was also examined on non-cancerous 3T3 cell lines (mouse fibroblast). Most of the compounds showed notable anticancer potential against both cancer cell lines however found more selective against HeLa cells. In addition, these compounds are mostly found non-toxic for 3T3 cells. Complete conversion of the ester derivative of thiadiazine-6-thione into its acidic counterpart under enzymatic hydrolysis further suggests their potential as prodrugs candidates. These results indicated that THTT derivatives especially ester derivatives can be a possible lead for future investigations as prodrug anticancer probes.

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