Original Article

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Synthesis and cytotoxic activity of N-(2,4-dichloro)benzoyl-N'-phenylthiourea against human breast cancer cell line

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ABSTRACT

Introduction: Urea- and thiourea-based compound has shown a potency to be further developed as anticancer compound. **Objective:** Another novel phenylthiourea analog (*N*-(2,4-dichloro) benzoyl-*N'*-phenylthiourea) was synthesized through Schotten-Baumann reaction followed by the assessment of its cytotoxic activity. **Methods:** *N*-(2,4-dichloro)benzoyl-*N'*-phenylthiourea was synthesized using *N*-Phenylthiourea and 2,4-dichlorobenzoyl chloride as starting materials. The reaction started at low temperature for 30 min followed by reflux for 8 h to yield target compound. Cytotoxicity assay was then performed against MCF-7, T47D, and Vero normal cell line. **Results:** The compound has been synthesized and its structure has been verified using infrared, ^{1}H-nuclear magnetic resonance (NMR), ^{13}C-NMR, various 2D NMR, and mass spectra. Furthermore, it is shown that the synthesized compound possesses better cytotoxicity profile than hydroxyurea. **Conclusion:** *N*-(2,4-dichloro)benzoyl-*N'*-phenylthiourea is a potential thiourea analog as anticancer agent, albeit further research is needed.

Keywords: Schotten-Baumann, phenylthiourea, anticancer activity

INTRODUCTION

Development of potent chemotherapeutic agents is still an important task to complete, since cancer has caused an enormous mortality case worldwide.[1,2] Since the first application of chemotherapy in the mid-20th century, 5-fluorouracyl was one of the most widely used compounds to treat various solid tumors.[3] The discovery of anticancer activity of hydroxyurea[4] several years later fosters the research and development of urea-based anticancer, one of which is thiourea analogs. Numerous studies have confirmed their activity against various target, namely, receptor protein tyrosine kinase, DNA-topoisomerases, sirtuins, carbonic anhydrase,[5] and aromatase.[6] This class of compound also acts as somatostatin agonist, which plays a key role in regulating proliferative process of cells.[5]

Benzoyl phenyl thiourea is one of an interesting scaffold in drug discovery, particularly as anticancer agent. It is argued that the presence of two aromatic moieties (phenyl and benzoyl) would enhance their lipophilicity, which, in turn, improving pharmacokinetics profile.[7] These moieties have also been proven to aid the binding process in several receptors such as sirtuin-1 as shown by tenovins and their analog,[8] and in epidermal growth factor receptor.[9] This study was performed in continuation of our attempt to design novel potent anti-breast cancer agent based on *N*-benzoyl-*N'*-phenylthiourea scaffold, where we have explored several halobenzoyl analogs.[10-12] Herein, we report the synthesis and structural characterization of *N*-(2,4-dichloro)benzoyl-*N'*-phenylthiourea. In addition, the synthesized compound was also tested against MCF-7, T47D, and Vero normal cell lines to assess its potential anti-breast cancer activity.[13]

MATERIALS AND METHODS

Materials

All chemical reagents were purchased from Sigma-Aldrich (Singapore). Thin-layer chromatography (TLC) was performed on silica gel 60 F254 (Merck Millipore, Jakarta, Indonesia). Melting point of the compound was determined

using Fisher-Johns Mel Temp apparatus. Infrared spectra were measured using Jasco FTIR-5300 (Tokyo, Japan). Nuclear magnetic resonance (NMR) spectra measurements were conducted using Agilent DD2(500 MHz ^{1}H; 125 MHz ^{13}C) (Agilent Technologies, Santa Clara, CA, USA) with the data reported as follows (chemical shift [δ, in ppm], multiplicity, coupling constant [J, in Hz], and integration). Mass spectra were recorded using Waters LCT-Premier XE orthogonal accelerated-time of flight (oa-TOF) (Waters MS Technologies, Manchester, England, UK).

Methods

Synthesis

Reaction was performed using method previously published.[11,12] A 8 mmol of *N*-phenylthiourea (1.2 g) was mixed with 10 ml tetrahydrofuran (THF) and 1.0 ml triethylamine (TEA). It was stirred constantly on the ice bath followed by dropwise addition of 1.0 ml 2,4-dichlorobenzoyl chloride (7 mmol) premixed with adequate amount of THF. After 30 min, the mixture was heated under reflux condition (100ºC). Reaction completion was monitored hourly using TLC. After 8 h, the reaction was deemed complete and subsequently followed by vacuum evaporation to remove THF. Crude solid obtained was then washed with saturated sodium bicarbonate solution and vacuum filtered, prior recrystallization using hot ethanol. Compound *N*-(2,4-dichloro)benzoyl-*N*'-phenylthiourea was obtained as white needle-shaped crystal.

Cytotoxicity assay

MCF-7, T47D, and Vero cell lines were seeded into 96-well plates and then incubated for 24 h in 5% CO_2 incubators. Furthermore, test solutions, positive and negative controls of various concentrations were added. Each concentration was replicated for 3 times. Wells containing no cells and only filled with medium were used as medium controls. At the end of incubation, each well was added with 100 µL of 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), followed by incubation for 3 h, and then, the MTT reaction was discontinued by adding 100 µL of 10% sodium dodecyl sulfate (SDS) in 0.01 N HCl into each well. The microplate was wrapped in paper and incubated at 37°C for 24 h. The live cells converted MTT into a dark blue formazan. Enzyme-linked immunosorbent assay (ELISA) reader was utilized to identify the absorption at λ = 595 nm. The IC_{50} values were obtained using probit analysis and compared to hydroxyurea as parent compound which possesses anticancer activity, to evaluate whether the modification improve its bioactivity.

RESULTS AND DISCUSSION

Synthesis

To the best of our knowledge, reports have in fact been published regarding the synthesis and characterization of *N*-(2,4-dichloro)benzoyl-*N*'-phenylthiourea[14] and its complex form with ruthenium.[15]. Our study has successfully synthesized *N*-(2,4-dichloro)benzoyl-*N*'-phenylthiourea using modified Schotten-Baumann reaction,[10-12] as opposed to the previous method[14] which utilized potassium thiocyanate and aniline as starting material. *N*-Phenylthiourea was reacted with 2,4-dichlorobenzoyl chloride [Figure 1]. This reaction followed the nucleophilic acyl substitution mechanism, where nucleophilic attack of primary amine of phenylthiourea was followed immediately by elimination of chlorine leaving group. Triethylamine acted as a base to neutralize the resulting hydrochloric acid [Figure 2]. Initially, the reaction took place exothermically so that it is necessary to put on an ice bath.[16] Afterward, the mixture was refluxed for 8 h to obtain target compound with yield of 34%. The structure of *N*-(2,4-dichloro)benzoyl-*N*'-phenylthiourea was then confirmed with infrared, NMR, and mass spectroscopy.

Infrared spectrum showed strong and very broad absorption peak at 3168 cm^{-1} indicating the presence of -NH group and strong peak at 1686 cm^{-1} which corresponds to carbonyl group. Both peaks were found in lower wavenumbers than usual (3350 and 1800–1700 cm^{-1}), which indicated the possible formation of intramolecular hydrogen bond in the benzoyl thiourea group. In addition, intense peak was also observed at 1537 cm^{-1} originated from -NH bending vibration, which further solidify the presumed hydrogen bond formation.[17-19] ^{1}H-NMR spectrum indicated the presence of singlet peak at 9.46 and 12.29 ppm with equal integration ratio, which corresponds to the formation of benzoyl thiourea bond. It is argued that the downfield chemical shift observed was due to intramolecular hydrogen bond.[19,20] Meanwhile, ^{13}C-NMR spectrum showed the existence of carbonyl and thione group at 165.3 and 177.7 ppm, respectively. Furthermore, 2D

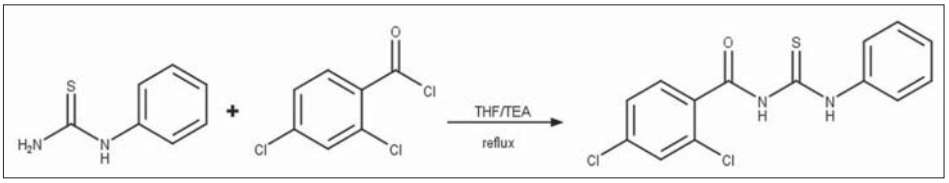


Figure 1: Synthesis of N-(2,4-dichloro)benzoyl-N'-phenylthiourea

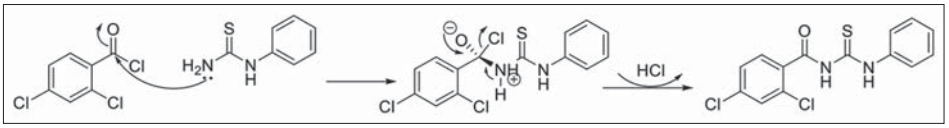
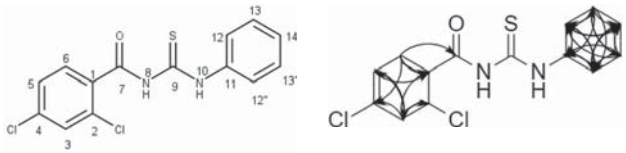


Figure 2: Reaction mechanism of synthesis of N-(2,4-dichloro)benzoyl-N'-phenylthiourea

NMR spectra were recorded (heteronuclear single quantum coherence/HSQC and heteronuclear multiple bond coherence/HMBC) to verify all hydrogen and carbon assignment of the compound [Table 1]. Ultimately, mass spectrum data confirmed the presence of the synthesized compound with m/z value of 322.9816 (M; 100%), 324.9795 (M+2; 65%), and 324.9795 (M+4; 10%). This pattern indicates the presence of two chlorine atoms.

N-(2,4-dichloro)benzoyl-*N*'-phenylthiourea (0.74 g) (34%), mp 118–119°C. IR (KBr disk): 3168 (-NH), 1686 (-C=O, amide), 1537 (-NH) cm^{-1}. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 12.29 (s, 1H), 9.46 (s, 1H), 7.69 (d, J = 7.6 Hz, 2H), 7.68 (d, J = 8.3 Hz, 1H), 7.50 (d, J = 2.0 Hz, 1H), 7.42 (t, J = 7.6 Hz, 2H), 7.40 (dd, J = 2.0. 8.3 Hz, 1H), 7.29 (t, J = 7.6 Hz, 1H). ^{13}C-NMR (125 MHz, $CDCl_3$): 177.7 (C=S), 165.3 (C=O), 139.2, 137.4, 132.2, 131.6, 130.9, 130.6, 129.0 (2C), 128.1, 127.2, 124.2 (2C). COSY, HMBC, and HSQC data as presented in Table 1 and Supplementary File. ESI oa-TOF MS: m/z calcd. $[M-H]^+$: 322.9813, found: 322.9816.

Table 1: Cross-peaks in HSQC and HMBC of synthesized compound



No.	HSQC		HMBC
	^{1}H-NMR	^{13}C-NMR	
1.	-	139.2	-
2.	-	130.6	-
3.	7.50 (1H, d, *J*=2.0 Hz)	130.9	C1; C2; C4; C5
4.	-	132.2	-
5.	7.40 (1H, dd, *J*=2.0 Hz ; 8.3 Hz)	128.1	C1; C3
6.	7.68 (1H, d, *J*=8.3 Hz)	131.6	C1; C4; C7
7.	-	165.3 (C=O)	-
8.	12.29 (s,-NH)	-	-
9.	-	177.7 (C=S)	-
10.	9.46 (s,-NH)	-	-
11	-	137.4	-
12	7.69	124.2 (2C)	C11; C14; C12'
12'	(2H, d, *J*=7.6)		
13	7.42	129.0 (2C)	C11; C12; C12'; C13'; C14
13'	(2H, t, *J*=7.6)		
14	7.29	127.2	C12; C12'
	(1H, t, *J*=7.6)		

[1]Numbering system does not reflect IUPAC nomenclature, only for the purpose of proton and carbon correlation

Cytotoxicity Assay

Previously, this type of compound has been assessed its anticancer activity against various cancer cell line. Using Ru^{II}(p-cymene)Cl_2 complexed derivative, *N*-(2,4-dichloro)benzoyl-*N*'-phenylthiourea showed micromolar inhibitory activity against colorectal (HCT-116 and SW480), lung (NCI-H460), and cervical (SiHa) cancer.[15] The result of anticancer activity against MCF-7 and T47D cell showed that the target compound showed better cytotoxicity profile compared to hydroxyurea in both cell lines (approximately 30 times and 5 times, respectively) [Table 2 and Figure 3]. This result is comparable to our previously synthesized halobenzoyl analogues.[11,12] Nevertheless, this compound poses negligible toxicity against Vero cell line and more selective toward malignant cell. It is argued that the addition of benzoyl, phenyl, and halogen moiety will increase the lipophilicity of the compound, thus enhancing its capability of cell penetration.[10,21] The microscopic images of MTT assay results are presented in Supplementary Materials.

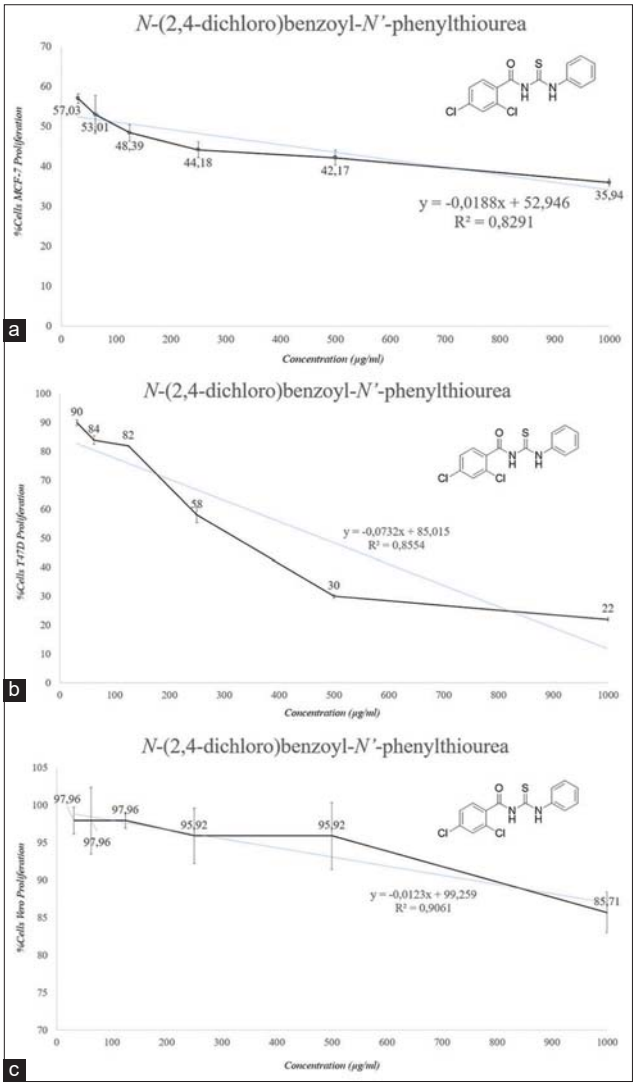
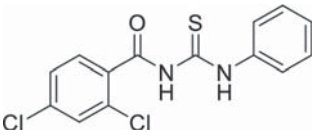
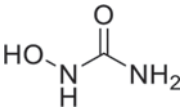


Figure 3: (a-c) Inhibitory concentration graphs of N-(2,4-dichloro) benzoyl-*N*'-phenylthiourea against MCF-7 cell (up), T47D cell (middle), and Vero normal cell (low)

Table 2: IC_{50} value and selectivity index of synthesized compound and hydroxyurea

Compound	IC_{50} (mM)		CC_{50} (mM)	Selectivity index	
	MCF-7	T47D	Vero	MCF-7	T47D
 *N*-(2,4-dichloro) benzoyl-*N*'-phenylthiourea	0.31±0.05	0.94±0.02	179.48±1.43	>10	>10
 Hydroxyurea	9.76±0.01	4.58±0.16	369.88±0.91	>10	>10

CONCLUSION

We have synthesized *N*-(2,4-dichloro)benzoyl-*N*'-phenylthiourea using Schotten-Baumann like reaction. This compound possesses better cytotoxic activity against hydroxyurea and high selectivity index against two cancer cell lines (MCF-7 and T47D), with the IC_{50} value of 0.31 and 0.94 mM, respectively. Further research can be focused on exploring another halogen-substituted derivative in finding a more potent anticancer candidate.

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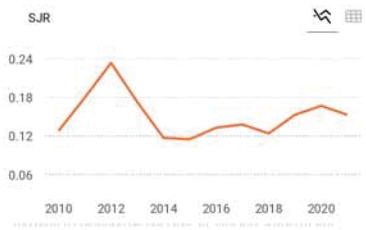

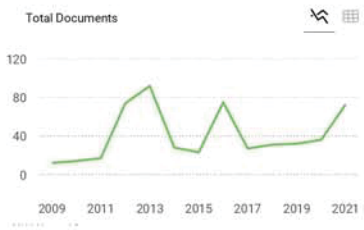

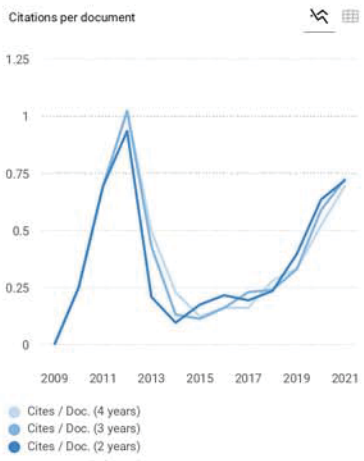

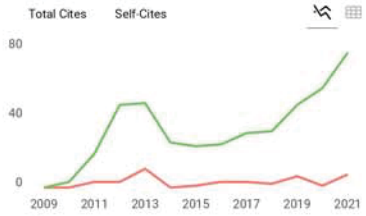

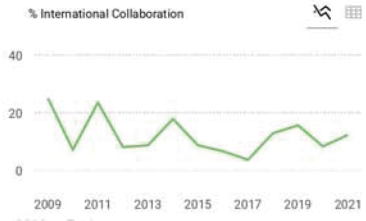

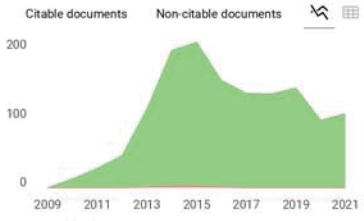

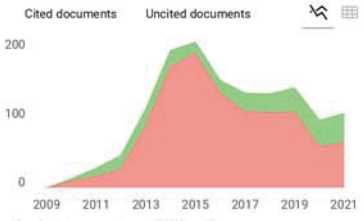

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