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SYNTHESIS OF *N*-4-METHOXYBENZOYL-*N*'-PHENYLUREA COMPOUND AND ACTIVITY TEST OF ANTICANCER AGAINST HeLa CELL LINE

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ABSTRACT

Research in the search for cancer drug compounds continues to be developed, considering that specific anticancer compound has not been obtained. Some of the urea derivative compounds are also being developed in the search for an anti-cancer compound which is potent with minimal side effects. Related to the explanation above, a urea derivative, which is *N*-phenylurea, would like to be developed. It would be reacted with 4-methoxybenzoyl chloride, thus *N*-4-methoxybenzoyl-*N*'-phenylurea compound would be obtained. The synthesis of *N*-4-methoxybenzoyl-*N*'-phenylurea was carried out by Schotten-Baumman method that had been modified, and then a test of purity was done by thin layer chromatography using 3 different kinds of eluent. The next phase was structure characterization which was carried out by using UV and IR spectrophotometry, then ^{1}H-NMR spectrometry also MS, so that the structure from *N*-4-methoxybenzoyl-*N*'-phenylurea would be obtained. Anticancer activation test was conducted on HeLa cell line by using MTT assay, and the value of IC_{50} would be obtained. The compound that has been successfully synthesized is *N*-4-methoxybenzoyl-*N*'-phenylurea, with a yield of 51,23%. The purity test of *N*-4-methoxybenzoyl-*N*'-phenylurea was done by thin layer chromatography with 3 different types of eluent (hexane: ethyl acetate: methanol = 2: 3: 1; Hexane: acetone = 4: 2; Hexane: ethyl acetate = 4: 2) one single spot was obtained, which its value of Rf is different than the original compound, *N*-phenylurea. While the melting point of the compound is 158^{o}C, thus it was seen that the compound has been formed and was different from the original compound, *N*-phenylurea. Anticancer activation test has been performed with MTT assay method by using HeLa cell line, the result obtained of IC_{50} is 6.50 mM more active than hydroxyurea is 170.57 mM as a standard. The conclusion is that

N-4-methoxybenzoyl-*N*'phenylurea, have been successfully synthesized and has anticancer activity.

KEYWORDS: synthesis; *N*-4-methoxybenzoyl-*N*'-phenylurea; anticancer activity.

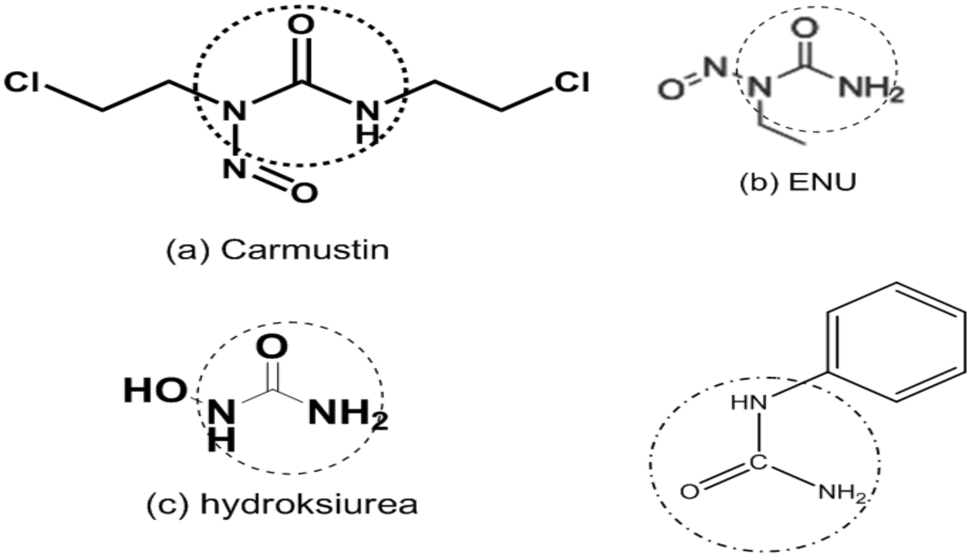
INTRODUCTION

Cancer is one of the diseases which is the main cause of death in developing countries and also worldwide. From 58 million worldwide death in 2005, there were 7.6 million (13%) which were caused by cancer. Death which is caused by cancer was expected to increase in 2015 and 11.4 million people in 2030. In Indonesia, cancer became the third largest contributor to death after heart disease.[1] Nowadays, the attempt to find new anticancer drug continue to be done because the old drugs which have long been used gradually become less effective and there is a tendency of resistance.[2]

Classical anticancer drugs which are used in chemotherapy are a group of the alkylating compound, antimetabolite, anthracycline antibiotics, plant alkaloids, topoisomerase inhibitors. The drugs in those groups inhibit the cell division or the synthesis and function of DNA in some ways. New anticancer drugs do not affect DNA directly, but they affect specific target molecule which is needed for carcinogenesis and the growth of cancer cells, known as *molecularly targeted therapy*.[3] Anticancer which are included in this group are tyrosine kinase inhibitors (*small molecule tyrosine kinase inhibitor*) and monoclonal antibodies. Tyrosine kinase inhibitors which have become the anticancer drugs are imatinib, gefitinib, and erlotinib. Oncologists believe that the target molecule therapy is the future chemotherapy. Hydroxy Urea (Hydrea®) also used as anticancer has distinctive features that are able to produce nitric oxide, which is a vasodilator may cause antitumor effects. RNR inhibition by hydroxy urea possibility for it has an unpaired electron that can extinguish tyrosine radicals.[4]

Modern drug development is conducted through a long process and also spends great costs because in the beginning, it was just a trial (trial and error). To minimize the nature of the trial, the drug designing is performed (drug design), which is developing drugs which molecular structure and biological activity have been known, based on the reasoning that is systemic and rational by Quantitative Relationships Structure and Activity method (QSAR).[5] In the era of a computer nowadays, the approach of structure and activity relationship can be performed more quickly and directed by "molecular modeling".[6]

The first step in drug designing is determining the lead compound which structure will be modified to obtain the desired activity, to increase the activity or selectivity, or to decrease the toxicity. After that the modification of the lead compound structure was done. Urea is one of the lead compounds, which is the parent of many derivatives which are potential as anticancer. Urea derivative, which is well known as an anticancer drug, is carmustine or BCNU (*bis-chloroethylnitrosourea*) and ENU (*N-ethyl-N-nitrosourea*) which include alkylating compounds, and hydroxyurea (HU) which works by inhibiting ribonucleotide reductase enzyme.[7] ENU can induce mutations and also toxic at the high dose. The structure of these compounds is shown in Figure 1.


(a) Carmustin

(b) ENU

(c) hydroksiurea

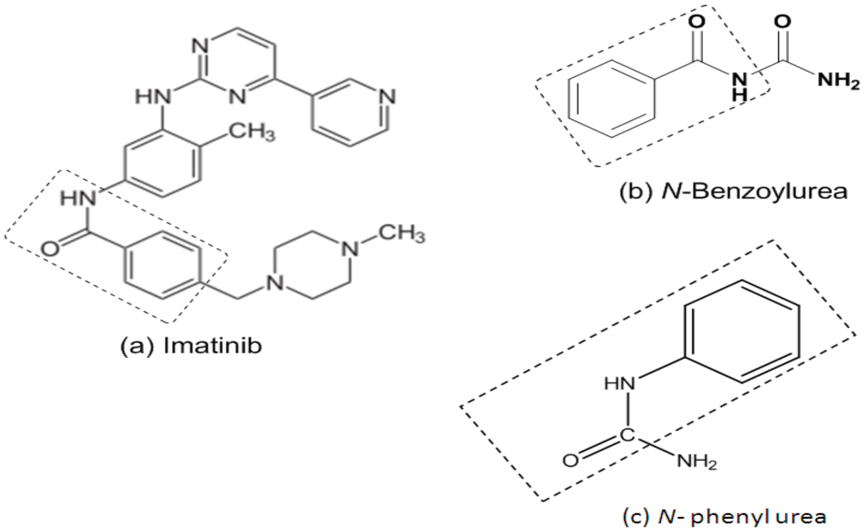
(d) *N*-Phenyl urea

Figure 1. Structure of Carmustine (a), ENU (b), hydroxyurea (c), and *N*-phenylurea (d)

Siswandono has done the synthesis of a new compound which is derived from benzoylurea that has activity on the central nervous system in the form of movement disorder in mice.[8] Susilowati and Diyah reported that *N*-benzoylurea, *N*-4-methoxy-benzoylurea, and 2-chlorobenzoylurea showed a cytotoxic effect based on BST (*Brine Shrimp Lethality Test*) so that it is potential as an anticancer.[9] The cytotoxic activity of those compounds is higher than hydroxyurea, which has been known as an anticancer drug. Hydroxyurea and its hydroxamic acid derivatives are reported to work by affecting the specific enzyme target involved in the cancer development such as histone deacetylase, matrix metalloproteinase (MMP), and RNR.[10,11] The cytotoxic activity by Brine Shrimp Test is also indicated of the cells die, this method is also used to determine the cytotoxic activity of the 11 bases ketone Schiff and 16 heterocyclic compounds synthesized.[12;13] Hardjono has made a modification of the structure of urea by synthesizing 9 derivatives 1- (benzoyloxy) urea and concluded that there is a linear relationship between in vitro cytotoxic activity against HeLa cell with the activity which is predicted to be conducted *in silico* through molecular modeling. The activity of cytotoxic derivative 1- (benzoyloxy) urea is higher than hydroxyurea.[14]

In the attempt to get a new anticancer compound, urea structural modifications are done. The structure of anticancer compounds contains NH-CO-NH moiety, which is a pharmacophoric group. In some compounds, such pharmacophore is in an aromatic heterocyclic ring or in the nonaromatic heterocyclic ring, as -NH or -N-.[15] By maintaining pharmacophore -NH-CO-NH- and also adding an alkyl group in form of ethyl and an aromatic group, *N*-phenylurea derivative compound will be made (figure 2).

For the optimization of anticancer activity, modification of the structure of phenylurea compound based on the change of the nature of lipophilic, steric and compound electronic is done. The modification of lipophilic and electronic properties is done based on the Topliss model[16], which is by including the substituent in the benzene ring, which is predicted to produce compounds with higher activity than the parent compounds. Substituent methyl ($-CH_3$) will increase the non-polar lipophilic properties, while the electronegative substituents such as methoxy, chloro, and trifluoromethyl will change the electronic properties of compounds. The modification of steric properties results from the change in the size of benzoyl group which has been substituted.[17] Compound and *N*-phenylurea derivatives can be synthesized through the reaction between *N*-phenylurea compound with benzoyl chloride based on Schotten-Baumann method.[18]



(a) Imatinib

(b) *N*-Benzoylurea

(c) *N*- phenyl urea

Figure 2. Structure of Imatinib (a), *N*-benzoylurea (b), and *N*-phenylurea (c)

To test the anticancer activity, the determination of cell growth inhibition activity by *in vitro* is done.[19] Tumor cell culture which is used is cervical carcinoma cell (HeLa cells). The activity of cell growth inhibition is expressed in IC_{50}, namely the concentration of drug that can result in the death of 50% of tumor cells.[20]

From this research, a new compound which is derived from *N*-phenylurea which can be used as an anticancer drug will be obtained.

RESEARCH METHOD

Materials

The materials for synthesis and physicochemical analysis have grades of pro analysis: *N*-phenylurea, pyridine, various organic solvents (acetone, ethyl acetate, *n*-hexane, chloroform, ethanol, and methanol), Kieselgel 60 F_{254}, DMSO-d_6. And were purchased from the Sigma Aldrich and E.Merck.

The materials for the anticancer activation test: synthesized compound, HeLa cell culture, Culture Media DMEM, DMSO solution, phosphate buffer saline (PBS), MTT (3- (4,5-dimetiltiazol-2-il) -2,5-diphenyltetrazolium bromide), SDS 10% in 0.1 N HCl.

Tools

The tools used for the synthesis and structural analysis of compounds: glassware in the laboratory, Spectrophotometer UV-vis Shimadzu HP, 8452 A, Spectrophotometer Jasco FT-IR 5300, Spectrometers, ^{1}H-NMR Hitachi R-1900, Electrothermal Mel-Temp, Corning Hot Plate P 351, Analytical balance Shimadzu LM-20. The tools for cytotoxic test: Micropipette 200, 1000 μL and tip, test tube, microplate, conical tube, ELISA-Reader.

Synthesis procedure of *N*-phenylurea derivate

In a round bottom flask of 200 ml, 0.03 mol of *N*-phenylurea is mixed with 40 ml of tetrahydrofuran and 4 ml of pyridine. At a temperature of 5° C, 4-methoxybenzoyl chloride derivative solution of 0.01 mol is added to 20 ml of tetrahydrofuran, bit by bit while stirred with a magnetic stirrer. After the benzoyl chloride derivative solution is out, the mixture is refluxed and stirred for 3 hours.

After the reaction is stopped, tetrahydrofuran is evaporated on a rotavapor (rotary evaporator). On the result of the reaction, saturated sodium bicarbonate solution is added while stirring until there is no air bubble (foam). The result is filtered through a Buchner funnel, the solids are washed with 50 ml of water 2 times, then they are washed with 10 ml of ethanol two times.

Recrystallization is done by dissolving the solids with ethanol as necessary while it is stirred above the heater (hot plate). The solution is filtered in hot condition, the filtrate is left at room temperature until it is cool and then left it for a night. A crystal which is formed is filtered through a Buchner funnel, and then it is washed with 10 ml of ethanol two times. Depending on the synthesized compounds obtained, recrystallization can be done with another solvent which is suitable, such as acetone-water. A crystal which is formed is moved into a petri dish, and then it is dried in an oven.

Purity Test and Compound Structure Identification

The purity test of the synthesized compounds is done by thin layer chromatography, through stationary phase Silica gel GF_{254} plate aluminum E. Merck and UV lamp stain appeared. A melting point determination is done by electrothermal melting point apparatus. The identification of the structure of the synthesized compound is performed by a spectrophotometer ultra-violet (UV) and infrared (IR), nucleus magnetic resonance spectrometry (^{1}H-NMR) and mass spectrometry (MS).[21]

Anticancer Activity Test

Anticancer activity is determined through the cytotoxic test *in vitro* by using cancer cells (HeLa cell line) with MTT method. Parent solution from 10 test compounds at the level of 5000 μg/ mL in DMSO solvent. From each of the parent solution, a series of working standard solution with concentrations of 250, 500, 750, 1000, 2000 μg/ mL are made by dilution. The standard solution of anticancer drug (hydroxyurea) is also made as a positive control and solvent blank as a negative control.

The culture of cancer cell and the normal cell is prepared in the form of a cell suspension with a density of 10^5-2.10^6. Then the cell is inserted into *Microplate* wells, each is 100 μL, except the well used as a control media. A total of 0.2 mL of each standard work, positive control, and negative control, is included in the *microplate*. For each concentration of the working standard solution, three replications are made. *The microplate* is incubated in an incubator of 5% CO_2 for 24 hours at a temperature of 37º C and pH of 7.4 to 7.7. MTT reagent is prepared for treatment (0.5 mg/ ml) by diluting 1 mL of stock MTT (50 mg MTT in 10 mL of PBS) with sufficient media.

After incubation, the cell media is removed, and the cell is washed with PBS. Then, 100 μL of MTT reagent is added to each well, including media controls (without cells). The plate is incubated for 2-4 hours in a CO_2 incubator. After formazan is formed, 100μL SDS 10% is added in 0.1 N HCl. *The plate* is wrapped in aluminum foil and incubated in a dark place at room temperature for a night. Furthermore, the absorbance of each of the wells is observed by ELISA reader at 595 nm. The more the number of cells live, the greater the absorbance. IC_{50} of the test compound is determined by probit regression analysis. The same procedure is also performed for normal cells.

RESULT

Synthesis of *N*-4-methoxybenzoyl-*N*'-phenylurea

Synthesis result in the form of white needle-shaped crystals with a yield of 51,23 %, it shows that the method of Schotten-Bauman was the elected method of the synthesis process to produced the *N*-4-methoxybenzoyl-*N*'-phenylurea compound. Identification of the structure of the active compound *N*-4-methoxybenzoyl-*N*'-phenylurea can be seen in the following analysis.

In the next stage test, thin-layer chromatography on compounds synthesized by using 2 different solvents (n-hexane: acetone = 4:2 and n-hexane: ethyl acetate = 4:2) gave a single spot with different Rf compound with the *N*-phenylurea as the parent compound. The above shows that the desired compounds synthesized have been formed and relatively pure compounds also have different from the parent compound. At the melting point analysis of test compound that had been synthesized had a melting point (158^{o}C) and had a difference with the parent compound which had the melting point (145^{o}C). In this test has proven that the compound which was synthesized has been formed and has a relative purity because there were no other impurities in it.

Characterization of the compound

N-4methoxybenzoyl-*N*'-phenylurea, λ max (nm)(Ethanol) = 252; IR (KBr pellet), 3446 cm^{-1} (NH secondary), 1685 cm^{-1} (-CO), 1538 cm^{-1} (C=C arom); $^{1-}$HNMR (DMSO-d_6), 6.90–8, 10, m, (C_6H_5), 12.00-13.00, d, (NH); 3.70-4.10, m, (OCH_3); MS (EI), 270 $(M)^+$, 151 $(CH_3O(C_6H_5)CONH)^+$

Anticancer activation test of *N*-methoxybenzoyl-*N*'-phenylurea compound and Hidroxy Urea

After the anticancer test is conducted in *N*-4-methoxybenzoyl-*N*'-phenylurea compound and hydroxyl urea by using MTT assay method, the result can be seen in Figure 1 and 2 below.

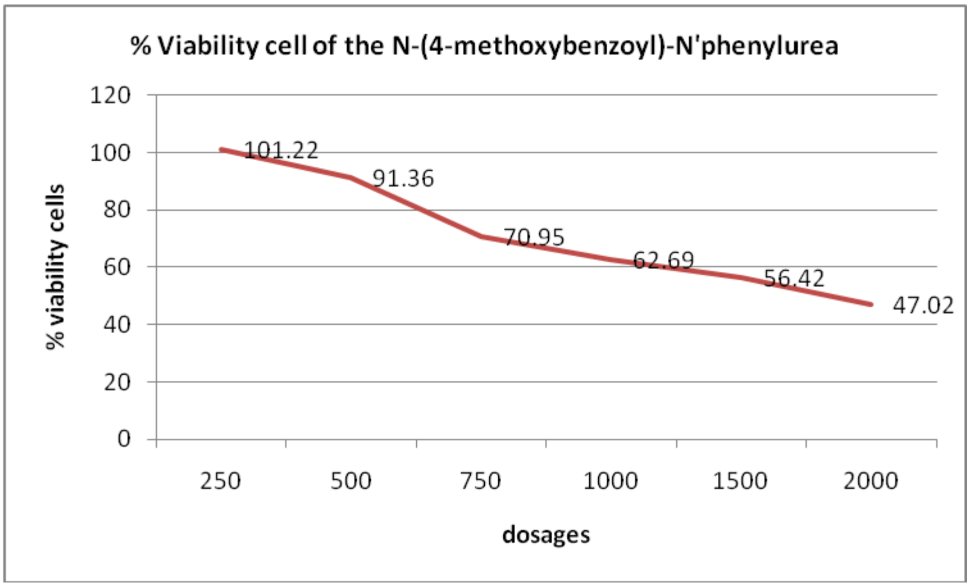


Figure 1: The result of anticancer activation test of *N*-4-methoxybenzoyl-*N*'-phenylurea compound.

IC_{50} = 1757 ug/mL = 6.50 mM

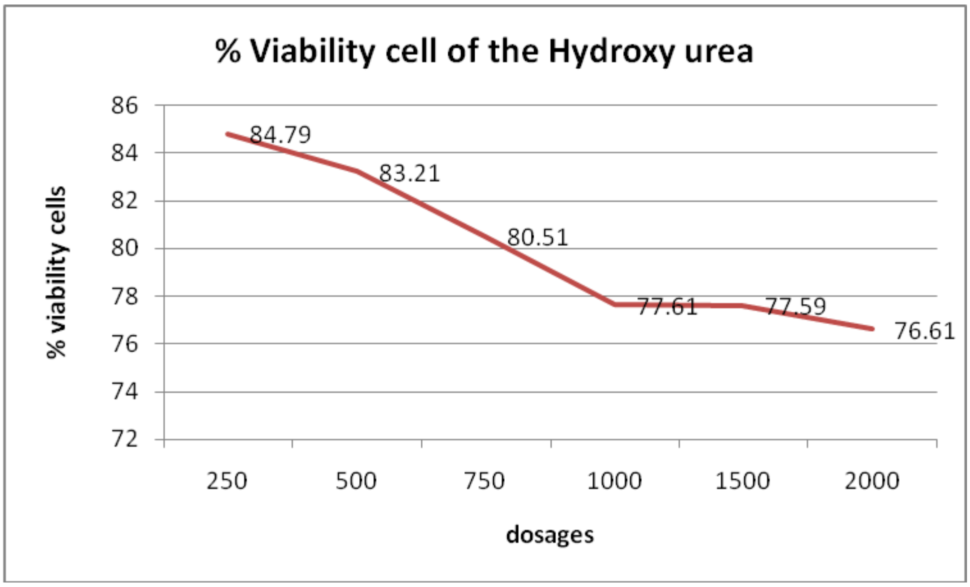


Figure 2: The result of anticancer activation test of hidroxy urea as a standard compound.

IC_{50} = 12972 ug/mL = 170.57 mM (Hidroxy urea)

DISCUSSION

The synthesis of *N*-4-methoxybenzoyl-*N*'-phenylurea is carried out by reacting the primary amine group of *N*-phenylurea with 4-methoxybenzoyl chloride group of benzoyl chloride. Several researchers have done some method of reaction between primary amine group with the derivative of benzoyl chloride. Reksohadiprodjo,1987 and Tjiptasurasa,1991 have done acylation reaction between the derivative of urea with an acyl chloride, the method used is by mixing and heating at the temperature of 60 - 80°C.[22;23] It turns out that the result obtained has a relatively small yield (9-31%), this can be due to the lack of contact between the compounds because there are no solvent media which is used. The method above is used by researchers because urea derivative has different solubility properties with acyl chloride derivative. Urea derivative is a compound that is water soluble, while the derivative of acyl chloride is not water soluble. On the implementation of acylation reaction, water is avoided because the presence of water can react with benzoyl chloride derivatives and it will form benzoic acid. Alcohol solvent also should not be used because it will form ester compound if it is reacted with benzoyl chloride derivative. In the acylation reaction, benzoyl chloride derivative can react quickly and perfectly with primary, secondary, or tertiary amine compounds in the use of pyridine solvent and gives the percentage of result which is relatively good.[24]

In the acylation reaction between urea compound with a derivative of acyl chloride, theoretically both primary amine groups can react with the derivative of acyl chloride, but the result of research that has been done by Reksohadiprodjo, 1987 and Tjiptasurasa, 1991 shows that only one primary amine group of urea compound reacts with acyl chloride derivative. This is due to the influence of the space obstacle of aromatic nucleus so that it will interfere the next reaction. In the acylation reaction, HCl will be released. It can interfere the process of the reaction because the amide group which is formed will be parsed back, therefore it can be overcome with the addition of 2 equivalents of the amine compound. Another way to overcome the problems above is by neutralizing HCl which is formed by adding a strong base such as sodium hydroxide or potassium hydroxide solution. The reaction between amine compound with acyl chloride using organic solvent is known as Schotten-Baumann reaction.[18;24]

Bambang Soekardjo (1989) has conducted the acylation reaction between ampicillin and benzoyl chloride derivative. Ampicillin, which has a primary amine group, will react with benzoyl group and formed the amide compound. The reaction solvent used is tetrahydrofuran, which is an inert solvent.[25]

The same thing was done by Bambang Tri Purwanto (1991), who has succeeded in doing the formation reaction of *p*-bromobenzoylampicillin by reacting ampicillin with *p*-bromobenzoylchloride by using the reaction solvent of tetrahydrofuran.[26]

Siswandono (1999) has conducted the acylation reaction between urea compound and a derivative of benzoyl chloride. It turns out that this reaction found a difficulty because urea compound is dissolved in polar solvents, while derivative of benzoyl chloride is dissolved in a nonpolar solvent. Therefore, the tetrahydrofuran solvent is used, that will form the suspension with urea compound, which is then reacted with a derivative of benzoyl chloride which is dissolved in tetrahydrofuran as well.[27]

Suzana (2004) has conducted a formation reaction of benzoylthiourea compound, which is reacting urea with thiocyanate compound to form a new thiourea, which is then reacted with benzoyl chloride derivative.[28]

Another researcher, Dini (2005), has successfully performed the formation reaction of the benzoylthiourea derivative by reacting thiourea with benzoyl chloride derivative by using tetrahydrofuran reaction solvent.[29]

On the formation of *N*-4-methoxybenzoyl-*N*'-phenylurea, the reaction is also carried out by reacting *N*-phenylurea compound with 4-methoxybenzoyl chloride by using tetrahydrofuran reaction solvent, and it is refluxed for 8 hours. After the reaction is finished, then saturated sodium carbonate solution is added to neutralize HCl and benzoic acid derivative which is formed. The next step is washing it with water to remove the remnants of the existing HCl salt, and then it is washed with methanol to remove the residue of benzoyl chloride derivative or its hydrolysis result, which is benzoic acid derivative. The next stage is doing recrystallization with hot methanol because *N*-4-methoxybenzoyl-*N*'-phenylurea derivatively is soluble in hot methanol but it is not soluble in cold condition. In general, the recrystallization result of *N*-4-methoxybenzoyl-*N*'-phenylurea derivative is in the form of small shiny white needle crystal or shiny pieces.

The purity of compounds which are the result of synthesis that have been formed then is tested by thin layer chromatography (KLT) by using three mobile phases, namely a mixture of hexane: ethyl acetate: methanol (2: 3: 1), a mixture of hexane: acetone (4: 2) and hexane: ethyl acetate (4: 2). The result of KLT shows that at various mobile phases used above, there is only one stain. This means that the compound which is the result of synthesis might be a single compound.

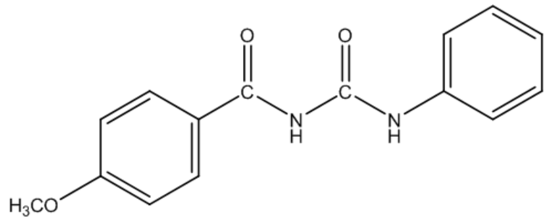
The next stage is to test the purity by determining the melting range of the compounds which are the result of synthesis. The result of melting range determination of compounds which are the result of synthesis shows that the melting range is relatively small, which is around two degrees Celsius. It means that the compounds which are the result of synthesis are pure.

Structure identification of *N*-4-methoxybenzoyl-*N*'-phenylurea compound

In determining the wavelength of *N*-4-methoxybenzoyl-*N*'-phenylurea by using UV spectrophotometer, it can be seen that the compounds which are the result of synthesis show two maximum wavelengths, which are 252 nm. The compounds which are the result of synthesis is estimated to have a conjugated double bond chromophore group or aromatic system and auxochrome group. From the UV spectra, there is also seen a shift in the maximum wavelength. *N*-phenylurea reagent shows two wavelengths, which are 204 nm and 238 nm, while the compounds which are the result of synthesis also show two maximum wavelengths, which are 252 nm. It shows that there is the addition of chromophore group in the benzoyl structure so that it can be said that the acylation reaction between *N*-phenylurea and 4-methoxybenzoyl chloride has been going as expected.

In the IR spectrum of *N*-4-methoxybenzoyl-*N*'-phenylurea it can be seen that there is a band in the area of 3446 cm^{-1} which indicates the secondary amine group of aromatic amine compounds. It is also supported by ^{1}H-NMR spectra with two sharp bands in the area of 12.00 and 13. 00 ppm, which indicate that there is 1 atom of H singlet from secondary amine group (group CONH). Two carbonyl groups are seen in IR spectrum at wave numbers of 1685 and 1603 cm^{-1}, while for the group -C=C- can be seen in wave numbers of 1538 and 1515 cm^{-1}. This is strengthened by a ^{1}H-NMR spectrum that shows the multiplicity of 10 H atoms from two aromatic nuclei in the chemical shift from 6.90 to 8.10 ppm.

From the analysis of the spectra above, it can be concluded that the compound is *N*-4-methoxybenzoyl-*N*'-phenylurea with chemical structural formula as follows.



From the data of anticancer activity against Hella cell line at the table and IC$_{50}$ form the *N*-4-methoxybenzoyl-*N*'-phenylurea is 6.50 mM, it can be seen that the test compounds can cause the death of 50% of cancer cells at the level of approximately 1000 to 2000 µg/ mL. While the hydroxy urea as a standard has IC$_{50}$ 170.57 mM.

CONCLUSION

1. *N*-4-methoxybenzoyl-*N*'-phenylurea compound has been successfully synthesized, and it shows the difference between one compound and the parent compound of *N*-phenylurea in the purity test by thin layer chromatography and also melting range test.
2. *N*-4-methoxybenzoyl-*N*'-phenylurea compound has anti-cancer activity against HeLa cell and higher than a standard hidroxyurea.

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