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ANTI-AMOEBIC ACTIVITIES OF SYNTHETIC AMINOTHIOUREA DERIVATIVES AGAINST *Acanthamoeba castellanii*

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ABSTRACT

This study was carried out to observe the cytotoxicity and genotoxicity properties of three novel compounds of synthetic aminothiourea derivatives on a pathogenic *Acanthamoeba castellanii* (IMR isolate) in order to search for a new anti-*acanthamoeba* agent especially for treatment of *Acanthamoeba* keratitis. In cytotoxicity study, viability of cells and CD_{50} of the compounds on *A. castellanii* were determined using eosin dye assay. The amoebae were treated with three aminothiourea derivatives compounds at different concentrations at 30ºC for 72h. The compounds used were *N*-(benzyl(carboxy)carbamothioyl)-2-methylbenzamide, 3-(1–carbamoyl–3-(4–chlorobenzoyl)thioureido)propanoic acid, and 2-[3-(Furan-2-carbonylthioureido]acetic acid that were labeled in this study as C1, C2 and C3, respectively to facilitate discussion. Genotoxicity effect of the synthetic aminothiourea derivatives on *A. castellanii* was performed at compound concentrations of CD_{10}, CD_{25} and CD_{50} using the Comet assay. The CD_{50} values of C1, C2 and C3 compounds obtained in this study were 45.0 µg/mL, 62.5 µg/mL and 45.0 µg/mL, respectively. The DNA damage observed in *A. castellanii* cells after treated with C1, C2 and C3 compounds varied depending on the compounds and their concentrations used. In general, DNA damage observed in individual cells of *A. castellanii* by the C2 compound treatment was more severe than the treatment with C1 and C3. Due to higher CD_{50} value in C2 compared to C1 and C3 based on the DNA damage imposed on the amoeba, C2 compound which is the 3-(1–carbamoyl–3-(4–chlorobenzoyl)thioureido)propanoic acid is considered the most potent compound with anti-*Acanthamoeb*a activities observed in the present study.

ABSTRAK

Kajian ini dilakukan untuk melihat sifat-sifat kesitotoksikan dan kegenotoksikan tiga sebatian terbitan aminotiourea ke atas patogen *Acanthamoeba castellanii* (isolat IMR). Dalam kajian kesitotoksikan, daya hidup sel ameba dan CD_{50} sebatian tersebut ditentukan dengan menggunakan cerakin pewarna eosin. Ameba dirawat dengan tiga sebatian terbitan aminotiourea pada kepekatan yang berbeza selama 72 jam pada 30ºC. Sebatian yang digunakan adalah asid *N*-(benzal(karboksi)karbamotiol-2-metilbenzamida, asid propanoik 3-(1-karboksi-3-(4-klorobenzoil)tioureido) dan asid asetik 2-(3-furan-2-karboniltioreido) yang dilabel sebagai C1, C2 and C3, masing-masing bagi memudahkan perbincangan. Kesan kegenotoksikan sebatian-sebatian terbitan ini ke atas *Acanthamoeba* dilakukan pada kepekatan CD_{10}, CD_{25} dan CD_{50} dengan menggunakan cerakin Comet. Nilai CD_{50} yang diperolehi untuk C1, C2 and C3 adalah 45.0 µg/mL, 62.5 µg/mL dan 45.0 µg/mL, masing-masing. Tahap kerosakan DNA pada sel *Acanthamoeba* adalah berlainan bergantung kepada sebatian dan juga kepekatan yang digunakan. Secara umumnya, tahap kerosakan DNA pada sel yang dirawat dengan C2 lebih teruk dibandingkan dengan sel yang dirawat dengan C1 dan C3. Walaupun nilai CD_{50} C2 lebih tinggi daripada C1 dan C3, berdasarkan kerosakan DNA ameba, sebatian C2 iaitu asid propanoik 3-(1-karboksi-3-(4-klorobenzoil)tioureido) dianggap sebatian yang paling kuat mempunyai aktiviti anti-*Acanthamoeba* dalam kajian ini.

Key words: Aminothiourea derivatives, *Acanthamoeba*, genotoxicity, cytotoxicity, CD_{50}

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INTRODUCTION

Acanthamoeba spp. can be found in various places including in salt water, swimming pools, fresh water, air and also eyewash stations (Alizadeh *et al.,* 1994). Although free living, *Acanthamoeba* may cause diseases infecting the eye, brain, skin and lungs (Radford *et al.,* 2002). One of the diseases is *Acanthamoeba* keratitis, an eye infection that may cause vision loss due to contact lens contaminated with this amoeba (Radford *et al.,* 2002).

Amoebicidal agents such as propamidine and hesamidine have been suggested to be used as treatment for serious types of keratitis (Saturnino *et al.,* 2003). Other anti-amoeba drugs are metronidazole, tinidazole, chloroquine and emetine (Bansal *et al.,* 2004). Both metronidazole and tinidazole are derived from 5-nitroimidazole and have the ability to kill the amoeba trophozoites but ineffective to kill their cysts (Bansal *et al.,* 2004). In addition, biguanides such as polyhexamethylene biguanide (PHMB) and chlorhexidine, diamidines such as propamidine and hexamidine have also been used as modern anti-amoeba agents (Lim *et al.,* 2008).

Thiourea is an organic compound consisting of carbon, hydrogen, nitrogen and sulphur with the formula CH_4N_2S. This compound has three functional groups which are amino, imino and thiol. Some compounds of thiourea have shown fungicidal, herbicidal, algaecidal and bactericidal activities (Khan *et al.,* 2008). A previous study on a series of bis-thioureidic derivatives against amoeba showed that these compounds were effective on *Acanthamoeba polyphaga, Hartmannella varinii* and *Vahlkampfia avara* (Saturnino *et al.,* 2003). According to Khan *et al.* (2008), one of the thiourea derivatives such as triazine showed antibacterial and antifungal activities against *Bacillus subtilis* and *Candida albicans*. There are no effective medicines available to treat patients infected with pathogenic *Acanthamoeba* spp. without long term effects. Therefore, in the present study, three synthetic aminothiourea derivatives were investigated for the presence of anti-acanthamoeba activities especially against *Acanthamoeba castellanii*, a clinical isolate. This investigation involves cytotoxicity and genotoxicity effects of the compounds towards this pathogenic *Acanthamoeba in vitro.*

MATERIALS AND METHODS

Source and Preparation of Compounds

Three synthetic aminothiourea derivatives compounds used in this study were *N*-(benzyl(carboxy)carbamothioyl)-2-methylbenzamide,3-(1–carbamoyl–3-(4–chlorobenzoyl)thioureido)propanoic acid, and 2-[3-(Furan-2-carbonylthioureido]acetic acid and were labeled as C1, C2 and C3 to facilitate discussion and their molecular structures are shown in Figure 1. Five milligram of each compound were first dissolved in dimethyl sulphoxide (DMSO) and then were diluted in amoeba fresh culture media to prepare the two fold dilution samples with concentrations of compounds from 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.3 µg/mL, 15.6 µg/mL to 0.0 µg/mL.

Cytotoxicity assay to determine cell viability

Cytotoxic assay of the compounds on *A. castellanii* was conducted in 24-well plates. Six different concentrations of synthetic aminothiourea derivatives ranging from 250 µg/mL to 15.6 µg/mL as prepared earlier were used to treat the amoeba. The treated and non-treated amoebae were incubated at 30ºC for 72 h before the cell viability was determined by eosin dye assay following the

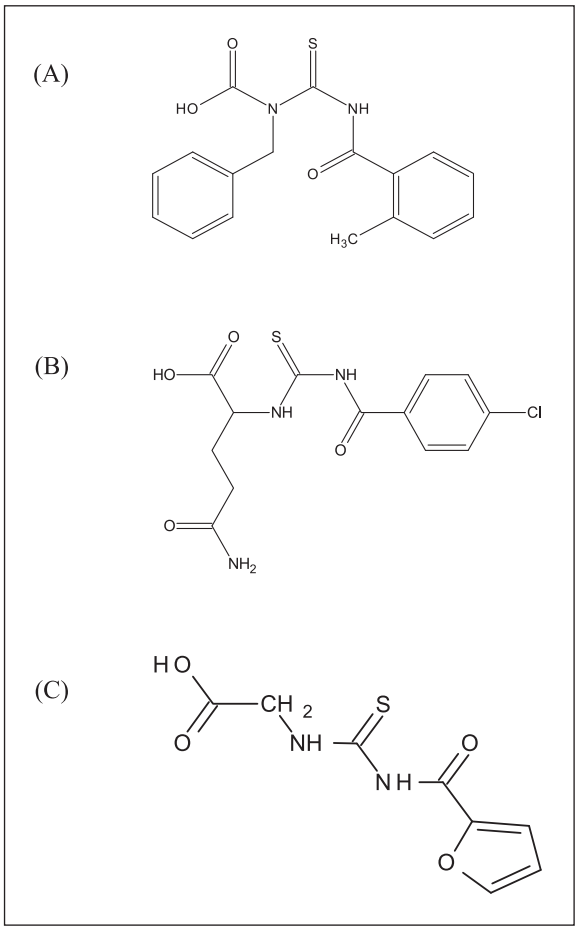

Fig. 1. Molecular structure of the derivative compounds used in this study. A. *N*-(benzyl(carboxy)carbamothioyl)-2-methylbenzamide (C1), B. 3-(1–carbamoyl–3-(4–chlorobenzoyl)thioureido)propanoic acid (C2) and C. 2-[3-(Furan-2-carbonylthioureido]acetic acid (C3).

method of Wrights *et al.* (1988). The percent viability of amoebae was calculated from the optical densities of treated wells relative to the untreated wells (control wells). A dose-response curve was plotted between data for percentage of cell viability against the concentration of compounds. The CD_{50}, CD_{25} and CD_{10} values for the three compounds against *A. castellanii* were deduced from this curve.

Alkaline Comet Assay

In this assay, the protocol by Lah *et al.* (2004) was followed. Briefly, the amoeba cells were treated with the three compounds at CD_{10}, CD_{25} and CD_{50} concentrations for 2 h. The amoebae were then harvested and centrifuged at 1000 rpm for 5 min. The supernatant was discarded and the pellet was washed once with Ca^{2+} and Mg^{2+} free PBS and re-centrifuged. Following that, the pellet formed was mixed thoroughly with 80 µl of 0.7% low melting agarose (LMA) and the mixture was spread over hardened 0.6% normal melting agarose (NMA) that was prepared earlier as the first layer gel on the slide. A cover slip was placed to spread the cells and the slide was left on ice to solidify. After the removal of the cover slip, the gel was covered with 200 µL of 0.5% LMA in order to prevent nuclear DNA from lysed cells from escaping during the electrophoresis (at 1 V/cm and 300 mA, for 5 min). After electrophoresis, the slide was neutralized with 400 mM Tris-HCl (pH 7.5) three times, 5 min each. The slide was later stained with ethidium bromide (20 µg/mL) and was left overnight in the dark at 4ºC before analyzing under fluorescence microscopy. One hundred cells (with three replicates of slides) were viewed, and the DNA damage was classified and quantified based on the method by Collins (2004).

RESULTS AND DISCUSSION

Percentage of viability of *A. castellanii* (IMR isolate) and CD_{50} determination

Viability of *A. castellanii* (IMR isolate) after treatment with the three derivative compounds at different concentrations was evaluated by using eosin staining assay and the data obtained are presented in Table 1 and Figure 2. In general, the percentage of cell viability was observed to decrease with the increase of compound concentration. This indicated that the growth of amoebae was affected when exposed to these compounds. Membrane permeability of this amoeba was also affected as shown by AOPI staining (data not shown) which

Table 1. Percentage of viability of *A. castellanii* (IMR isolate) at various concentrations of C1, C2 and C3 compounds

Compound Concentration (µg/mL)	% Viability (Mean ± SD)		
	C1	C2	C3
0	100.00 ± 0.52	100.00 ± 0.52	100.00 ± 0.52
15.6	97.66 ± 0.65	88.83 ± 1.59	91.17 ± 1.00
31.3	85.41 ± 0.53	76.40 ± 2.16	81.80 ± 1.51
62.5	13.69 ± 0.42	50.99 ± 1.29	10.63 ± 0.51
125	10.09 ± 0.47	24.32 ± 0.26	7.39 ± 0.15
250	9.01 ± 0.55	9.37 ± 0.68	7.03 ± 0.17

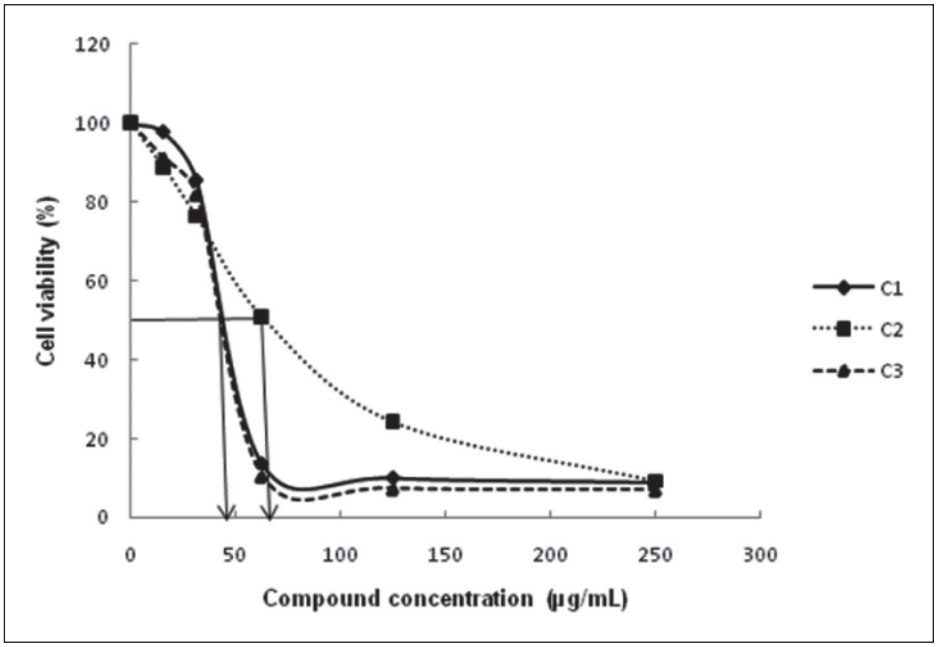

Fig. 2. Percentage of viable *A. castellanii* plotted against different concentrations of C1, C2 and C3. The CD_{50} obtained for each compound was derived from this figure and the value for C1, C2 and C3 was 45 µg/mL, 62.5 µg/mL and 45 µg/mL, respectively.

results in the death of amoeba. Interestingly, the decrease in percentage of amoeba viability when treated with different concentrations of C1 and C3 compounds showed a sudden drop when the concentration used to treat the amoeba reached 62.5 µg/mL. After this value, their dose-response curves almost reached a plateau indicating that there were not many amoeba cells left in the experimental wells. Subsequent treatment of the two compounds on the amoeba did not cause an apparent drop in percentage of cell viability.

Since the curve for percentage of amoeba viability against various concentration of compounds for C1 and C3 are almost identical (Figure 2), their anti-*Acanthamoeba* activities are suggested to be comparable, therefore, their CD_{50} values derived from this figure were the same i.e. 45 µg/mL. On the other hand, for C2, the decrease of amoeba viability with increasing compound concentration, was gradual (Figure 2). The CD_{50} value for C2 derived from this figure was 62.5 µg/mL. Based on their CD_{50} values alone, both C1 and C3 are more potent with anti-*Acanthamoeba* activity towards *A. castellanii* than C2 since their CD_{50} values are smaller (45 µg/mL) than the value for C2 (62.5 µg/mL). These values (CD_{50}) together with their CD_{25} and CD_{10} values were used to investigate the genotoxicity effect of these compound on DNA of this amoeba. Lower concentrations of compounds were suggested to be used in genotoxicity study to avoid reporting false positive results (Singh *et al.,* 1988).

Genotoxicity of compounds towards the DNA of *A. castellanii*

The genotoxicity effect of synthetic aminothiourea derivatives on *A. castellanii* (IMR isolate) by the three compounds at concentration of their CD_{10}, CD_{25} and CD_{50} against the amoeba is shown in Figure 3 where various scores for damage in the amoeba DNA at these concentrations are illustrated. DNA damage at scores 3 and 4 were only observed when the amoeba was treated with higher concentration of compounds such as at their CD_{25} and CD_{50}, particularly for C2 compound. At concentration of CD_{50}, the highest concentration of compound used in this study, only C2 induced the DNA damage at score 4. Neither C1 nor C3 were capable to induce the DNA damage at score 4. The DNA damage at scores 3 and 4 are irreversible and the damage is permanent, and in contrast to the DNA damage at scores 1 and 2, the damage is reversible. This type of DNA damage was also observed in the control amoeba. The frequency of the DNA damage at these scores in the control amoeba was low, only less than 20% as indicated in Figure 3 thus suggesting that the DNA damage at these levels occured naturally.

Among the synthetic compounds used, 3-(1–carbamoyl–3-(4–chlorobenzoyl)thioureido) propanoic acid, labeled as C2 had the most severe effect on the DNA of *Acanthamoeba* although its cytotoxicity (based on the CD_{50} value) on this amoeba was relatively low as compared to C1 and C3. The CD_{50} value for C2 was 62.5 µg/mL, thus the amount of this compound used in the genotoxicity study was relatively higher than the other two compounds (their CD_{50} values was 45 µg/mL). The difference in the amount of the C2 compound used may contribute to severe damage in the DNA of *Acanthamoeba* observed in the present study. Other than this, among the three compounds, only C2 consists of Cl, a halogen group in its structure that is lacking in the other compounds (Figure 1). Therefore, it is also suggested in this study that the chlorin atom that exists in the C2 compound might contribute to its strong genotoxicity properties against

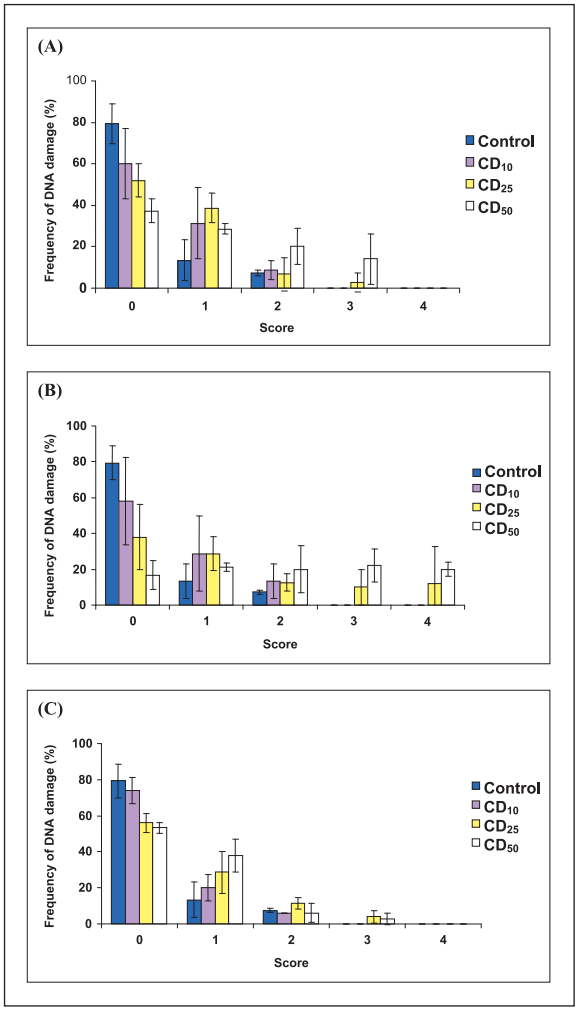

Fig. 3. DNA damage scoring in *Acanthamoeba* after 2 h treatment with compounds at their CD_{50}, CD_{25} and CD_{10} concentrations. Explanation for panels: A) C1; B) C2; C) C3. The results are the mean and standard deviation from three separate experiments.

Acanthamoeba as observed in the present study. To understand more the relationship between the compound structure and their anti-*Acanthamoeba* activity, more compounds with similar structures should be tested for their anti-*Acanthamoeba* activitiy. Detail studies involving quantitative structure-activity relationship (QSAR) approach should also be carried out.

CONCLUSION

Based on CD_{50} values alone, C1, *N*-(benzyl(carboxy) carbamothioyl)-2-methylbenzamide, and C3, 2-[3-(Furan-2-carbonylthioureido]acetic acid have stronger anti-*Acanthamoeba* activity against *A. castellanii* (IMR isolate) compared to C2, 3-(1–carbamoyl–3-(4–chlorobenzoyl) thioureido) propanoic acid. But based on the damage imposed on the DNA of *Acanthamoeba*, C2 compound is considered the most potent synthetic aminothiourea derivatives with anti-*Acanthamoeba* activity.

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