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The Effect of Fatty Alkanolamide Structure on Cytotoxic and Antimicrobial Activity

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Abstract

This research was conducted to explore the prospect of fatty acid derivatives as candidates for anticancer and antimicrobial drugs. Synthesis of fatty alkanolamides was carried out by direct amidation of fatty acids with alkanolamines. Several fatty acids and alkanolamines were used as starting materials to investigate the effect of the chain length, the presence of C=C bond, and the number of hydroxyl group of alkanolamides on the cytotoxic activity against HeLa cell line and also antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*. The structure of synthesized alkanolamides was characterized using FTIR and ^{1}H-NMR spectroscopy, the cytotoxic activity was evaluated using a standard MTT assay, and the antimicrobial activity was determined using the disc diffusion method. The HeLa cell line was cultured in DMEM medium supplemented with fetal bovine serum (FBS), penicillin, and streptomycin for 48 h during the cytotoxic assay. Chloramphenicol was used as positive control dan DMSO as a negative control for antimicrobial activity. Antimicrobial activity was conducted at 37 °C for 24 h. All fatty alkanolamines have been successfully synthesized in this research. All compounds demonstrated cytotoxic and antimicrobial activity as indicated and displayed accurately. A long-saturated alkyl chain was advantageous for the activities. Alkanolamide stearate-monoethanolamine showed the highest cytotoxic activity (IC_{50} = 37.5 μM) and antimicrobial activity. The presence of C=C bond decreased the activities. However, the polyunsaturated alkyl chain expressed greater activities than the monounsaturated alkyl chain. The number of hydroxyl groups on alkanolamines residue indicated a small contribution to the activity. Therefore, fatty alkanolamide is promising to be developed as an anti-cancer and antimicrobial drug.

Keywords: Fatty alkanolamide; direct amidation; cytotoxic activity; antimicrobial activity; lipoamide.

1. Introduction

The unique amphiphilic properties of alkanolamides are responsible for their wide applications as surfactants in various industries [1–5]. The abundant number of fatty acids in nature makes it suitable as an alkanolamides precursor. The presence of amide bond in fatty alkanolamides structures is a key feature for the pharmaceutical industry [6]. Even in 1999 databases, more than 25% of clinically approved drugs contain amide bonds [7]. Amide derivatives of fatty acids have been reported biologically active for signaling [8], antimicrobial agent [9–10], anticancer agent [11–12], oxidative stress therapeutic candidate [13], and improving mitochondrial function [14–15]. Fatty alkanolamide naturally becomes one of the endocannabinoid compounds [16] that have been reported to induce apoptosis in cancer cells [17–19]. Moreover, several amide derivatives of fatty acids have been shown to be equivalent to doxorubicin, a known anticancer drug, in controlling tumor cells [20].

Diverse methods have been developed for amide bond formation from a carboxylic acid. Normally carboxylic acids are converted to more reactive derivatives first [21] such as acyl chloride, anhydride [22], ester [23], or using a coupling agent (e.g. *N*,*N*'-dicyclohexylcarbodiimide). Therefore, the direct amidation on carboxylic acid attracts the attention of many researchers because this route has better atom economic. A wide variety of catalysts are employed to support the direct amidation of non-reactive carboxylic acid. The use of lipase in non-aqueous

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media for enzymatic direct amidation has been recorded [24–25]. However, the delicate of enzymes limits their applications. Thus, convenient handling of heterogeneous catalysts from inorganic solids (e.g. zeolite, silica) and organic-inorganic solids (e.g. graphene, organosilica) are more engaged [6, 26, 27].

This work described direct amidation of fatty acids using alkanolamine to obtain fatty alkanolamides (**Fig. 1**). Silica gel was appointed as the catalyst. The structures of fatty alkanolamide were carefully selected to clarify the effect of the chain length, the presence of C=C bond, and the number of hydroxyl group of alkanolamides on the cytotoxic activity against HeLa cell line and also antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*. New anticancer and antimicrobial drugs are urgently required. Cancer and the evolution of antibiotic-resistant bacteria are the two leading causes of death worldwide.

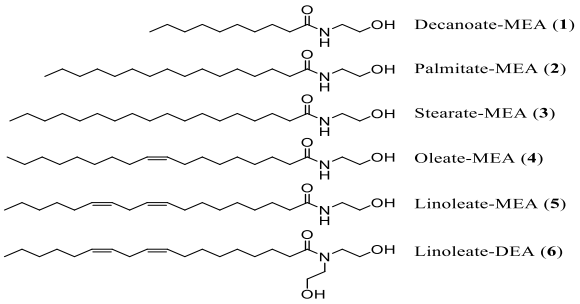

Fig. 1 The structure of fatty alkanolamides prepared from direct amidation of fatty acids (decanoic acid, palmitic acid, stearic acid, oleic acid, and linoleic acid) using alkanolamines (MEA = monoethanolamine, DEA = diethanolamine).

2. Experimental

Chemicals and all reagents and solvents have been used as received from the suppliers Merck, Sigma-Aldrich, Pudak Scientific, and Loba Chemie without further purification. The reaction progress was monitored on TLC aluminium sheets coated with silica gel 60 F_{254}. Melting points were measured on an SMP1 Stuart Scientific in open capillary tubes and are uncorrected. FTIR spectrum were analyzed on KBr using Shimadzu 2450 Prestige. ^{1}H-NMR spectrum were recorded in $CDCl_3$ on a Bruker Avance Neo 500 (500 MHz) spectrophotometer using TMS as the internal standard. Commercially available fatty acids (decanoic acid, palmitic acid, stearic acid, oleic acid, and linoleic acid) and alkanolamines (monoethanolamine and diethanolamine) were employed as the starting materials.

2.1. Synthesis

Fatty alkanolamides were synthesized from fatty acid according to the previously described method [Ojeda-Porras et al. 2015]. A reaction mixture of fatty acid (15 mmol), alkanolamine (16.5 mmol), and silica gel (20%) in *p*-xylene was heated for 3 h under reflux condition equipped with a Dean-Stark trap. The formation of alkanolamides was identified by TLC. The reaction mixture was cooled to room temperature, filtered, and washed with ethyl acetate. The organic solvent was evaporated and the residue was purified by silica gel column chromatography using *n*-hexane/ethyl acetate to get the desired compounds.

Decanoate-MEA (1) : white crystal, mp 69–70 °C, **FTIR** υ (cm^{-1}, neat) : 3347 (O-H), 3296 (N-H), 2918, 1640 (C=O), 1215 (C-N). **^{1}H-NMR** δ ppm : 0.88 (3H, t, *J* = 7.3 Hz), 1.28 (12H, m), 1.63 (2H, m), 2.20 (2H, t, *J* = 8.2 Hz), 3.41 (2H, q, *J* = 6.6 Hz), 3.71 (2H, t, *J* = 6.6 Hz), 6.22 (1H, broad s).

Palmitate-MEA (2) : white crystal, mp 94–95 °C, **FTIR** υ (cm^{-1}, neat) : 3347 (O-H), 3296 (N-H), 2920, 1640 (C=O), 1295 (C-N). **^{1}H-NMR** δ ppm : 0.88 (3H, t, *J* = 6.9 Hz), 1.29 (24H, m), 1.63 (2H, m), 2.20 (2H, t, *J* = 7.9 Hz), 3.43 (2H, q, *J* = 5.3 Hz), 3.73 (2H, t, *J* = 5.0 Hz), 5.89 (1H, broad s).

Stearate-MEA (3) : white crystal, mp 90–91 °C, **FTIR** υ (cm^{-1}, neat) : 3353 (O-H), 3305 (N-H), 2918, 1641 (C=O), 1052 (C-N). **^{1}H-NMR** δ ppm : 0.88 (3H, t, *J* = 7.0 Hz), 1.29 (28H, m), 1.63 (2H, m), 2.20 (2H, t, *J* = 7.8 Hz), 3.43 (2H, q, *J* = 5.6 Hz), 3.72 (2H, t, *J* = 5.0 Hz), 5.91 (1H, broad s).

Oleate-MEA (4) : colorless oil, **FTIR** υ (cm^{-1}, neat) : 3423 (O-H), 3306 (N-H), 2930, 1652 (C=O), 1047 (C-N). **^{1}H-NMR** δ ppm : 0.88 (3H, t, *J* = 7.0 Hz), 1.29 (20H, m), 1.62 (2H, m), 2.01 (4H, q, *J* = 6.5 Hz), 2.19 (2H, t, *J* = 7.9 Hz), 3.40 (2H, q, *J* = 5.5 Hz), 3.70 (2H, t, *J* = 5.0 Hz), 5.34 (2H, td, *J* = 3.9, 1.7 Hz), 6.25 (1H, broad s).

Linoleate-MEA (5) : colorless oil, **FTIR** υ (cm^{-1}, neat) : 3433 (O-H), 3290 (N-H), 2930, 1650 (C=O), 1047 (C-N). **^{1}H-NMR** δ ppm : 0.88 (3H, t, *J* = 6.8 Hz), 1.31 (14H, m), 1.62 (2H, m), 2.04 (4H, q, *J* = 6.3 Hz), 2.21 (2H, t, *J* = 7.5 Hz), 2.77 (2H, t, *J* = 6.7 Hz), 3.41 (2H, q, *J* = 5.5 Hz), 3.71 (2H, t, *J* = 5.2 Hz), 5.35 (4H, m), 6.15 (1H, broad s).

Linoleate-DEA (6) : colorless oil, **FTIR** υ (cm^{-1}, neat) : 3525 (O-H), 2930, 1630 (C=O), 1069 (C-N). **^{1}H-NMR** δ ppm : 0.88 (3H, t, *J* = 7.1 Hz), 1.29 (14H, m), 1.62 (2H, m), 2.04 (4H, q, *J* = 7.0 Hz), 2.39 (2H, t, *J* = 7.8 Hz), 2.77 (2H, t, *J* = 6.8 Hz), 3.50 (2H, t, *J* = 5.1 Hz), 3.55 (2H, t, *J* = 5.1 Hz), 3.78 (2H, t, *J* = 5.3 Hz), 3.84 (2H, t, *J* = 5.1 Hz), 5.35 (4H, m).

2.2. Cytotoxic activity

HeLa cell line (ATCC CCL 2) was cultured in the DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. A 100 μL cell suspension was seeded at 5000 cells/well and kept at 37 °C in a humidified

atmosphere of 5% CO_2 for 24 h to reach ~50% confluency. The cells' morphology and confluency were observed under a microscope prior to the addition of a series concentration of samples. The treated cells were incubated for 48 h at 37 °C. The viable cells were determined by MTT assay using 10 µL MTT solution (5 mg/mL)/well and incubated for 4 h at 37 °C. The MTT reagent was reduced to purple formazan by mitochondrial reductase in viable cells. The formazan was dissolved in ethanol and the absorbance was measured at 595 nm. The IC_{50} value was determined using GraphPad Prism 9.5.0.

2.3. Antimicrobial assay

The bacterial suspension was prepared according to McFarland 0.5 turbidity standards. A 200 µL of bacterial suspension was added to a sterile petri dish followed by the addition of 20 mL of sterile nutrient agar at 40-45 °C, homogenized, and solidified. Then, the petri dish was divided into four to six sections and sterile disc paper was placed on top to fit the predetermined division. After that, 6 µL of the tested compounds were added on a paper disc with a diameter of 6 mm. Incubation was conducted at 37 °C for 24 h. The diameter of the clear zone was measured. Chloramphenicol was used as a positive control and DMSO as a negative control.

3. Result and Discussion

The carboxylic group of five different fatty acids was converted into an amide group through a one-step reaction in this research. The reaction was managed at around 135 °C by using a *p*-xylene reflux system. Yang *et al*. reported that the amidation reaction in xylene provided a better yield than in toluene [11]. Xylene has a higher boiling point than toluene. The Si–OH groups on the surface of silica gel as a catalyst played an important role in the dehydration process during the formation of an amide bond [6].

Saturated and unsaturated fatty acids were involved in this study. The saturated fatty acids were decanoic acid (10-carbon), palmitic acid (16-carbon), and stearic acid (18-carbon). Oleic acid (has one C=C) and linoleic acid (has two C=C) were also chosen because they are the most abundant 18-carbon unsaturated fatty acids in nature.

Among the six fatty alkanolamides, three fatty alkanolamides with a saturated alkyl group (compound **1**–**3**) were obtained as a white crystal at room temperature. This finding indicated that heating the fatty acids at around 135 °C during the direct amidation protocols did not change the configuration of the *cis*-C=C bond. The *trans*-unsaturated fatty acid will be solid at room temperature because their geometry is similar to saturated fatty acids [28].

3.1. FTIR spectral data

The FTIR spectra confirmed the conversion of fatty acids to the corresponding fatty alkanolamides (**Fig. 2**). The FTIR spectra of the starting material, the fatty acids, displayed a typical very broad and intense peak of O–H stretching in the region of 3000–3750 cm^{-1} due to the formation of the carboxylic acid dimer. On the other hand, fatty alkanolamides exhibited a broad and shallow peak in that region as a hint for the existence of O–H alcohol stretching. A single intense peak absorption of N–H alkanolamides occurred in the same region. The transformation of fatty acid to fatty alkanolamides shifted the peak of C=O stretching to a lower frequency [29]. The C=O of amide appeared as multiple intense peaks in the region of 1300–1800 cm^{-1} [30].

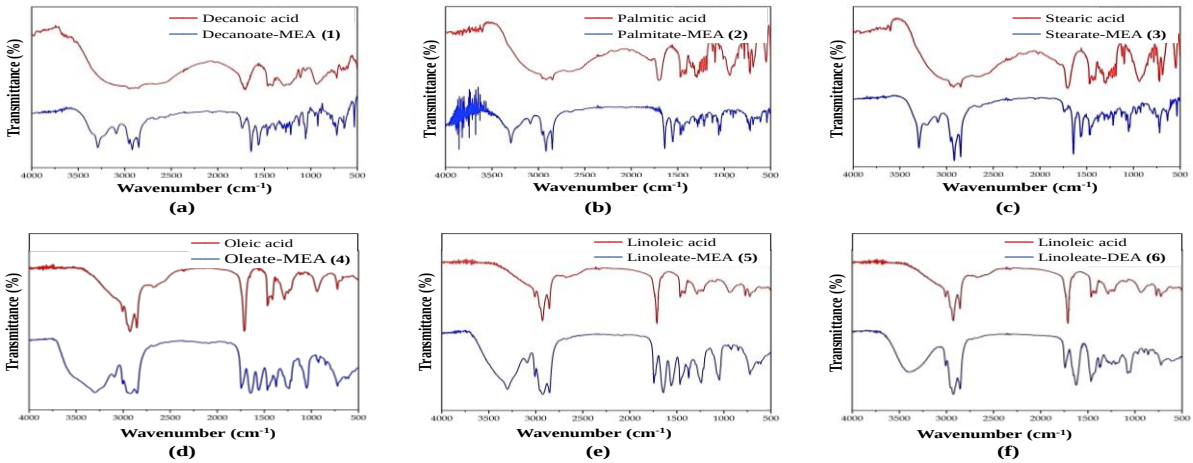

Fig. 2 FTIR spectra of fatty alkanolamide decanoate-MEA **1** (a), palmitate-MEA **2** (b), stearate-MEA **3** (c), oleate-MEA **4** (d), linoleate-MEA **5** (e), and linoleate-DEA 6 (f). (Note: MEA = monoethanolamine, DEA = diethanolamine).

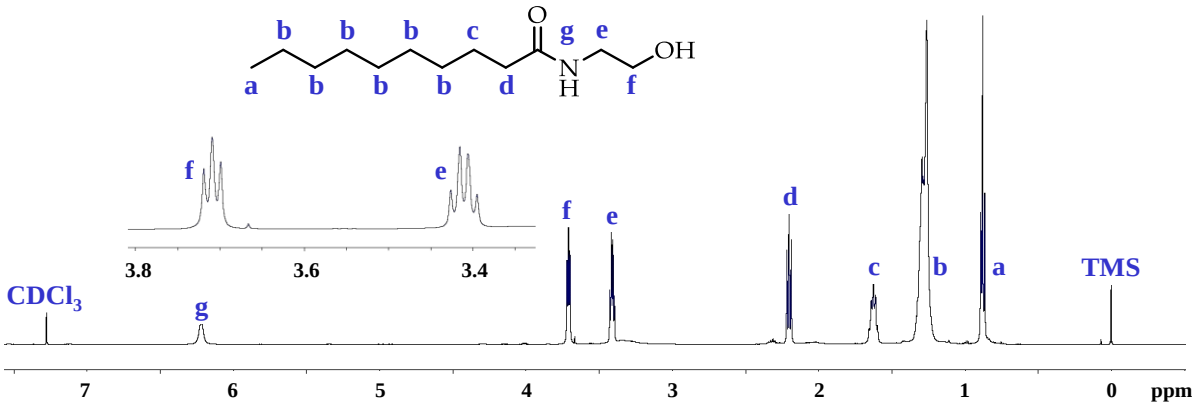

Fig. 3 ^{1}H-NMR spectra of fatty alkanolamide decanoate-monoethanolamine **1**.

3.2. ^{1}H-NMR spectral data

Fig. 3 shows the ^{1}H-NMR spectra of alkanolamide decanoate-monoethanolamine **1**. Detailed ^{1}H-NMR data for other fatty alkanolamides are presented in the Materials and Methods Section. ^{1}H-NMR data of all fatty alkanolamides consistently displayed the peaks in the regions of 3.4–3.5 and 3.7–3.8 ppm. These peaks demonstrated the attendance of protons on alkanolamine alkyl residues (**Fig. 3**, proton **e** and **f**). In addition, a broad peak in the region of about 6 ppm (**Fig. 3**, proton **g**) proved the presence of N–H proton from the secondary amide (compound **1**–**5**) [19]. Hence, the ^{1}H-NMR data convinced that all fatty alkanolamides have been successfully synthesized. The reaction between fatty acid and alkanolamine favored the formation of an amide product rather than ester product indicating that the amine group of alkanolamine had higher nucleophilicity than the hydroxyl group due to the lower electronegativity of the N atom.

3.3. Cytotoxic activity

The cytotoxic effect of six fatty alkanolamides on HeLa cell line was investigated by MTT (methyl thiazolyl tetrazolium) assay. The MTT assay is a colorimetric method based on the formation of formazan. The mitochondrial reductase of viable cells reduces the yellow MTT reagent to purple formazan.

All fatty alkanolamides exhibited cytotoxicity effects. Alkanolamides of palmitate **2** and stearate **3** demonstrated moderate cytotoxic activity with IC_{50} value between 10 and 50 µM [31], while the others had weak activity (**Fig. 4**). In general, unsaturated fatty alkanolamides expressed higher toxicity to HeLa cell line than unsaturated fatty alkanolamides. **Fig. 4** also revealed that the chain length of saturated fatty alkanolamide (compound **1**–**3**) had a positive correlation with the cytotoxic activity. The alkanolamide of stearate **3** had 1.7-fold stronger activity than the alkanolamide of decanoate **1**. On the other hand, the attendance of one C=C bond declined the activity. The alkanolamide of oleate **4** was found as the least toxic against HeLa cell line. However, the alkanolamide of linoleate **5** with two C=C bonds had greater activity than the alkanolamide of oleate **4**. In 2016, Arouri et al. stated that 250 µM of long saturated fatty acid decreased the number of viable MCF-7 cells after 48 h incubation [32]. Plötz et al. denoted caspase-3 activation was directly proportional to the length of fatty acid chain but lowered by monosaturated fatty acid [33]. The membrane permeability of tested compounds is crucial for cytotoxic activity. The geometry of the tested compound will be responsible for the membrane permeability.

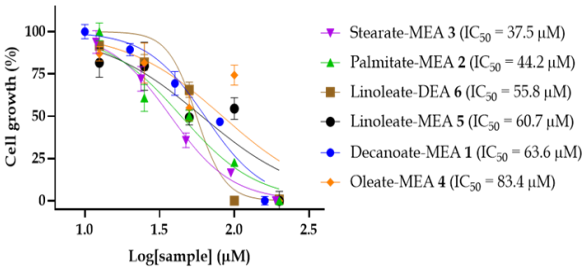

Fig. 4 Cytotoxic activity of six fatty alkanolamides against HeLa cell line. IC_{50} value was determined after 48 h treatment of cells with fatty alkanolamides (Note: MEA = monoethanolamine, DEA = diethanolamine)

The number of hydroxyl groups affected the cytotoxic activity. The cytotoxicity of alkanolamide **6** bearing two hydroxyl groups was slightly stronger than alkanolamide **5** bearing one hydroxyl group. Compounds that have more functional groups can interact better with the active site of the enzyme.

3.4. Antimicrobial activity

Disc diffusion method was utilized to evaluate the antimicrobial activity of fatty alkanolamides. The clear zone was observed and measured as inhibition zone diameter. Data in the **Table 1.** outlines the antimicrobial activity of six fatty alkanolamides against Gram-negative (*Escherichia coli*) and Gram-positive bacteria (*Staphylococcus aureus*). *E. coli* was more sensitive to tested compounds than *S. aureus*. The cell wall of Gram-positive bacteria has a thicker peptidoglycan layer than that of Gram-positive bacteria [34].

In line with cytotoxic activity, alkanolamide stearate **3** demonstrated the most potent antimicrobial activity while alkanolamide **4** was the weakest. A long unsaturated alkyl group was beneficial for antimicrobial activity. The number of hydroxyl groups also displayed a positive impact on antimicrobial activity (compounds **5** and **6**). Since the structure of fatty alkanolamide mimics the structure of phospholipid bilayer of the cell membrane, disruption of the cell membrane by fatty alkanolamide may related to their antimicrobial activity. The amphiphilic property of fatty alkanolamide can solubilize the membrane [35].

Table 1. Inhibition zone diameter (mm) various concentrations of six fatty alkanolamides against *Escherichia coli* and *Staphylococcus aureus*

Fatty alkanolamide	*E. coli*		*S. aureus*	
	500 ppm	1000 ppm	500 ppm	1000 ppm
Decanoate-MEA **1**	6.0	7.7	6.4	6.6
Palmitate-MEA **2**	6.0	ND*	6.4	ND*
Stearate-MEA **3**	7.4	7.5	0	7.9
Oleate-MEA **4**	6.4	7.3	0	6
Linoleate-MEA **5**	7.1	7.2	6.1	6.9
Linoleate-DEA **6**	7.3	7.3	6.3	7.2
DMSO	0	0	0	0

* ND = not determined

4. Conclusion

Six fatty alkanolamides have been successfully synthesized in this research. All compounds demonstrated cytotoxic and antimicrobial activity. Both cytotoxic and antimicrobial activity favored long-saturated fatty alkanolamides. The presence of C=C bond was disadvantageous for the activities. The number of hydroxyl groups displayed a small contribution to both cytotoxic and antimicrobial activity.

5. Conflict of interest

The authors declared no conflict of interest.

6. Acknowledgements

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