Moleküler Biyoloji ve Genetik / *Moleculer Biology and Genetic,* **DOI: 10.21597/jist.695018**
Araştırma Makalesi / *Research Article*

Iğdır Üniversitesi Fen Bilimleri Enstitüsü Dergisi, 10(3): 2021-2027, 2020
Journal of the Institute of Science and Technology, 10(3): 2021-2027, 2020
ISSN: 2146-0574, eISSN: 2536-4618

Antiproliferative and Antimicrobial Effects of Tris(2-hydroxyethyl)ammonium-Based Protic Ionic Liquids with Some Fatty Acids

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ABSTRACT: Protic ionic liquids (PILs) that emerge as promising compounds are of great interest in industry and academia due to their easy synthesis and unique physical and chemical properties. This study aims to evaluate the antimicrobial activities against various microorganisms and the antiproliferative effects of four PILs namely, the PILs tris(2-hydroxyethyl)ammonium dodecanoate (TALA), tris(2-hydroxyethyl)ammonium tetradecanoate (TAMA), tris(2-hydroxyethyl)ammonium palmitate (TAPA) and tris(2-hydroxyethyl)ammonium stearate (TASA). Antiproliferative effects of PILs were investigated *in vitro* on breast cancer cell line (MDA-MB 231), colon cancer cell line (HT29) and prostate cancer cell line (PC3). Furthermore, minimum inhibitory concentrations (MIC) were established for PILs tested against rods, cocci and fungi. The antimicrobial activities of the PILs are strongly related to the alkyl chain length of the anion.

Keywords: Antibacterial effect, Antiproliferative effect, Protic ionic liquids

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Geliş tarihi / *Received:* 26-02-2020
Kabul tarihi / *Accepted:* 12-05-2020

INTRODUCTION

Ionic liquids (ILs) are defined as salts synthesized by combining organic cations with a wide variety of anions (Vekeriya, 2017; Akbaş, 2020). ILs also have many advantages physicochemical properties, such as low vapor pressure, high ionic conductivity, excellent thermal and chemical stability, a low melting point and favorable solvation properties (Tawfik, 2015). One of the most important features of ILs is their environmentally friendly and non-hazardous structures due to their negligible vapor pressures. ILs have gained great attention lately as green chemicals rather than traditional solvents (Earle and Seddon, 2000).

ILs are of great interest among the scientific community in the race to synthesize new pharmaceuticals, because of its various potential biological, pharmacological and pharmaceutical applications (Hough et al., 2007; Egorova et al., 2017; Egorova and Ananikov, 2018). In addition, ILs can be used as potential antimicrobial agents against gram-positive and gram-negative bacteria in the control of contamination and infection by potential microorganisms (Garcia et al., 2013). There are also studies on the anti-cancer activity of ILs against a panel of human cell lines, including melanoma, leukemia and cancers of the prostate, lung, breast and colon cell lines (Malhotra and Kumar, 2010; Rezki et al., 2018). Significant antiproliferative effects were also observed against five different human cancer cell lines with ILs based on ampicillin and thiabendazolium salts (Ferraz et al., 2015; El Bourakadi et al., 2019).

In this study, the PILs (TALA, TAMA, TAPA and TASA) were synthesized by using an equimolar amount of tris(2-hydroxyethyl)amine and long alkyl chain carboxylic acids (fatty acids). In addition to using classical chemotherapeutic in cancer treatment, new drug targets are being investigated and new molecules are trying to be defined in order to increase the effectiveness of the treatment. *In vitro* research on cell lines is of great importance in determining the effect of the agents used on cancer cells and using them as candidate molecules for cancer treatment. Antiproliferative effects of these PILs with long alkyl chains were investigated *in vitro* on MDA-MB-231, PC-3 and HT-29 which is one of the most commonly used breast cancer cell lines in medical researches. We have also applied experimental tests to determine their antimicrobial activity, towards two Gram-negative and one Gram-positive bacteria.

MATERIALS AND METHODS

Tris(2-hydroxyethyl) amine, lauric, myristic, palmitic and stearic acids were purchased from commercial sources (Sigma-Aldrich, St. Louis) and used without further purification.

All PILs were prepared as previously reported and analytical data were in accordance with the literature (Toledo Hijo et al., 2017). The PILs were synthesized from stoichiometric amounts of the tris(2-hydroxyethyl)amine as cation and the organic acids with different numbers of carbon atoms (lauric, myristic, palmitic and stearic acids) as anions. In order to eliminate traces of water either coming from the starting materials or from the atmosphere, the resulting mixtures were heated at 80 ^{o}C for 48 h under vacuum.

Determination of Antiproliferative Effects of PILs

Antiproliferative effects of PILs were investigated on MDA-MB-231, PC3 and HT29 cell lines using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. This test was performed at eleven concentrations (50-0.05 (μg mL^{-1})) and cells were left in contact with synthesized compounds for 24 and 48 hours. MDA-MB-231 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, BiochromAg, Germany) which was supplemented with 10% heat inactivated FBS and 1% l-glutamine, 100 IU mL^{-1} penicillin and 10 mg mL^{-1} streptomycin. Additionally, PC3 and HT29

cells were cultivated with RPMI 1640 (BiochromAg, Germany) containing 10% heat inactivated FBS. Cultured cells were grown in 96 well plates (Greiner Bio-One, Austria) and in a humidified atmosphere containing 5% CO_2 at 37 ºC. 5-Fu was used as a positive control reagent. A stock solution of the compounds was filter sterilized prior to addition to the culture plate. In each experimental set, cells were plated in triplicates and exposed to synthesized molecules at 37 ºC in a humidified atmosphere of 5% CO_2. After this incubation period, cells were incubated for three hours with an MTT solution (0,5 mg mL^{-1}). Following the MTT removal after these incubation period, DMSO (100 mL) was added and complete dissolution of formazan crystals was achieved. After dissolution of purple formazan products, the optical density of the colored solution was measured at 570 nm using a microplate reader (Tecan, Switzerland). In order to determine the inhibitory effects of compounds, the cell viability of control has been accepted as 100%, and the other results were proportioned according to the control value. Percentage of viable cells were calculated with the following formula: cell viability % = average absorbance of the treated group/ average absorbance of untreated group x 100 (İnan et al. 2018; İspir et al. 2019).

Differences in the mean values of measured activities were evaluated statistically using the SPSS 17.0 program (Univariate Variance Analyses and Pearson Correlation). Probability values of $p < 0.05$ were considered to be significant.

Determination of Antimicrobial Properties of PILs

The antimicrobial effects of PILs were examined with the aim of discovering new antimicrobial agents. In order to further explore the antimicrobial activity; we studied the minimal inhibitory concentrations (MIC) (Gökşen, 2016). The PILs of antibacterial activity was studied two gram-negatives (G-) bacteria and one gram-positive (G+) bacterium. Bacterial strains of *Staphylococcus aureus* (ATCC 25923 (G+)), *Pseudomonas aeruginosa* (ATCC 27853 (G-)), *Klebsiella pneumonia* (ATCC 15380 (G-)) were used. Cultures were grown in exponential phase in nutrient broth at 37 ºC for 18 h, adjusted to a final concentration of 10^4 CFU mL^{-1} by diluting fresh broth medium. The MIC method was utilized for evaluating *in vitro* antimicrobial activity. The MIC is the lowest concentration of an antimicrobial agent that can inhibit the visible growth of a microorganism after overnight incubation. The study of solvent control indicated that 10% DMSO did not inhibit the growth of microorganisms. Also, in the present experiment, the concentration of DMSO was gradually decreased because of the two fold serial dilution assay (the working concentration was 5% and lower). So, the PILs synthesized for this study were solved in 10% DMSO and diluted from 50 mg mL^{-1} to 0.78 mg mL^{-1} in sterile 96-well microtiter plates containing broth medium for bacteria. After dilution of samples, intensity of bacteria was standardized to equal a 0.5 McFarland standard (approximately $5x10^7$ organisms mL^{-1}). The bacteria were then inoculated 96 well-plates and were incubated at 37 °C for 24 h. After 24 h, the optical density of each well was recorded at 600 nm using a microplate reader (Tecan, Switzerland). Each test included growth control and sterility control. The experiments were made three times, and the mean values were used.

RESULT AND DISCUSSION

The Antiproliferative Properties of PILs

Potential pharmaceutical applications of ILs were first demonstrated by studies on their toxicity and antimicrobial activity (Frizzo et al., 2013). The anticancer activity of ILs on breast cancer, prostate cancer and colon cancer cell lines has been studied. The effect on cell viability was observed depending on time and dose (Kumar and Malhotra, 2009) .

As a result of MTT cell viability experiments, it was found that PILs did not significantly affect cell viability on MDA-MB 231 (Figure 1). Only TAMA and TALA were inhibited cancer cell viability weakly at low doses (3.125 µM and 1.5625 µM) at first 24 hours. On the second day, compounds have been shown to lose their antiproliverative effects. At the 48th hour, TAPA compound was effective at low concentrations. Thus, it was determined that the antiproliferative effects of the tested compounds were much lower than the positive control group (5-Fu). On the other hand, they inhibited cancer cell proliferation weakly compared to the negative control (NC).

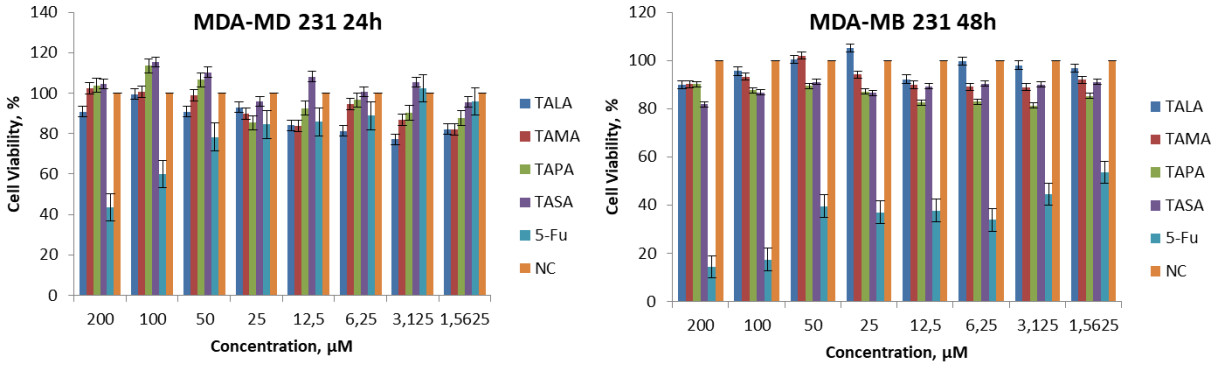

Figure 1. The effect of different concentration of PILs on the viability of MDA-MB 231 at 24 an 48 hours (5-Fu: 5- fluorouacil, NC: Negative control).

In the vitality analysis for HT-29 cell, it was seen that it has antiproliferative effect at the 24th hour, but it was too weak, while it was observed that test compounds were increased cell viability at the 48th hour. Based on these data, the differences between the groups were not significant (Figure 2).

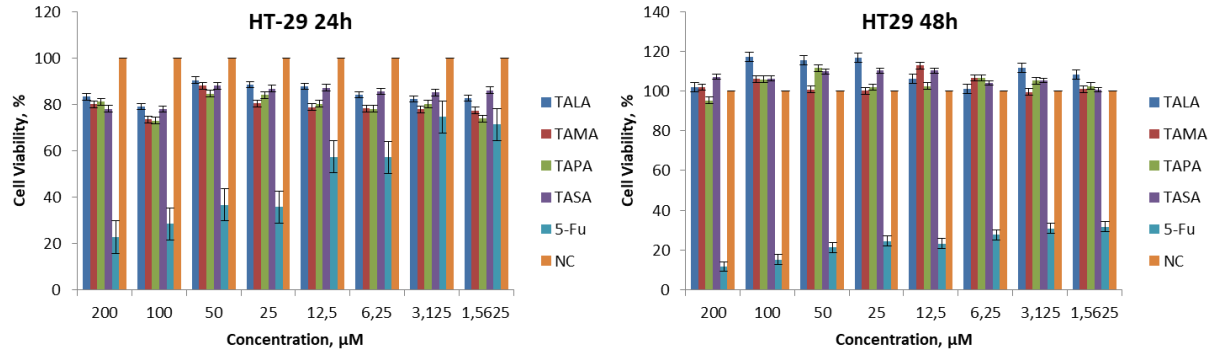

Figure 2. The effect of different concentration of PILs on the viability of HT-29 at 24 an 48 hours (5-Fu: 5- fluorouacil , NC: Negative control).

In the MTT analysis for PC3 cell, there was an increase and decrease in cell viability at 24 hours. While the first three concentrations showed a weak antiproliferative effect, it was observed that this antiproliferative effect decreased when low doses were used. When compared with the negative control (NC) at 48th hour, this situation varied considerably and it was seen that the antiproliferative effect of substances increases, and cell viability decreased up to 45% at 50 µM (Figure 3). The most effective concentration of 50µM for these PILs in the PC3 cell was determined. It was determined that the antiproliferative effects of the tested compounds were much lower than the positive control group (5-

Fu). If ranking is made for the antiproliferative effects of the substances, it is interpreted as TASA, TAPA, TAMA and TALA.

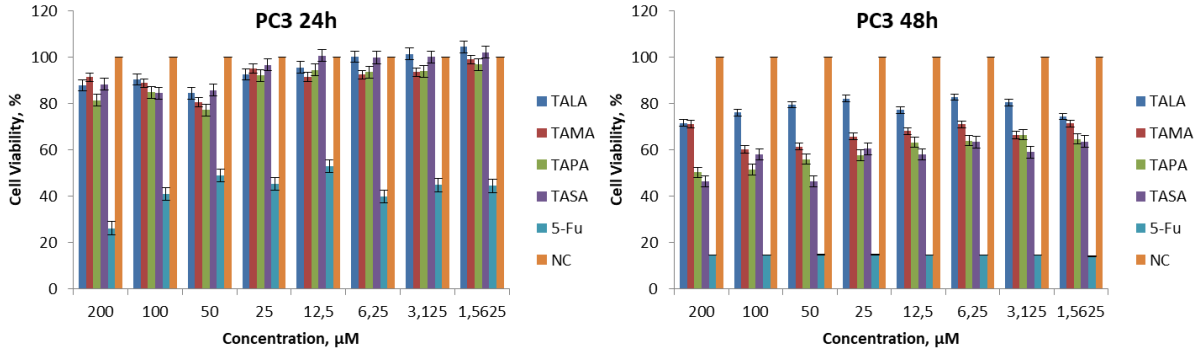

Figure 3. The effect of different concentration of PILs on the viability of PC-3 at 24 an 48 hours (5-Fu: 5- fluorouacil, NC: Negative control).

The Antimicrobial Activity of the PILs

ILs are classically liquid at room temperature, but some of them are solid which can be dissolved in an appropriate solvent to form aqueous solutions. However, aqueous solutions of any ILs are no 'true' IL because they no longer consist exclusively of the parent ions, however such solutions retain potent antimicrobial activity (Freemantle, 2009). Anions play a subsidiary role in antimicrobial activity and the anions of antimicrobial effects can be negligible in most reported studies. As anions are known 'green solvent' (Pendleton and Gilmore, 2015), it is very important to discover and synthesize anions that will exhibit antimicrobial activity.

In the present study, the antimicrobial activity test was based on the MIC recommended by Gökşen (2016). The antibacterial effects of the PILs were examined with the aim of discovering new possible antimicrobial agents for use against some bacteria. One of the synthesized compounds were inhibited cell growths of both G (+) and G (-) bacteria. The compound coded TALA was exhibited antimicrobial activity on all of the bacterial strain. TAMA, TAPA and TASA compounds were no exhibited antimicrobial activity at used concentration range. The results of MIC showed in Table 1.

Table1. The determined MIC values (mg mL^{-1}) of compounds ATCC bacterial strains.

Compounds	*S. aureus (ATCC 25923)*	*P. aeruginosa (ATCC 27853)*	*K. pneumonia (ATCC 15380)*
TALA	50	50	50
TAMA	-	-	-
TAPA	-	-	-
TASA	-	-	-

CONCLUSION

The biological activation of four PILs colon cancer, prostate cancer and breast cancer cell lines was investigated. These compounds appear to not significantly affect cell viability of colon and breast cancer cells. However, studies in prostate cancer cells show that its antiproliferative effect is quite good compared to the negative control.

Comparing the activity status of the substances in each cell line in these studied PILs, the results for MDA-MB 231 were found to be insignificant, and in this case there was no comparison of the efficacy status of any substances. For the HT-29 cell line, it can be listed as TAMA> TAPA> TASA>

TALA. The activity order of the items at the 24th and 48th hours in the PC-3 cell line changes. The chronic effectiveness of TASA, which has an acute efficacy compared to other substances, ranks first, with the opposite difference. When evaluated in general, it is seen that substances make more sense in chronic prostate cancer.

Without TALA compound, the PILs analyzed in the present study have no shown antimicrobial activity at used concentration range. The compound of TALA exhibited antimicrobial activity all of bacteria strain at a concentration of 50 mg mL^{-1}.

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