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Original article

Formulation, characterization, cytotoxicity and *Salmonella*/microsome mutagenicity (Ames) studies of a novel 5-fluorouracil derivative

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ABSTRACT

5-Fluorouracil is one of the first line drugs for the systemic therapy of solid tumors like breast, colorectal, oesophageal, stomach, pancreatic, head and neck.

It could be shown that sugars can improve the absorption across cell membranes and can help to bypass some pharmacokinetic problems. Carbohydrates as most common organic molecules are an important issue of plant and animal metabolisms. They are non toxic and have important duties in the body like participating in DNA and RNA synthesis and being responsible for energy production. In addition, they have many hydroxyl, aldehyde and ketone groups that attract attention for synthesis as a potential drug derivative. 1,2,3,-Triazole compounds have also important role in heterocyclic chemistry because of their pharmaceutical properties and their high reactivity, which could be used as a building block for complex chemical compounds. In this study, following the "Click Reaction" of 5-FU and tetra-*O*-acetylglycose the 5-fluorouracil derivative 1-[{1′-(2″,3″,4″,6″-tetra-*O*-acetyl-*β*-D-glycopyronosyl)-1′*H*-1′,2′,3′-triazole-4′-yl} methyl]5-fluorouracil was synthesized.

Following, a micellar formulation of 5-Fluorouracil derivative was prepared and characterized in terms of particle size, polydispersity index, zeta potential, refractive index and pH. Furthermore, the cytotoxicity and mutagenicity of the 5-fluorouracil derivative was investigated using an *in vitro* cell culture model and the AMES test. According to the results of this study, the novel 5-fluorouracil derivative could be a drug candidate for the therapy of cancer and needs further *in vivo* investigations.

1. Introduction

Cancer has been a major and global health threat and one of the leading causes of death worldwide. According to the World Cancer Report, more than 14 million people were diagnosed with cancer in 2012 (McGuire, 2015).

Chemotherapy, radiotherapy and surgery are current options for cancer treatment. Drug targeting and immune therapy are some of the novel, promising approaches (Li et al., 2017) for the therapy of cancer. Due to the fact of increasing cancer incidence (Cancer Research UK, 2016), the need for novel anticancer drugs with preferred less side effects is growing.

Carbohydrates like cellulose, starch and sucrose are probably the most common, natural organic molecules. They play important roles in plant, animal and human metabolism (Loftsson and Duchêne, 2007). They are not toxic and have important duties in the body like being part of the DNA and RNA, and responsible for energy production. Several hydroxyl, aldehyde and ketone groups present on their structure make them attractive for synthesis of potential drugs or drug derivatives. Besides, nucleobases are nitrogen-containing compounds having at least as much importance as the carbohydrates in various biological processes and they have attracted much attention of chemists and biologists. In this regard, both nucleobases and carbohydrates have been basic units of biologically active heterocyclic moieties called 1,2,3-triazoles that can be readily synthesized via copper(I)-catalyzed alkyne-azide cycloaddition "click" reaction (Kumar, 2015; Galmarini et al., 2002).

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In addition to their usage in a broad range of disciplines including materials chemistry and combinatorial chemistry, 1,2,3-triazoles have been proved to possess various pharmaceutical properties and high reactivity, which could be used as a building block for complex chemical compounds (Dheer et al., 2017; Obchoei et al., 2016; Ferreira et al., 2010; Carvalho et al., 2010).

5-Fluorouracil (5-FU) is one of the first-line systemic antineoplastic drugs. As a pyrimidine analog 5-FU belongs to the family of drugs called antimetabolites (Sweetman, 2009). 5-FU is irreversibly inhibiting the thymidylate synthase, which in turn blocks the synthesis of a nucleoside required for DNA replication, namely the pyrimidine thymidine. The function of thymidylate synthase is to methylate deoxyuridine monophosphate to thymidine monophosphate. Application of 5-FU is leading to a lack of thymidine monophosphate in the cell. Consequently, rapidly dividing cancerous cells undergo cell death (Longley et al., 2003).

5-FU is a uracil analogue which has a fluorine atom at the fifth position and is metabolized in a similar manner to uracil (Prabha and Raj, 2016). 5-FU is indicated for the systemic therapy of various cancers like breast, colorectal, oesophageal, stomach, pancreatic and head and neck cancer (Danesi et al., 2012; Metterle et al., 2015; Rossi, 2013).

In this study, a 5-fluorouracil derivative (5-FUD) was synthesized via "Click Reaction" using tetra-*O*-acetylglycose. First, tetra-*O*-acetylglucose was converted to its azide derivative by reacting with sodium azide in DMF. Then 5-FU was reacted with propargyl bromide to obtain its propargyl derivative. Finally the azide derivative and propargyl derivative of tetra-*O*-acetylglucose and 5-FU reacted in THF in the presence of copper (II) sulphate and sodium ascorbate as catalysts to obtain 1-[{1′-(2″,3″,4″,6″-tetra-*O*-acetyl-*β*-D-glycopyronosyl)-1′*H*-1′,2′,3′-triazole-4′-yl}methyl]5-fluorouracil (5-FUD).

It could be shown that sugars can improve the absorption across cell membranes and this could be a way to bypass pharmacokinetic problems (Ana, et al., 2015). A micellar formulation of 5-FUD was prepared and characterized in terms of particle size, polydispersity index, zeta potential, refractive index and pH. Furthermore, the cytotoxicity and *Salmonella*/Microsome Mutagenicity (Ames) tests of 5-FUD micellar formulation were performed.

Our final approach was to establish a novel anticancer drug candidate 5-FUD in a suitable formulation and with an appropriate cytotoxicity.

2. Materials and methods

2.1. Materials

All chemicals used for the synthesis were supplied from Merck (Darmstadt, Germany). Ethanol (CAS No: 64-17-5, absolute ≥99.8%) and DMSO (CAS No: 67-68-5) were obtained from Sigma-Aldrich (St. Louis, MO, Germany). Lutrol-F68 (CAS No: 9003-11-69) was gifted by BASF (Ludwigshafen, Germany). Ames MPF^{tm} mutagenicity assay kit was obtained from Xenometrix Inc. (USA). The MCF-7 (human breast adenocarcinoma) cells were purchased from the American Type Culture Collection (ATCC-LGC, Rockville, MD).

2.2. Methods

2.2.1. Synthesis

1-[{1′-(2″,3″,4″,6″-tetra-*O*-acetyl-*β*-D-glycopyronosyl)-1′*H*-1′,2′,3′-triazole-4′-yl}methyl]-5-fluorouracil (5-FUD) was synthesized from 2,3,4,6-tetra-*O*-acetyl-*β*-D-glycopyranosyl bromide *via* azidation, and then substitution reaction with *N*-propargylated 5-FU (Fig. 1). The detailed synthesis procedure was given in the previous study by Halay et al., 2017.

2.2.2. Preparation of the 5-FUD micellar formulation

Lutrol F68:ethanol:water 2.25:2.25:5.50 (w/w) was selected for preparing the solution based on preliminary experiments showing that 5-FUD is completely dissolved at 1 mg/ml concentration, and no precipitates are observed. For preparation of the 5-FUD micellar solutions, 1.0, 1.5 and 2.0 mg of the compound were accurately weighed in sterile glass vials. Subsequently, 2 ml of Lutrol F68: ethanol:water 2.25:2.25:5.50 (w/w) was added to each vial and the contents were stirred at 1000 rpm in room temperature for 48 h until complete dissolution of 5-FUD.

2.2.3. Characterization of the 5-FUD micellar formulation

The micellar formulation was characterized in terms of particle size, particle size distribution, zetapotential, refractive index, and pH. Particle size and zetapotential were measured with Zetasizer NanoZS (Malvern, USA) using the non-invasive back scattering (NIBS) technique and laser doppler micro-electrophoresis technique, respectively. Size measurements were performed in disposable polystyrene microcuvettes, and zetapotential measurements were carried out in standard zeta cuvettes. Samples were measured after 30-fold dilution with ultrapure water. Refractivity of samples was measured with DR301-95 refractometer (A.KRÜSS Optronic GmbH, Germany). Viscosity of Lutrol F68:ethanol:water mixture was measured with SV-10 Vibro Viscometer (A&D Co. Ltd., Japan).

2.2.4. Cell culture and in vitro cytotoxicity assay

The cytotoxicity of 5-FUD was determined by XTT cell proliferation assay using MCF-7 cells (Mosmann, 1983). The MCF 7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 μg/mL) and 2 mM L-glutamine in a 37 °C incubator under 5% CO_2 atmosphere. Prior to treatment, cells were trypsinized and seeded in a final volume of 100 μL (5×10^4 cells/mL) into each well of 96-well plates, and incubated for 24 h. At the day of treatment the medium was aspirated, cells were washed with PBS and 50 μL of fresh medium was added to each well. Treatment formulations were diluted in the growth medium and added as 50 μL portions to the corresponding wells (final volume 100 μL). The concentration of 5-FUD was between 5 and 200 μM. Cells were incubated for 24 h in a 37 °C incubator under 5% CO_2 in the presence of the formulations. Blank micellar dispersion was tested as a vehicle control. After the incubation the medium was aspirated, cells were washed with PBS and 50 μL of fresh medium was added to each well. Subsequently 50 μL of XTT reagent prepared as per manufacturers instructions was added and incubated for 2 h at 37 °C. The absorbance of formed orange-colored formazan compound was measured by using an automatic microplate reader (Varioskan Flash microplate reader, Thermo Fisher Scientific, USA) at 450 nm. All experiments were performed in triplicate.

2.2.5. The Ames Salmonella/microsome mutagenicity assay

The *Salmonella*/microsome mutagenicity assay (Ames test) as a short-term bacterial reverse mutation assay was used to analyze the potential of 5-FUD to cause genetic damage. The mutagenicity (bacterial growth) is measured colorimetrically by a color change (pH drop) from purple to yellow. The Ames test was performed in four histidine-requiring strains of *Salmonella typhimurium*, TA98, TA100, TA1535 and TA1537, according to the OECD Guideline 471 (1997) and Maron and Ames (Maron and Ames, 1983). The strains TA98 and TA1537 are used for the detection of

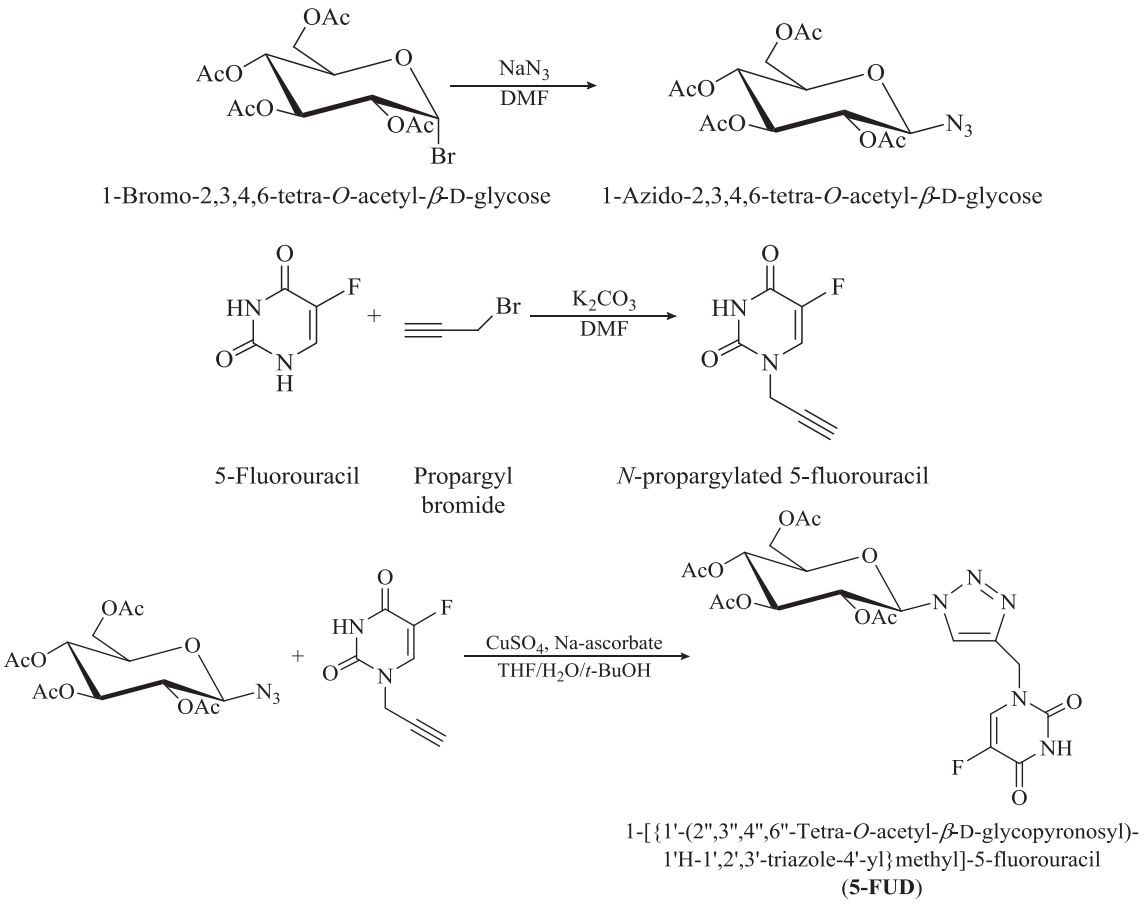

Fig. 1. Synthetic route of 5-FUD.

frameshift mutations, while the strains TA100 and TA1535 can reveal base pair substitutions. Genotypes of the Ames MPF^{tm} *S. typhimurium* strains were checked with help of Xenometrix. Experimental design was performed using Aroclor™-1254 induced rat liver S9, strain-specific positive control chemicals (2-nitrofluorene, 4-nitroquinoline-*N*-oxide, N4-aminocytidine, 9-aminoacridine), the solvent DMSO as the negative control and bacteria, at all concentrations of 1000 μg/mL, 2000 μg/mL and 5000 μg/mL 5-FUD.

2.2.6. Statistical analysis

Cell culture data and Ames test results were analyzed by GraphPad Prism 6.0 statistics software applying Mann-Whitney *U* test. Results were accepted as significant in the case of $p < .05$. Student's *t*-test (1-sided, unpaired) used to determine significance at the a = 0.05 level for Ames test.

3. Results and discussion

3.1. Preparation of the 5-FUD micellar formulation

The solubility investigations revealed that 5-FUD could not be solved in ultrapure water, oleic acid, plurol oleique, propylene glycol, Tween 80 and ethanol. Only a mixture consisting of Lutrol F68:ethanol:water (2.25:2.25:5.50, w/w) was suitable for dissolving 2 mg/mL 5-FUD. Due to the fact that this micellar formulation containing 2 mg/mL 5-FUD showed a slight precipitation after 24 h, the 5-FUD concentration was decreased to 500 (M1), 750 (M2) and 1000 μg/mL (M3), respectively.

3.2. Characterization of the 5-FUD micellar formulation

The characterization of the micellar formulation in terms of particle size (7.2–11.2 nm), zetapotential (−3 to −8.6 mV), refractive index (1.3782–1.3791), and pH (7.15–7.98) revealed that there was not a significant difference between the formulations M0 (empty) M1, M2 and M3, except for the polydispersity index, expressing the particle size distribution. This difference of the PDI is due to the increasing 5-FUD concentration in the micellar formulation. Increasing the 5-FUD concentration from 0 (M0) to 1000 μg/mL (M3) resulted in increasing PDI values between 0.218 (narrow distribution) and 0.441 (broad distribution) (Table 1). The viscosity of the micellar formulation was 38.5 cP.

For further investigations like cytotoxicity tests the micellar formulation M1 with a 5-FUD concentration of 500 μg/mL was used because of a slight turbidity for the micellar formulations M2 and M3.

3.3. Cytotoxicity assay

The choice of the cell line for the cytotoxicity studies is crucial, hence in some cases cytotoxic effects are requested and in some cases not. Here, human breast adenocarcinoma MCF-7 cells were used, due to the fact that 5-FU is also used for the therapy of breast cancer, among others. Investigation of the intrincic activity of the new compound on MCF-7 cells is beyond the scope of this study. However, the cytotoxicity of 5-FUD was comparable to that of 5-FU which demonstrates that the new compound has meaningful activity on the tested cell line (Fig. 2). Although at the tested doses there was no statistical significance between cytotoxicity results of

Table 1
Results of physicochemical characterization of 5-FUD micellar formulations.

Formulation	5-FUD content (μg/mL)	D[a] (nm)	PDI[b]	ZP[c] (mV)	RI[d]	pH
M0	–	7.2 ± 1.4	0.24 ± (0.07	−3.0 ± 2.8	1.3784	7.98
M1	500	7.3 ± 1.0	0.29 ± (0.02	−3.2 ± 2.4	1.3791	7.15
M2	750	8.6 ± 4.9	0.22 ± (0.08	−7.8 ± 3.5	1.3788	7.36
M3	1000	11.2 ± 1.1	0.44 ± 0.03	−8.6 ± 1.7	1.3782	7.36

[a] Particle size.
[b] Polydispersity index.
[c] Zeta potential.
[d] Refractive index.

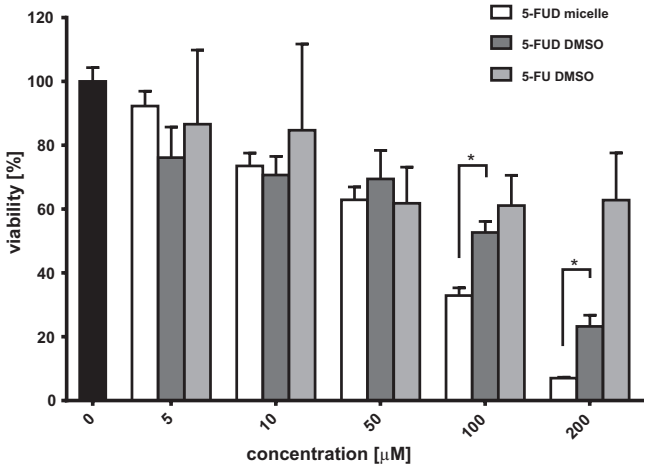

Fig. 2. The cell viabilities of the 5-FUD micellar solution and 5-FUD in DMSO on MCF-7 cells.

5-FU and 5-FUD, at higher doses (i.e. 100 and 200 μM), the new derivative shows remarkable decrease in cell viability as compared to the parental drug. In this case a high cytoxicity is requested and obtained.

As shown in Fig. 2, MCF-7 cells treated with 5 μM 5-FUD in DMSO revealed a decrease of the viability to 76%. Although increasing the 5-FUD concentration to 10 and 50 μM resulted in further decrease of the viability to 71 and 69%, respectively, there is no significant difference between the viabilities after the application of 5, 10 and 50 μM FUD DMSO solutions. More distinctive cytotoxic effect was observed for 100 and 200 μM FUD DMSO solutions, namely viabilities of 53 and 23%, respectively. In contrast, the viability of MCF-7 cells treated with the FUD micelle formulations is continuously decreasing from 92 to 74, 63, 33 and 7%, respectively, with the increasing 5-FUD concentration.

However, there were no significant differences in viability of the 5-FUD micelle formulation – treated group compared to the DMSO solution – treated group up to 50 μM ($p > .05$). Further increasing the concentration to 100 and 200 μM showed a significant difference. The cytotoxicity of the FUD micelle was significantly higher compared to the DMSO solution ($p = .0286$). This is definitely an advantage, because the very low solubility of 5-FU makes it difficult for administration in clinically relevant concentrations. In this view, the 5-FUD micelle formulation is a promising delivery system for further investigating the effects of this new compound using *in vivo* cancer models.

3.4. Salmonella/microsome mutagenicity assay (Ames test)

The Ames test was performed with and without pre-incubation in the absence and presence of metabolic activation (S9-Mix). The spontaneous mutant frequency of the strains was made according to control levels and remained constant during the test period. The mutagenic effect of 5-FUD-M1 in the strains TA98, TA100, TA1535 and TA1537 is reported in Tables 2–5. No mutagenic potential of 5-FUD-M1 could be observed for the tested strains. The results obtained revealed that the mutagenic potential of 5-FUD and 5-FUD-M1 were significantly lower than the corresponding positive controls.

The mean number of positive yellow wells per 1000 μg/mL, 2000 μg/mL, and 5000 μg/mL were calculated for each dose of 5-FUD and its micellar formulation (5-FUD-M1). According to these results no mutagenic potential was observed for all 5-FUD concentrations (in pure form and as a micellar formulation) in each *S. typhimurium* strain. The highest and non genotoxic dose of 5-FUD was determined as 5000 μg/mL and mutagenicity test results at this concentration were given in Tables 2–5. Anticancer drugs are not selective enough to have an impact only on cancer cells. Thus, anticancer drugs also affect the viability of healthy cells and tissues, with an increased possibility of genotoxicity during the chemotherapy (Novak et al., 2017). Genotoxic effects of 5-FU were known due to the fact that 5-FU is a chemotherapeutic agent which targets thymidylate synthase to exert anticancer effects through blocking the normal synthesis of DNA and disrupting RNA processing (Kovacs et al., 2015). Additionally, 5-FUD and its micellar for-

Table 2
S. typhimurium TA98 Mutagencity test results.

Treatment	Concentration	Number of revertant colonies				Mean ± SD
		S9	Replicates			
			#1	#2	#3	
5-FUD	5000 μg/mL	–	2	1	1	1.33 ± 0.58
		+	1	1	1	1.00 ± 0.00
5-FUD-M1	5000 μg/mL	–	2	2	0	1.33 ± 1.15
		+	1	1	0	0.67 ± 0.58
Solvent control (DMSO)	4%	–	0	0	1	0.58 ± 0.33
		+	1	1	1	1.00 ± 0.00
Positive control (2-nitfrofluorene)	2 μg/mL	–	47	48	48	47.33 ± 0.58*
Positive control (2-aminoanthracene)	25 μg/mL	+	48	48	48	48.00 ± 0.00*

* $p < .01$.

Table 3
S. typhimurium TA100 Mutagencity test results.

Treatment	Concentration	Number of revertant colonies				Mean ± SD
		S9	Replicates			
			#1	#2	#3	
5-FUD	5000 μg/mL	–	3	1	2	2.00 ± 1.00
		+	1	2	1	1.33 ± 0.58
5-FUD-M1	5000 μg/mL	–	1	1	0	0.67 ± 0.58
		+	0	3	2	1.67 ± 1.53
Solvent control (DMSO)	4%	–	1	1	1	1.00 ± 0.00
		+	0	2	1	1.00 ± 0.44
Positive control (4-nitroquinoline-*N*-oxide)	0.1 μg/mL	–	45	47	48	46.67 ± 1.53*
Positive control (2-aminoanthracene)	62.5 μg/mL	+	48	46	48	47.33 ± 1.15*

* $p < .01$.

Table 4
S. typhimurium TA1535 Mutagencity test results.

Treatment	Concentration	Number of revertant colonies				Mean ± SD
		S9	Replicates			
			#1	#2	#3	
5-FUD	5000 μg/mL	–	1	2	3	2.00 ± 1.00
		+	0	2	2	1.33 ± 1.15
5-FUD-M1	5000 μg/mL	–	1	1	0	0.67 ± 0.58
		+	0	0	1	0.58 ± 0.33
Solvent control (DMSO)	4%	–	2	2	1	1.67 ± 0.58
		+	1	1	0	0.67 ± 0.58
Positive control (N^4-aminocytidine)	100 μg/mL	–	46	48	48	47.33 ± 1.15*
Positive control (2-aminoanthracene)	125 μg/mL	+	47	47	48	47.33 ± 0.58*

* $p < .01$.

Table 5
S. typhimurium TA1537 Mutagencity test results.

Treatment	Concentration	Number of revertant colonies				Mean ± SD
		S9	Replicates			
			#1	#2	#3	
5-FUD	5000 μg/mL	–	1	0	1	0.67 ± 0.58
		+	0	0	2	1.15 ± 0.67
5-FUD-M1	5000 μg/mL	–	3	1	2	2.00 ± 1.00
		+	3	2	1	2.00 ± 1.00
Solvent control (DMSO)	4%	–	1	0	0	0.33 ± 0.58
		+	3	1	2	2.00 ± 1.00
Positive control (9-aminoacridine)	15 μg/mL	–	48	47	48	47.67 ± 0.58*
Positive control (2-aminoanthracene)	125 μg/mL	+	47	47	46	46.67 ± 0.58*

* $p < .01$.

mulation are innocuous because of the molecule modification caused by carbohydrates.

The results corroborate that both 5-FUD and its micellar formulation caused no mutagenicity in the tested *S. typhimurium* strains, in absence and presence of S9 fraction, which was shown by the negative results obtained in Ames MPF^{tm} assay. This implies that 5-FUD and its micellar formulation did not produce frame shift mutations in the strains TA98 and TA1537 and base pair mutations in the strains TA100 and TA1535 of *S. typhimurium*. Similar results were obtained in a previous study using imatinib microemulsion formulations (Başpınar et al., 2016). The use of S9 mix as a source of enzymes was decisive in order to uncover the conceivable risk of 5-FUD and its micellar formulation after metabolic activation (Fluckiger-Isler et al., 2004). Thus, the present study gives a good result about the absence of direct/indirect genotoxic potential of both 5-FUD and its micellar formulation. Taking this as basis for the future, carbohydrate modified anticancer drugs could be promising candidates for safer treatment of cancer patients.

4. Conclusion

The global concern about cancer is expected to increase in the following years due to a variety of grounds (Sleire et al., 2017). However, only a limited number of novel synthetic anticancer drug candidates progress to the next phase in laboratory experiments and the approval rate of novel synthetic anticancer drugs seems not to be promising. Therefore, not a completely novel synthesis method, but rather some sophisticated modifications like coupling known anticancer drugs with carbohydrates could be clever move for cancer treatment, without genotoxic activities on healthy cells. As the result, we demonstrated that the newly synthesized 5-fluorouracil derivative has comparable activity on MCF-7 cells. Furthermore, it tends to be more active than 5-fluorouracil at higher doses and its micellar formulation shows superior activity than DMSO solution. The genotoxicity studies showed that the newly synthesized 5-fluorouracil derivative does not possess mutagenic activity. Therefore, the new compound formulated in the micellar

formulation can be employed in future animal studies in order to investigate its *in vivo* activity and toxicity profile.

Conflict of interest

The authors have declared that they have no conflict of interest.

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