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Original article

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

Çinel Köksal Karayildirim [a,*], Mustafa Kotmakçi [b], Erkan Halay [c], Kadir Ay [d], Yücel Başpinar [b]

[a] Center for Drug Research & Development and Pharmacokinetic Applications, Ege University, 35100 Bornova, İzmir, Turkey
[b] Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Ege University, 35100 Bornova, İzmir, Turkey
[c] Scientific Analysis Technological Application and Research Center, Uşak University, 64200 Uşak, Turkey
[d] Department of Chemistry, Faculty of Science & Letters, Manisa Celal Bayar University, 45140 Yunusemre, Manisa, Turkey

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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.

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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).

* Corresponding author.
E-mail address: cinel.koksal.karayildirim@gmail.com (Ç. Köksal Karayildirim).

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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

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