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Regular Article

Solubility enhancement and targeted delivery of a potent anticancer flavonoid analogue to cancer cells using ligand decorated dendrimer nano-architectures

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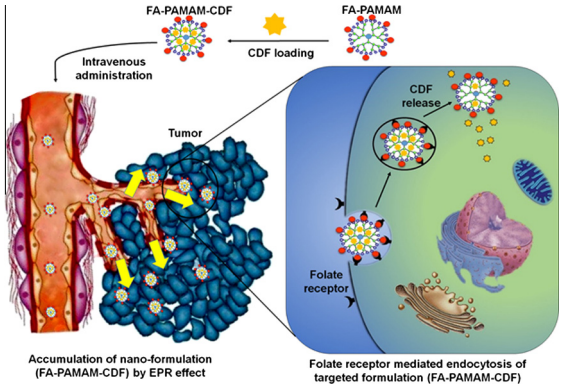
GRAPHICAL ABSTRACT



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ABSTRACT

Conventional chemotherapy using small molecule drugs is marred by several challenges such as short half-life, low therapeutic index and adverse systemic side effects. In this regard, targeted therapies using ligand directed polyamidoamine (PAMAM) dendrimers could be a promising strategy to specifically deliver anticancer drugs to cancer cells overexpressing complementary receptor binding domains. The aim of this study was to utilize folate decorated PAMAM to enhance the aqueous solubility of a highly hydrophobic but very potent anticancer flavonoid analogue, 3,4-difluorobenzylidene diferuloylmethane (CDF) and to deliver it specifically to folate receptor overexpressing cervical cancer cells (HeLa) and ovarian cancer cells (SKOV3). As compared to the non-targeted formulation, the targeted formulation exhibited significant anticancer activity with higher accumulation in folate receptor overexpressing cells, larger population of apoptotic cancer cells, elevated expression of tumor suppressor phosphatase and tensin homolog

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34-Difluorobenzylidene diferuloylmethane (CDF)
Flavonoid analogue
Targeted drug delivery

(PTEN), and inhibition of nuclear factor kappa B (NFκB) which further confirmed the targeting ability and the promising anticancer activity of the folate based nanoformulation.

1. Introduction

Cancer is the second most common cause of death in the United States, exceeded only by heart diseases. According to the American Cancer Society, approximately 1.7 million new cancer cases are expected to be diagnosed and about 600,000 Americans are expected to die of cancer in 2016. However, with the dramatically increased number of cancer cases, there are not many improvements in cancer treatment efficacy [1]. Currently, chemotherapy is the most common treatment given to patients diagnosed with cancer. Unfortunately, chemotherapeutic agents are generally distributed non-specifically in the body and kill both cancer cells and normal cells indiscriminately. Consequently, conventional chemotherapy is associated with severe side effects with relatively low therapeutic index which causes the treatment to be ineffective and lead to the recurrence of tumor after initial treatment. The problem could be better addressed if chemotherapeutic agents could be delivered in a targeted fashion towards cancer cells [2–4].

Recently, studies have shown that nanoparticles can enhance the intracellular concentration of anticancer compounds in cancer cells while decreasing toxicity in normal cells by using both passive [5–7], and active targeting strategies [8,9]. In passive targeting approach, the delivery of nanoparticles is determined by factors associated with the enhance permeability and retention (EPR) effect due to leaky tumor vasculature and poor lymphatic clearance. In addition, the natural size, shape and surface charge of nanoparticles also dictate their preferential accumulation (or clearance). Targeting approach has taken a step further to enhance the selective internalization of nanoparticles into the tumor cells by attaching bio-recognition molecules to the surface of the nanovectors, to target specific markers that are overexpressed by cancer cells. Among many types of nanoconstructs such as polymeric nanoparticles, liposomes, polymeric micelles, dendrimers, solid-lipid nanoparticles, silica nanoparticles and carbon nanotubes, dendrimer nano-platform has demonstrated to be a promising approach to deliver anticancer agents with both passive and active targeting approach [10–13].

Polyamidoamine (PAMAM) dendrimers are hyper-branched, nano-sized, well-defined macromolecules with numerous reactive surface functional groups. PAMAM dendrimers are known for their ability to enhance aqueous solubility of hydrophobic compounds by encapsulating them into their hydrophobic cavities [14–16]. On the other hand, PAMAM dendrimers can achieve both passive and active targeting strategies due to their inherited nano-size and the ability to carry bio-recognition molecules on the surface by conjugation with the peripheral reactive amine groups. In this regard, one of the most commonly used targeting ligands is folic acid (FA), which can be used to target folate receptors found on the membranes of many tumor cells including ovarian, colon, lung, breast and cervical cancers [17–20]. The binding of FA ligands with folate receptors can efficiently enhance the cellular uptake via receptor mediated endocytosis. In previous studies, PAMAM dendrimers conjugated with FA (FA-PAMAM) have shown better cellular uptake by target cancer cells; thus, resulting in better drug accumulation with minimum toxicity to normal cells [21,22].

In our earlier studies 3,4-difluorobenzylidene diferuloylmethane (CDF), a synthetic analogue of a potent flavonoid anticancer compound has shown 16-fold increased half-life and high anticancer activity compared to its natural analogue, diferuloylmethane, when tested on pancreatic cancer cells [23–25]. The observed improvement in properties of CDF were attributed to its much higher stability and bioavailability compared to the natural counterpart. CDF could inhibit the growth of cancer cells through down-regulation of multiple miRNAs, up-regulation of phosphatase and tensin homolog (PTEN), and attenuation of histone methyltransferase EZH2 [26–28]. These findings strongly indicate that CDF could be a good candidate for several cancers, including cervical cancer. However, one significant problem limiting CDF from further preclinical and clinical testing is its very poor aqueous solubility. In our previous studies, we successfully overcame CDF's solubility problem by using dendrimer and micellar nanocarriers [23,29]. In those studies, CDF has shown a significant anticancer activity on tested cancer cells including triple marker positive stem like pancreatic cancer cells. With the earlier observed impressive results of CDF formulations in pancreatic cancer, the aim of this study was to further explore the anticancer potential of CDF in cervical cancer cells. In addition, by conjugating folic acid to dendrimer nano-architectures, the nanoformulation loaded with CDF is expected to specifically deliver the drug to cervical cancer cells overexpressing folate receptors, thus enhancing its anticancer activity (Fig. 1).

2. Material and methods

2.1. Materials

CDF was synthesized as described earlier [23,29]. Fourth generation (4.0G) PAMAM dendrimer, N-(3-(dimethylamino) propyl)-*N*-ethylcarbodiimide hydrochloride (EDC), and 3-[4,5 di methylthiazol-2-yl]-2,5diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO). FA was purchased from Fisher Scientific. Alexa Fluor® 488 Annexin V/Dead cell apoptosis kit was purchased from Thermo Fisher Scientific. All other chemicals were of reagent grade and used without any modification.

2.2. Synthesis and characterization of FA-PAMAM conjugate

FA was conjugated to PAMAM dendrimers through carbodiimide coupling chemistry [30,31] (Fig. 2a). Briefly, FA (55 mg, 0.125 mmol) was dissolved in dimethylsulfoxide (DMSO) (10.0 mL). The solution was added to *N*-hydroxysuccinimide (NHS) (28.65 mg, 0.249 mmol) and EDC (47.73 mg, 0.249 mmol), and was stirred for 24 h at room temperature. The insoluble byproduct was filtered via whatman filter paper. The filtrate was precipitated in acetone. The active ester of FA was collected by filtration, washed with dry tetrahydrofuran (THF) and dissolved back in 10 mL of DMSO. PAMAM dendrimer (50 mg) was added to the solution and stirred for 2 days at room temperature. The resulting FA-PAMAM conjugate was purified by dialysis with the molecular weight cut-off 3.5 kDa (Spectrapor, Spectrum Labs, SD) in 4 L of deionized water (DIW) thrice, followed by lyophilization. Fourier Transform Infrared Spectroscopy (FTIR) and proton Nuclear Magnetic Resonance Spectroscopy (^{1}H NMR) were used to confirm formation of FA-PAMAM conjugate.