

Evaluation of Nanocarrier Targeted Drug Delivery of Capecitabine-PAMAM Dendrimer Complex in a Mice Colorectal Cancer Model

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Abstract- Capecitabine, an effective anticancer drug in colorectal cancer chemotherapy, may create adverse side effects on healthy tissues. In the present study, we first induced colon adenocarcinoma with azoxymethane, a carcinogen agent, and then investigated the potentiality of polyamidoamine (PAMAM) dendrimer to improve capecitabine therapeutic index and decrease its adverse side effects on healthy tissues like liver and bone marrow. Other variables such as nanoparticle concentrations have also been investigated. Drug loading concentration (DLC) and encapsulation efficiency (EE) were calculated for capecitabine/dendrimer complex. Experimental results showed an increase in DLC percentage resulted from elevated capecitabine/dendrimer ratio. Capecitabine/dendrimer complex could reduce tumor size and adverse side effects in comparison with free capecitabine form.

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Introduction

Colorectal cancer is the third most common malignancy in the world and is the fourth most commonly diagnosed cancer and the second leading cause of cancer related death in the United States (1). Development of colorectal cancer is a process sequentially acquiring a number of genetic changes in normal epithelium, which enables precancerous cells to change into an adenomatous polyp and progress into an invasive tumor (2,3). Although the development of colorectal cancer is usually sporadic, risk factors include increasing age, male gender; diseases such as diabetes, inflammatory bowel syndrome and environmental factors including high fat/low fiber diets, excessive alcohol consumption, smoking, obesity and sedentary lifestyle are closely connected with the disease (4-10). Surgical resection is the primary modality for the colorectal cancer treatment, though preoperative (neoadjuvant) and postoperative (adjuvant) chemotherapies have important roles to increase survival (11,12). Chemotherapy drugs in colorectal cancer include oxaliplatin, irinotecan,

Avastin[®] (bevacizumab), 5-fluorouracil (5-Fu) and Xeloda[®] (capecitabine) (13-17). Capecitabine, an oral chemotherapeutic prodrug for breast and colorectal cancer, enzymatically converts to 5-Fu in the tumor, inhibits DNA synthesis and slows the growth of tumoral tissues (18,19).

Although chemotherapeutic agents can reduce tumor size and cancer remission and have high potential to destroy cancer cells, they are not organ specific and can damage proliferative cells (20). Sensitive cells to chemotherapeutic agents include blood cells, hair cells and the cells lining the intestine which are prone to commonly induced side effects such as myelosuppression, diarrhea, alopecia, and liver malfunction. On the other hand, whole body drug distribution necessitates the use of high doses and repeated frequency of their administration.

In the last decade, specifically targeted drug delivery has been the main objective of the cancer treatment to decrease chemotherapeutic side effects, doses, and frequency of administration. Extensive researches have also been done to reach this aim among which different nanocarriers use for targeted drug delivery is one of the

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striking means (21-25). Polymeric systems are among most widely used drug carrier delivery protocols. One of the most common applications of conjugated polymer-drug is anti-cancer therapeutic delivery (26-28). Chemotherapeutic conjugation with polymeric carriers has the capacity to improve therapeutic index by enhanced drug accumulation at the tumor with fewer side effects in other organs (29).

One of the most widely studied properties of polymeric drug delivery is the enhanced permeability and retention (EPR) (30-33) effect. The EPR effect describes the propensity of macromolecules to accumulate in solid tumors (34). Because of rapid angiogenesis associated with solid tumor formation, tumors have dense and leaky vasculature which allows easy permeation of molecules. Microvasculature and lack of lymphatic drainage lead to retention and accumulation of the delivered compounds in a tumor. In EPR targeting, the therapeutic agent is locally released and taken up by tumor cells to achieve its target. Thus, passive targeting by the EPR effect allows drug accumulation at the tumor site and improves therapeutic index compared to the free drug.

The ability of polymers to serve as backbones for drug conjugation, targeting moieties and imaging agents make them powerful multifunctional delivery system (35). Any degradation of the carrier or premature release of the free drug before reaching to the desired location defeats the carrier purpose and reduces targeting efficacy and finally increases side effects. For this reason, a stable system is required for any macromolecular carrier system for targeted drug delivery.

In the search for an ideal carrier system, dendrimers may have significant potentials. Dendritic polymers or dendrimers have been proposed as one type of carrier in targeted drug delivery (36). The unique characteristics of dendrimers such as uniform and controlled size and modifiable surface group functionality make these molecules appealing for biomedical applications (37). The ability to functionalize their terminal groups with various targeting, therapeutic and imaging agents in a specific and controllable manner provides the potential to use the dendrimers as suitable carriers for targeted drug delivery. The utility of the internal void volume of dendrimers to encapsulate hydrophobic guest molecules and drugs has been demonstrated by several research groups (37). Aqueous solubility, biocompatibility, and availability, however, lead to limited drug delivery investigations to find very few classes of dendrimers (38,39). The PAMAM dendrimers, which fulfill most

requirements for use in in vivo applications, are extensively being considered for medical applications and are under vast investigations for their use as carriers in gene transfection, MRI contrast agents and drug delivery applications (36,40,41). The internal tertiary amines of PAMAM dendrimers are available for acid-base interactions and hydrogen bonding as well as other non-covalent interactions. Encapsulated guest molecules make the polymers effective agents for solubilizing hydrophobic drugs. PAMAM dendrimers have been shown to exhibit minimum cytotoxicity up to generation 4 (42,43). The potential of PAMAM dendrimers as oral drug delivery carriers was first reported in 2000 by Wiwattanapatapee (44). Colorectal cancer incidence is increasing over the world and also in Iran. On the other hand, cancer treatment is expensive and rather difficult to follow. In addition, whole body distribution of chemotherapeutics requires frequent doses which accompanied by disparate adverse effects on healthy tissues. Conceivably, searching new methods are inevitable. In this study, we investigate PAMAM dendrimer application with capecitabine, a chemotherapeutic agent, for target drug delivery in neoadjuvant colorectal chemotherapy and compare the effects of free and conjugated capecitabine form on tumor size and blood cell lines abnormalities.

Materials and Methods

1.1. Materials

PAMAM-G4-NH₂ (molecular weight=14,215 g/mol, 64 amine end groups), azoxymethane and capecitabine, were purchased from Aldrich chemical company.

1.2. Animals

Male NMRI inbred albino mice (20-25 g) were kept in a temperature controlled environment on a 12:12 light/dark cycle with free access to food and water. The procedures were in accordance with the guidelines for the care and use of laboratory animals of Tehran University of Medical Sciences.

1.3. Study design

According to the study protocol, fifty male NMRI inbred albino mice were equally divided into five groups of 10 as per follows (i) vehicle control, animals in this group, received normal saline (0.5 ml, i.p, once a week for 6 weeks), (ii) positive control, received azoxymethane (45) (10 mg/kg i.p, once a week for 6 weeks) for cancer induction, (iii) capecitabine, animals first received azoxymethane like group 2, and then 20

weeks after the last azoxymethane injection, they received capecitabine (700 mg/kg/day) through oral gavages for 3 cycles (each cycle include 1 week drug administration than 1 week rest), (iv) PAMAM dendrimers, animals first received azoxymethane for cancer induction and then 20 weeks after the last azoxymethane injection, they received PAMAM dendrimer through oral gavages (244 mg/ml) twice a day for 3 cycle, (v) capecitabine-PAMAM dendrimer complex, animals first received azoxymethane for cancer induction and then 20 weeks after the last azoxymethane dose, they received capecitabine-PAMAM dendrimer complex for 3 cycle. The examination finished on week 32.

1.4. Capecitabine calibration curve

An excess of capecitabine (10 mg) dissolved in 10 ml deionized water. The mixture then stirred with a shaker to make the stock solutions (1-5 µg/ml). Prepared solutions kept in the amber colored bottle to avoid dendrimer and capecitabine degeneration.

Capecitabine calibration curve at different concentration (1-5 µg/ml) were obtained using specific absorbance peak at 240 nm. Maximum absorbance of PAMAM dendrimer was also determined.

1.5. Capecitabine dendrimer encapsulation

Capecitabine dissolved in deionized water (5 µg/ml). The capecitabine/PAMAM dendrimer ratio of 5:1, 5:2, 5:3, 5:4 and 5:5 obtained from several trials. The mixture stirred in a dark environment at 37°C for 24 hours with a shaker. After equilibration prepared suspensions centrifuged at 3000 rpm for 10 minutes. One ml of each centrifuged solution was taken to estimate the amount of dendrimer incorporated drug using Uv-vis spectrometer. Maximum absorbance and calibration curve of capecitabine at different concentrations (1-5 µg/ml) were determined using specific absorbance peak at 240 nm.

1.6. Drug loading concentration (DLC) and encapsulation efficiency (EE)

Encapsulation efficiency is defined as the percentage of capecitabine loading content that can be entrapped into PAMAM dendrimer.

DLC and EE were calculated from the following equations:

Drug loading concentration (%) =

$$\frac{\text{Drug weight in nanoparticle}}{\text{Nanoparticles weight}} * 100$$

Encapsulation Efficiency (%) =

$$\frac{\text{Total capecitabine} - \text{free capecitabine}}{\text{Total capecitabine}} * 100$$

According to Encapsulation Efficiency and Drug Loading Concentration percentage, the appropriated ratio of capecitabine-PAMAM dendrimer was selected.

1.7. Laboratory tests

At the end of the study, animals were anesthetized with ketamine (50 mg/kg, i.p). The abdominal cavity was opened with a midline incision. Thoracic cavity was also opened for cardiac blood sampling. One ml of each sample centrifuged for 5 min in 7000 rpm for serum collections. Serological tests including alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were then measured. Red blood cell (RBC) and platelet (PLT) were also counted using extra 1 ml blood sample.

1.8. Histological assay

Colorectal tumoral and adjacent non-tumoral mucosal tissues were excised from the collected specimens and fixed in 10% formaldehyde, passaged and embedded in paraffin. Paraffin blocks were then sectioned into 3-5 µm thickness for H and E staining. Nine serial sections were prepared from each case for hematoxylin and eosin (H and E) staining.

1.9. Statistical analysis

Results were expressed as means±SEM. Analysis of variance (ANOVA) and post hoc Tukey test was used for comparison between groups. Fisher's exact probability test was used to show different tumor incidences expressed as a percentage of animals with colon adenocarcinoma. Ratio comparison was determined by *chi-square* test. *P*-values below 0.05 were considered statistically significant.

Results

2.1. Determined encapsulation efficiency (EE) and drug loading concentration (DLC)

The capecitabine-PAMAM dendrimer ratio used throughout this study was 5:1, 5:2, 5:3, 5:4 and 5:5 that obtained from several trials (Table 1).

Table 1. Capecitabine nanoparticle ratio

Formulation	Capecitabine (µg/ml)	PAMAM dendrimer (µg/ml)
F1	5.0	1.0
F2	5.0	2.0
F3	5.0	3.0
F4	5.0	4.0
F5	5.0	5.0

The EE and DLC of capecitabine nanoparticles were measured using spectrophotometry method (Table 2). Capecitabine standard curve was obtained by plotting the concentration from 1 µg/ml to 5 µg/ml at 240 nm (Figure 1)

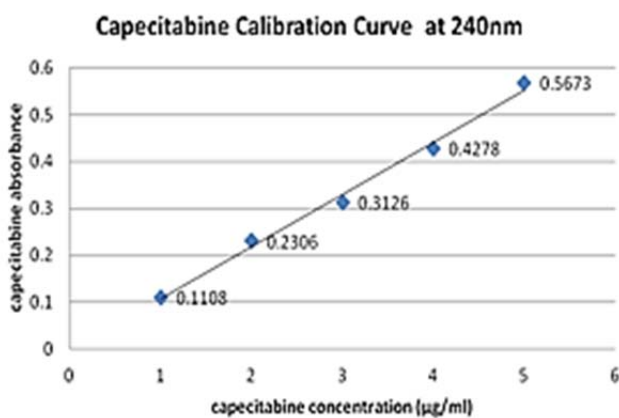


Figure 1. Capecitabine Standard Calibration Curve using spectrophotometer

The effects of capecitabine-dendrimer ratio on encapsulation efficiency and drug loading concentration

were demonstrated. Increasing PAMAM dendrimer concentration during encapsulation process provoking more protonated form while boosting surface charge leading to the stronger electrostatic interaction between capecitabine and PAMAM dendrimer. Encapsulation efficiency of capecitabine decrease with higher PAMAM dendrimer concentration (Table 2). Capecitabine drug loading concentration was also increased when PAMAM dendrimer concentration boosted (Table 2). Reversed correlation was seen between encapsulation efficiency and capecitabine loading concentration.

According to DLC and EE percentage, the capecitabine-PAMAM dendrimer ratio selected in this study was 5 to 1. With increasing capecitabine-PAMAM dendrimer ratio, EE percentage decreased. In this study, we selected 5 to 1 ratio capecitabine-PAMAM dendrimer because in this ratio EE percentage is higher than others.

According to EE and DLC percentage, we can to determined how much of capecitabine loaded in capecitabine-PAMAM dendrimer complex. According to this data, the amount of PAMAM dendrimer was required for formation complex containing 700 mg/ml of capecitabine was determined.

Table 2. Encapsulation efficiency and drug loading concentration of capecitabine/PAMAM dendrimer

Formulation (cap/den)	Drug loading concentration (%)	Encapsulation efficiency (%)
F1(5:1)	57.4	28.73
F2 (5:2)	62.5	24.43
F3 (5:3)	68.2	19.1
F4 (5:4)	72.3	16.46
F5 (5:5)	78.6	13.34

2.2. Tumor type

Azoxymethane changed normal colon tissue and induced colon adenocarcinoma in NMRI mice (Figure 2e). In this study, we observed normal mucosa (Figure 2a) adenovillus polyp (Figure 2b) dysplastic polyp (Figure 2c) dysplastic gland (Figure 2d) and invasive

adenocarcinoma (Figure 2e) in groups under carcinogenic administration.

In the capecitabine group, we saw decrease tumor size and tumor tissue was better than positive control. (Figure 2f). In PAMAM dendrimer group we saw invasive adenocarcinoma-like positive control group

(Figure 2g). In the animals that received capecitabine-dendrimer complex colon tissue was better than

positive control and capecitabine group (Figure 2h).

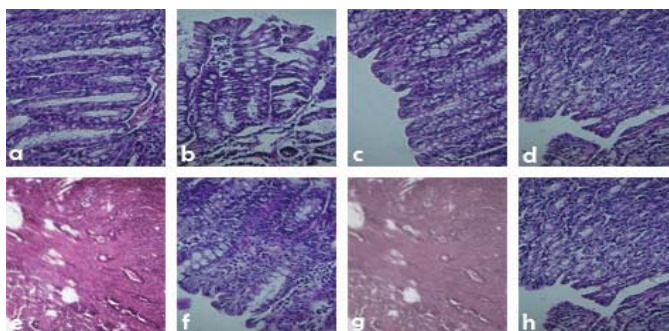


Figure 2. Colorectal histological changes after H and E staining in different experimental groups*400

a; vehicle control: normal colorectal histology* 400, b; positive control: received azoxymethane (10 mg/kg, i.p) for cancer induction (adenovillus polyp), c; positive control: received azoxymethane (10 mg/kg, i.p) for cancer induction (dysplastic polyp), d; positive control: received azoxymethane (10 mg/kg, i.p) for cancer induction (dysplastic glands), e; positive control: received azoxymethane (10 mg/kg, i.p) for cancer induction (invasive adenocarcinoma), f; capecitabine: capecitabine-fed (700 mg/kg/daily, gavage) post cancer induction, g; PAMAM dendrimer: PAMAM dendrimer received post cancer induction, h; capecitabine conjugated PAMAM dendrimer: capecitabine-PAMAM dendrimer complex received post cancer induction

2.3. Effect of capecitabine and capecitabine-PAMAM dendrimer complex on tumor size

Compared to vehicle control and positive control groups, animals received capecitabine-PAMAM dendrimer complex had smaller colon lesion ($P < 0.05$). Administration of capecitabine alone did not significantly reduce adenocarcinoma growth; whereas capecitabine-PAMAM dendrimer complex significantly reduced adenocarcinoma growth. Capecitabine alone decreased adenocarcinoma volume per mouse to 15.9 mm^3 , versus 17.6 mm^3 in AOM group (Figure 3). However, capecitabine-PAMAM dendrimer complex

resulted in a marked and significant reduction in adenoma volume per mouse to 6.8 mm^3 . We observed a decrease in tumor size in animals treated with capecitabine and capecitabine PAMAM dendrimer complex compared to positive control.

RBCs decreased in capecitabine group compared with others (Figure 5). In this study, we showed that neoadjuvant chemotherapy with capecitabine could decrease RBC and platelet counts in male inbred NMRI albino mice, but complex form with PAMAM dendrimer had fewer effects on blood cell lines.

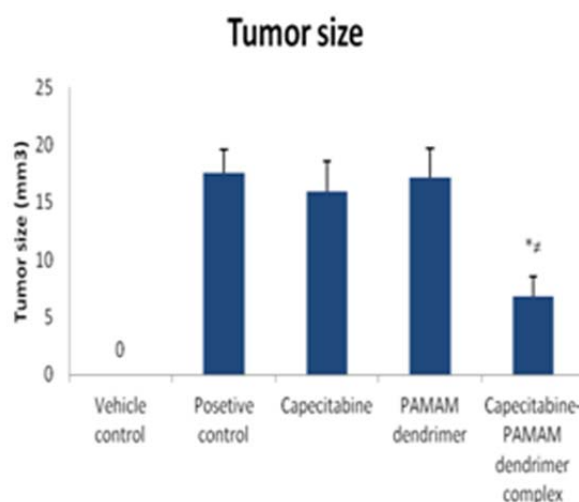


Figure 3. Tumor size in different experimental groups

* $P < 0.05$ compared to positive control group; # $P < 0.05$ compared to capecitabine group

2.4. Blood cell study

Platelet counts were significantly higher in control than capecitabine received animals (Figure 4).

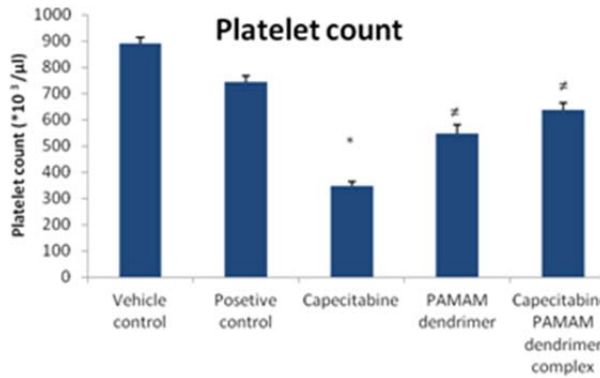


Figure 4. Platelet counts in different experimental groups
* $P < 0.05$ compared to positive control group; # $P < 0.05$ compared to capecitabine group

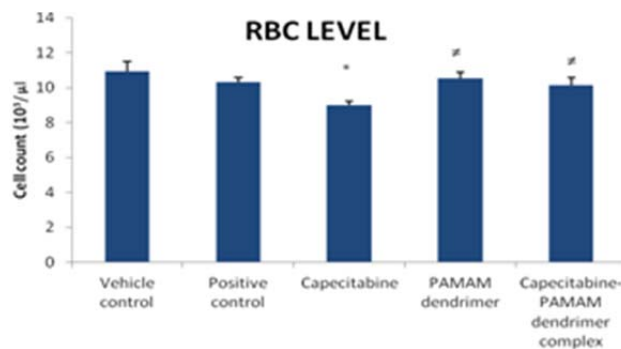


Figure 5. RBC count in different experimental groups.
* $P < 0.05$ compared to Positive control group; # $P < 0.05$ compared to capecitabine group

2.5. Liver enzymes

Capecitabine received animals (group 3) showed significant elevation of AST and ALT compared to the control animals (Figure 6 and 7), but in azoxymethane received animals (group 2), AST and ALT levels showed no significant changes compared to the control group. ALT and AST levels strikingly decreased in capecitabine-PAMAM dendrimer complex received animals compared to the capecitabine group ($P < 0.001$) but when compared with group 1 and 2, no significant difference was seen. PAMAM dendrimer received animals (group 4), showed statistically lowered ALT and AST level compared to group 3 ($P < 0.001$), however in group 4, a significant difference in AST level was noticed compared with control animals.

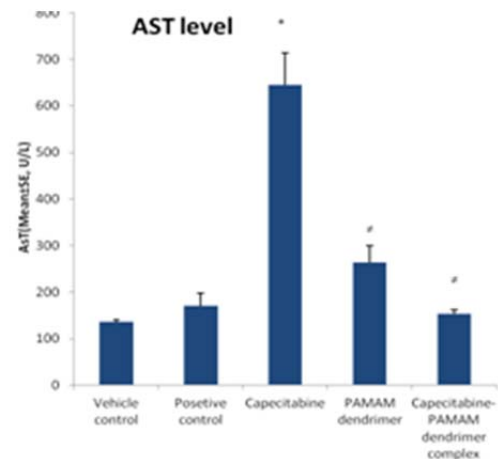


Figure 6. AST level in different experimental groups
* $P < 0.001$ compared to vehicle control groups; # $P < 0.001$ compared to capecitabine group

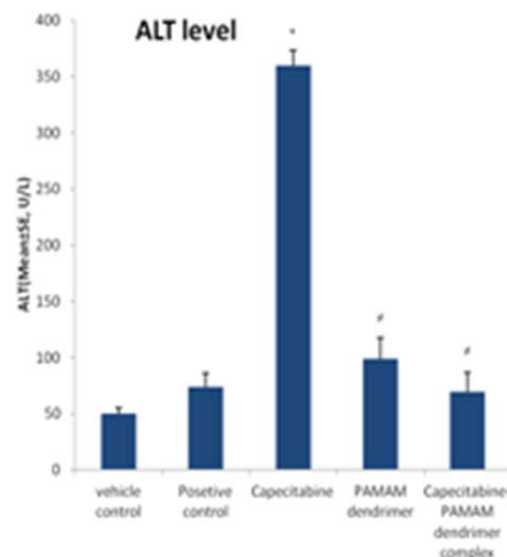


Figure 7. ALT level in different experimental groups
* $P < 0.001$ compared to vehicle control group; # $P < 0.001$ compared with capecitabine group

Discussion

One of the major goals of the cancer chemotherapy is to decrease chemotherapeutic agents' side effects in the clinical atmosphere. In the current study, first we made a colon cancer model by azoxymethane, a carcinogenic agent, administration, and then investigated if we could use capecitabine-PAMAM dendrimer complex to improve drug delivery and attenuate high chemotherapeutics' side effects. Predictably, we noticed that the capecitabine complex form could fairly reduce

side effects in neoadjuvant chemotherapy and decrease tumor size.

In this study, the effects of capecitabine/dendrimer ratio on encapsulation efficiency and drug Loading concentration was demonstrated. Increasing PAMAM dendrimer concentration during encapsulation process provoked more protonated form while boosting surface charge led to stronger electrostatic interaction between capecitabine and PAMAM dendrimer. Encapsulation efficiency of capecitabine increased with higher PAMAM dendrimer concentration. Capecitabine drug loading concentration was also increased when PAMAM dendrimer concentration boosted. Reversed correlation was seen between encapsulation efficiency and capecitabine loading concentration. When capecitabine loading concentration increased, more capecitabine molecules electrostatically adsorbed onto the surface of PAMAM dendrimer and in turn PAMAM dendrimer separation was easily done after centrifugation.

Azoxymethane administration led to carcinogenicity as expected. After injection, it traveled to the liver and colon via the arterial system and induced colonic tumors when reached to the epithelium from the circulatory system (46-48). A greater percentage of invasive colonic adenocarcinoma has also been induced by multiple weekly injections (49,50). In this study, azoxymethane changed normal colon tissue and induced colon adenocarcinoma in NMRI mice. We observed normal mucosa, adenovillus polyp, dysplastic polyp, dysplastic gland and invasive adenocarcinoma in groups under carcinogenic administration.

In capecitabine group tumor size and lesion development were lower than positive control. In PAMAM dendrimer group, invasive adenocarcinoma has been induced like the positive control group. In the animals received capecitabine/dendrimer complex, colon tissue changes were significantly improved compared with positive control and capecitabine group. Compared to vehicle control and positive control groups, animals received capecitabine-PAMAM dendrimer complex had smaller colon lesion. Administration of capecitabine did not significantly reduce adenocarcinoma growth; whereas capecitabine-PAMAM dendrimer complex significantly reduced it. We observed a decrease in tumor size in animals treated with capecitabine and capecitabine PAMAM dendrimer complex compared to positive control. In capecitabine group, the drug distributed post administration in the whole body, and consequently its tumor accumulation rate together with the drug side effects would be reduced. Capecitabine

could modestly reduce tumor size compared with a positive control group which may be due to the frequency of chemotherapy treatment. Increasing chemotherapy cycles may significantly reduce tumor size in capecitabine group compared to positive control.

Decreased tumor size in animals received capecitabine-PAMAM dendrimer complex was also significant. EPR effect caused capecitabine-PAMAM dendrimer complex accumulation in tumoral tissues and decreased tumor size compared to other experimental groups.

In this study, we showed that neoadjuvant chemotherapy with capecitabine could decrease RBC and platelet counts in male inbred NMRI albino mice, but complex form with PAMAM dendrimer had fewer side effects on blood cell lines. Capecitabine adverse decremental response on blood cell lines may be due to bone marrow depression. Capecitabine PAMAM dendrimer complex provoked low distribution of free capecitabine in non-tumoral tissues and furnished protective effects on blood cell lines.

We showed that capecitabine could increase AST and ALT level in NMRI mice compared to positive control groups, but capecitabine PAMAM dendrimer complex had a weaker side effect on liver enzymes compared to control and capecitabine groups. PAMAM dendrimer enhanced capecitabine accumulation at the tumor site and decreased capecitabine untoward sequel on liver enzymes.

We showed that capecitabine-PAMAM dendrimer complex could decrease tumor size and adverse side effects on healthy tissues compared to free capecitabine in NMRI mice colorectal cancer model.

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