COLON SPECIFIC DELIVERY OF COMBINATION OF 5-FLUOROURACIL AND
CELECOXIB: PREPARATION, CHARACTERIZATION, AND IN VITRO CYTOTOXICITY ASSAY

VIKAS BANSAL1,2*, ANJOO KAMBOJ1, JITENDER MADAN3

1Department of Pharmaceutics, Chandigarh College of Pharmacy, Mohali, Punjab, India. 2I.K. Gujral Punjab Technical University, Jalandhar, Punjab, India. Email: vikasbansal16@gmail.com

ABSTRACT

Objective: 5-Fluorouracil (5-FU) and celecoxib (Cel) combination offered additive effect in the treatment of colon cancer. However, physicochemical and biopharmaceutical attributes of both drugs deliver suboptimal concentration at the site of action. The objective of the current study is the development of a microparticulate drug delivery system loaded with a combination of 5-FU and Cel to achieve prolonged drug delivery in colon cancer.

Methods: 5-FU and Cel combination were loaded in Eudragit coated chitosan (CH) microspheres (MSs) and characterized.

Results: The average particle size of the MSs was in the range of 2.7±0.9µm to 4.8±1.1µm. A substantial drug encapsulation efficiency of 71.30±2.3% was obtained for 5-FU as compared to 35.20±1.9% of Cel in the tailored microparticles. The drug loading capacity of 6.5 mg/10 mg and 2.3 mg/10 mg was obtained for 5-FU and Cel, respectively. By Eudragit S100 (Ed) coating, significant pH-dependent release profile was achieved, and no drug release was observed in simulated gastric and intestinal fluids. The developed MSs exhibited the release of 92.1±2.9% of 5-FU in 8h whereas 18.9±0.7% Cel was found to be released from the developed MSs. The drug-loaded MSs exhibited appreciable potency against HT-29 cells with an IC50 value of 35.9 µM.

Conclusion: The results indicated that these microparticles are a promising vehicle for selectively targeting drugs to the colon in the chemotherapy of colon cancer.

Keywords: 5-Fluorouracil, Celecoxib, Chitosan, Eudragit coating, Colon targeting, Cytotoxicity.

INTRODUCTION

Colorectal cancer (CRC) is the third leading cause of death in all kinds of cancer after lung and liver cancer followed by stomach and breast cancer [1]. The occurrence of CRC varies globally, and more number of cases is reported in countries such as North America, Japan, Australia, Western Europe, and New Zealand [2]. Very few cases of CRC are connected with genetic disorders [3,4].

5-Fluorouracil (5-FU), a synthetic pyrimidine antimetabolite is the most effective and first line drug for the treatment of CRC [5]. Despite its efficacy against CRC, 5-FU suffers from several drawbacks including a short biological half-life, invariable oral bioavailability, the requirement of multiple dosages for successful chemotherapy, and gradual increase in the drug resistance [6]. This chemotherapeutic agent is administered as continuous intravenous infusion which causes side effects in non-targeted organs also.

5-FU also has cardiotoxic potential including angina pectoris, myocardial infarction, arrhythmias, heart failure, and cardiogenic shock [7]. 5-FU also reduces the heart pump capacity which leads to heart insufficiency due to scattered necrosis with loss of cardiomyocytes [8].

Various studies have demonstrated the role of nonsteroidal anti-inflammatory drugs in decreased risk of CRC [9]. Epidemiologic, clinical and laboratory-based investigations distinguished cyclooxygenase-2 (COX-2) as a potential molecular target, responsible for the production of PGs which stimulates cancer-associated angiogenesis [4]. Furthermore, it is also documented that the COX-2 levels are increased in 85% of human colorectal adenocarcinomas [10,11]. Therefore, selective COX-2 inhibitors like celecoxib (Cel) hold a very good application prospect for the prevention and treatment of colon cancer [6]. A study was conducted on the role of Cel in familial adenomatous polyposis (FAP) which shows twice daily dose of 400 mg of Cel significantly reduced the number and size of colorectal polyps [12]. Cel is approved by US Food and Drug Administration in the patients of FAP as add-on therapy to inhibit the growth of adenomatous polyps [13,14].

Studies conducted in the past revealed the synergistic effect of Cel when combined with 5-FU to retard the growth of colon tumor. Induced apoptosis of colon tumor cells and considerable tumor regression along with increased COX-2 inhibition was manifested in a subcutaneous implantation tumor model of HT-29 colon cancer cell line. Treated group of nude mice exhibited induced expression of cytochrome C, caspase-3, and caspase-9 indicative of apoptosis [6]. Chemosensitivity of 5-FU resistant cells was improved when exposed to the combination of 5-FU and Cel [11].

Various approaches have been developed for site-specific drug delivery of anticancer molecule in the colon, which may lead to a substantial decrease in the dose and dose-related side effects. Techniques such as pH-dependent systems, pressure dependent formulations, time-dependent approaches, and bacteria dependent systems are reported in literature [15,16].

Chitosan (CH), a cationic polysaccharide is a natural polymer obtained by treating chitin with sodium hydroxide and gained tremendous importance in the field of colon delivery. Due to its unique properties such as biodegradability, biocompatibility, and low toxicity, it has attracted the attention of formulation scientists for the fabrication of colon targeted tablets, beads, microparticles, and nanoparticles enable them to release the drug in colonic milieu [17,18]. It has been used...
successfully in the delivery of drugs such as valdecoxib, 5-FU, curcumin,  
icharin, and vancomycin [19-24].

Ed, a pH-sensitive polymer protects the loss of drug in the upper  
gastrointestinal tract and subsequently results in the successful delivery of  
drugs in the colon only. Ed coating is already been used by formulation  
scientist for the successful delivery of 5-FU and valdecoxib [19,20].

Therefore, in the present investigation, a combination of 5-FU and Cel  
was loaded in Ed coated CH-microspheres (MSs) as a novel approach to  
deliver the “cocktail of therapeutic moities” for the management of  
colon cancer. 5-FU and Cel combination loaded Ed coated CH-MSs  
were prepared by two methods, namely emulsion polymerization and  
nonaqueous solvent evaporation methods for investigating the effect  
on particle size and entrapment efficiency (EE). Both types of MSs  
were characterized with particle size analyzer, transmission electron  
microscope (TEM), EE, drug loading capacity, Fourier transform  
infrared spectroscopy (FTIR), X-ray diffraction (XRD), and differential  
scanning calorimetry (DSC). The optimized formulation was further  
characterized for in vitro drug release and cytotoxicity analysis against  
human colon cancer cell line, HT-29 following standard cell proliferation  
assay.

MATERIALS AND METHODS

Materials

5-FU was purchased from CDH Laboratory, Chemicals India, whereas Cel  
was obtained as a gift sample from Cadila Pharmaceuticals, Ahmedabad,  
India. HT-29 human colon cancer cell lines were purchased from NCS,  
Pune, India. CH and Ed were procured from HiMedia Laboratories,  
Mumbai, India. Glutaraldehyde (GA) and span 80 were purchased from  
Loba Chemie, Mumbai, India. Acetonitrile and water (high-performance  
liquid chromatography [HPLC] grade) were purchased from Fisher  
Scientific, Mumbai, India. All other reagents and chemicals were of  
analytical grade.

Cell culture and reagents

Human colon cancer cell lines (HT-29) were maintained at 37°C in  
DMEM enriched with fetal bovine serum (10%).

Methods

Preparation of drug loaded CH-MSs by an emulsion polymerization method

Emulsion polymerization method was employed for the development of  
CH-MSs loaded with 5-FU and Cel. In brief, a weighed quantity of  
CH (200 mg) was dissolved in 15 ml of 5% acetic acid, and 475 mg of  
5-FU and 200 mg of Cel was added to it. This drug-polymer dispersion  
was added dropwise with the help of hypodermic syringe in a 150 ml  
liquid paraffin (75 ml light liquid paraffin + 75 ml heavy liquid paraffin)  
containing 1% w/w span 80 and it was stirred with the help of  
mechanical stirrer for 1 h at 2500 revolutions per minute (RPM) and  
a temperature of 70°C. After 1 h a saturated solution of cross-linking  
agent (10 ml GA: 30 ml toluene) was added and stirred continuously  
until 2 h at 55°C. Suspension of cross-linked CH-MSs in paraffin oil thus  
obtained was allowed to stand for 20 min to let the MSs settle down  
until 2 h at 55°C. Suspension of cross-linked CH-MSs in paraffin oil thus  
obtained was allowed to stand for 20 min to let the MSs settle down  
and subsequently heated at a temperature range of 30–300°C and cooling  
rate of 10°C/min in an inert environment of nitrogen gas.

Coating of drug loaded CH-MSs by an emulsification-solvent evaporation method

Emulsification-solvent evaporation method was employed for the coating  
of 5-FU and Cel loaded CH-MSs with Ed. The prepared drug-coated CH-  
MSs (100 mg) were coated with 10 ml of 10% w/v ethanolic solution  
of Ed. Light liquid paraffin (70 ml) and span 80 (1% v/v) was added  
for emulsification followed by stirring for 3 h at 1000 RPM at room  
temperature for removal of the solvent by evaporation. The prepared drug- 
loaded Ed-CH-5-FU-Cel-MSs were filtered and washed with n-hexane and  
dried at room temperature and stored in desiccators [21,27].

Characterization of drug loaded MSs

FTIR spectroscopy

FTIR spectrum of 5-FU, Cel, physical mixture of 5-FU and Cel (5-FU- 
Cel-PM), Physical mixture of CH and Ed with both the drug molecules  
under investigation (Ed-CH-5-FU-Cel-PM) and Ed-CH-5-FU-Cel-MSs  
was recorded using infrared spectrophotometer (Alpha-E) utilizing KBr  
disc method (2 mg sample in 200 mg KBr). The scanning range selected  
for the study was 4000–400 cm⁻¹ at a resolution of 4 cm⁻¹.

DSC

DSC thermograms were recorded for 5-FU-Cel-PM and Ed-CH-5-FU- 
Cel-MSs using DSC (Mettler Toledo, 822e, Greifensee, Switzerland).  
All samples were hermetically sealed in crimped aluminum pan and  
subsequently heated at a temperature range of 30–300°C and cooling  
rate of 10°C/min in an inert environment of nitrogen gas.

Powder XRD (PXRD)

X-ray pattern of the selected samples was recorded using X-ray  
diffactometer (XPert PRO, Panalytical Company, Netherlands). Nickel  
filtered, CuKα radiations generated at a voltage of 60 kV and 50 mA  
current were employed for study at a scanning rate of 1°/min over the  
diffraction angle (2θ) range of 10°–60°. PXRD diffractograms of 5-FU,  
Cel, and Ed-CH-5-FU-Cel-MSs were recorded and analyzed.

Particle size analysis

The mean particle size distribution of prepared Ed-Ch-5-FU-Cel-MSs  
was calculated using MasterSizer 2000, Melvern Instruments Ltd. for dry  
powder measurement. The accurately weighed MSs (20 mg) were dispersed in  
HPLC water and visualized for particle size distribution [28,29].

TEM

The shape and surface morphology of the developed Ed coated CH-MSs  
was investigated using TEM (FTI Tecnai F20). An aqueous suspension  
of MSs (20 mg) was dispersed in HPLC water and visualized for particle  
size distribution [28,29].

Drug EE and drug loading capacity

Drug EE% of 5-FU and Cel in the developed MSs was determined using  
an HPLC method. For this purpose, precisely weighed (10 mg) of coated  
MSs were added into 10 ml of acetonitrile-water (50:50) mixture and  
subjected to centrifugation at 1500 RPM for 10 min. The resulting  
clear centrifuge (2 ml) was pipetted out and filtered through 0.45 μm  
membrane filter. 20 μL of this filtered solution was then injected into  
HPLC column and analyzed at 260 nm [28,29].

The percent drug EE and drug loading capacity were calculated using  
the following equations:
In vitro drug release studies

Ed-CH-5FU-Cel-MSs were evaluated for in vitro drug release in pH progression medium at 37°C±0.5°C. The dissolution study was performed using the paddle method. Accurately weighed coated MSs (100 mg) were deliberately spread over the surface of 900 ml of dissolution medium. The content was rotated at 50 RPM. To compare the release of 5-FU and Cel under different GI transit condition, the pH of the dissolution medium was altered at different time intervals. The experiments were performed in an acidic buffer (pH 1.2) for 2 h, which simulates the condition of the fasted stomach. After that, the study was conducted in the phosphate buffer (pH 7.4) to simulate duodenum for 2 h followed by in phosphate buffer (pH 6.8), which simulates mid jejunum. Finally, drug release study was continued in phosphate buffer (pH 7.4) to simulate ileo-colonic conditions for the next 2 h. Sample (5 ml) was withdrawn at specific time intervals (after 5, 15, 30, and 60 min for 1st h, afterward every hour till the end of the study) and replaced with fresh dissolution medium to mimic finite sink conditions. The samples were passed through a microfilter and analyzed by HPLC for estimation of drug release. The dissolution study was performed in triplicate [20,21,29,30].

In vitro cytotoxicity assay

3-[4,5-diethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay, a standard colorimetry based assay was selected for the determination of cytotoxicity. HT-29 human colon cancer cell line was cultured in a 96 well microtiter plate. Briefly, HT-29 cells were implanted in 200 µl of the serum DMEM medium at a density of 5×10⁵ cells per well. After the incubation period of 24 h, the medium was replaced with serum-free-DMEM. After 24 h, the HT 29 cells were exposed to 5-FU, Cel, 5-FU-Cel, Ed-CH-5-FU-Cel-MSs and blank MSs (Bl-MSs) equivalent to 10–70 µM of 5-FU and Cel for 72 h. At the end of the treatment, the medium was removed and the cells were treated with 0.5 mg/ml of MTT dye for 4 h at 37°C. The HT 29 cells were lysed and thus formed formazan crystals were dissolved in 100 µL of DMSO and quantified by ELISA reader at 570 nm and 630 nm. The experiments were performed in triplicate (n=3) [31-33].

RESULTS AND DISCUSSION

Preparation and characterization of MSs

Ed-CH-5-FU-Cel-MSs were prepared by emulsion polymerization method and characterized under a set of stringent parameters [25].

Characterization of MSs

FTIR spectroscopy

The results of the FTIR spectrum of 5-FU, Cel, 5-FU-Cel-PM, Ed-CH-5-FU-Cel-PM and Ed-CH-5-FU-Cel-MSs are shown in Table 1. FTIR spectrum showed characteristic peak due to pure 5-FU at 1641 and 1229 cm⁻¹ correspond to the C=O and C-X, respectively. The spectrum of Cel showed a characteristic peak at 3331, 1345, and 1138 cm⁻¹ due to N-H stretching and S=O stretching vibrations of sulfonamide. In 5-FU-Cel-PM a slight shift was observed at 1226 and 1138 cm⁻¹ from the original peaks of 5-FU and Cel, respectively. The spectrum of Ed-CH-5-FU-Cel-MSs indicated that characteristic peaks of 5-FU, Cel, and Ed are present in the PM of drugs and excipients as well as in the drug-loaded MSs. However, broadening and decrease in peak intensity were observed in the spectrum of drug loaded MSs which indicated no chemical interaction between drug and polymer [21].

DSC

DSC thermograms of 5-FU-Cel-PM along with Ed-CH-5-FU-Cel-MSs are presented in Fig. 1. A sharp endothermic peak at 162.02°C was observed for 5-FU, close to its investigated melting point (158°C). On the other side, Cel exhibited an endothermic peak at 275.87°C which is well matched with its reported melting point range of 280–282°C. Ed-CH-5-FU-Cel-MSs presented a depressed and relatively broad endothermic peak at 90.90°C which corroborated amorphization of the crystal structure of the drugs [34].

PXRD studies

PXRD studies were carried out to determine the crystalline nature of the drugs under investigation. The XRD diffractograms (Fig. 2) of 5-FU and Cel exhibited the sharp and intense peaks, indicating the crystalline structure. While the diffractograms of Ed-CH-5-FU-Cel-MSs demonstrated, low intensity peaks designated to the amorphous lattice. This was consistent with the previous reports [35].

Particle size analysis and surface morphology

It is apparent from the results obtained from laser diffractionmetry, the mean particle size of Ed-CH-5-FU-Cel-MSs was found to be 4.8±1.1µm and 2.7±0.9µm, respectively, for MSs prepared by emulsion

<table>
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<tr>
<th>Sample</th>
<th>Peak</th>
<th>Assignment</th>
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<tbody>
<tr>
<td>5-FU</td>
<td>1641</td>
<td>C=O</td>
</tr>
<tr>
<td></td>
<td>1229</td>
<td>C-X</td>
</tr>
<tr>
<td>Cel</td>
<td>3331</td>
<td>N-H stretching</td>
</tr>
<tr>
<td></td>
<td>1345 and 1138</td>
<td>S=O stretching</td>
</tr>
<tr>
<td>5-FU-Cel-PM</td>
<td>1345 and 1147</td>
<td>S=O stretching</td>
</tr>
<tr>
<td></td>
<td>1226</td>
<td>C-X</td>
</tr>
<tr>
<td>Ed-CH-5-FU-Cel-PM</td>
<td>1345</td>
<td>S=O stretching</td>
</tr>
<tr>
<td></td>
<td>1227</td>
<td>C-X</td>
</tr>
<tr>
<td></td>
<td>1148</td>
<td>Eduragit S 100</td>
</tr>
<tr>
<td>Ed-CH-5-FU-Cel-MSs</td>
<td>2912</td>
<td>OCH3/CH2</td>
</tr>
<tr>
<td></td>
<td>1647</td>
<td>C=O</td>
</tr>
<tr>
<td></td>
<td>1241</td>
<td>C-X</td>
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</tbody>
</table>

FTIR: Fourier transform infrared spectroscopy, 5-FU: 5-Fluorouracil, Cel: Celecoxib, CH: Chitosan, MSs: Microspheres, PM: Physical mixture.
polymerization method and nonaqueous solvent evaporation method with an insignificant difference (Unpaired t-test, p>0.05). The photomicrographs obtained from TEM indicated the spherical and uniform shape of the coated MSs without any distortion of surface texture (Fig. 3).

**Drug EE and drug loading capacity**

The results of drug EE and drug loading capacity of the Ed-CH-5-FU-Cel-MSs are expressed in Table 2. The drug EE for MSs developed by emulsion polymerization method was calculated to be 71.30±2.3% and 35.20±1.9% with significant difference (Unpaired t-test, p<0.05) whereas the drug loading capacity was found to be in the range of 6.5 mg/10 mg and 2.3 mg/10 mg for 5-FU and Cel respectively. On the other hand, in case of MSs developed by nonaqueous solvent evaporation method, slight decrease in drug EE as well as in drug loading capacity was observed.

Each experiment was carried out in triplicate (n=3)

**In vitro drug release studies**

The formulation approach used in the development of Ed-CH-5-FU-Cel-MSs was to delay the release of the combination of drugs in the stomach and to get a controlled drug release for a relatively long duration, especially at the site of action, i.e., colon. Results obtained from in vitro drug release study met with the expected outcome. Dissolution study was carried out in pH progression medium at 37±0.5°C. It is evident from the drug release study that at 0.5 h, 97.5±1.1 % 5-FU was released as compared (two-way ANOVA test, p<0.01) to 15.3±0.8% release of Cel from 5-FU-Cel-PM. Ed-CH-5-FU-Cel-MSs exhibited the release of 92.1±2.9% of 5-FU in 8 h as compared (two-way ANOVA test, p<0.01) to 18.9±0.7 release of Cel after the same duration of time (Fig. 4).

Ed-CH-5-FU-Cel-MSs showed that there was no substantial amount of drug release (5-FU and Cel) in the initial 2 h in simulated gastric fluid (pH 1.2). The release of both drugs under investigation started in intestinal pH and the maximum release observed in the colonic milieu, the site of action.

**In vitro cytotoxicity assay**

To investigate the synergistic effect of 5-FU and Cel combination in CRC, the standard cell proliferation assay was performed on pure drugs as well as on the developed formulations. Cytotoxicity of the 5-FU, Cel, 5-FU-Cel-PM, Ed-CH-5-FU-Cel-MSs, and Bl-MSs was studied against HT-29 cell line and expressed in terms of percentage cell viability (Fig. 5). The drug-loaded MSs exhibited appreciable potency against HT-29 cells with an IC\textsubscript{50} value of 35.9±4.2 µM, which is notably lower than (One-way ANOVA test, p<0.05) IC\textsubscript{50} value of pure Cel (48.7±2.1 µM), 5-FU (45.4±3.6 µM), and the combination of both drugs (47.3±2.5 µM). Bl-MSs did not produce any cytotoxicity against HT-29 cells. The results exhibited the possible synergistic effect when Cel and 5-FU were used in combination. This strategy could be used as a potential approach for the management of CRC due to controlled release phenomena [35,36].

**CONCLUSION**

In the present study, we have offered the oral drug delivery system in the form of MSs of 5-FU and Cel combination for the management of CRC. Carbohydrate polymer, CH was employed for the fabrication of MSs to enable the drug release in the colonic milieu. In addition, Ed coating has supported the tailored system to release the drug at the target site.

Our Eudragit coated carbohydrate based polymeric MSs of 5-FU and Cel combination will potentially improve the drug delivery in the colon while maintaining the high stability in the stomach and small intestine.

**ACKNOWLEDGMENT**

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**Table 2: Particle size, EE, and drug loading capacity of the drug-loaded MSs (Ed-CH-5-FU-Cel-MSs)**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Emulsion polymerization method</th>
<th>Nonaqueous solvent evaporation method</th>
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<tbody>
<tr>
<td>Particle size (µm)</td>
<td>4.8±1.1</td>
<td>2.7±0.9</td>
</tr>
<tr>
<td>EE</td>
<td>71.30±2.3% (5-FU)</td>
<td>70±1.7% (5-FU)</td>
</tr>
<tr>
<td></td>
<td>3 5.20±1.9% (Cel)</td>
<td>34.64±1.5% (Cel)</td>
</tr>
<tr>
<td>Drug loading capacity</td>
<td>6.5 mg/10 mg (5-FU)</td>
<td>6.4 mg/10 mg (5-FU)</td>
</tr>
<tr>
<td></td>
<td>2.3 mg/10 mg (Cel)</td>
<td>2.52 mg/10 mg (Cel)</td>
</tr>
</tbody>
</table>

EE: Entrapment efficiency, 5-FU: 5-Fluorouracil, Cel: Celecoxib, CH: Chitosan, MSs: Microspheres
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AUTHORS’ CONTRIBUTIONS

Vikas Bansal: Conceptualized the article, compiled full literature search and drafted the manuscript. Anjoo Kamboj: Supervisor of the research work, provided guidance in the preparation of a standard paper. Jitender Madan: Evaluated the manuscript and helped in statistical analysis.

CONFLICTS OF INTEREST

There are no conflicts of interest among authors.

REFERENCES


Fig. 3: Particle size distribution and transmission electron microscope of Ed-CH-5-FU-Cel-MSs prepared by (a) nonaqueous solvent evaporation method and (b) emulsion polymerization method. Each Experiment was carried out in triplicate (n=3). 5-FU: 5-Fluorouracil, Cel: Celecoxib, CH: Chitosan, MSs: Microspheres, Ed: Eudragit S 100

Fig. 4: (a) In vitro release profile of Ed-CH-5-FU-Cel-MSs in simulated gastric fluid followed by simulated intestinal fluid and finally in the simulated colonic fluid. The study was performed in triplicate (mean±standard deviation, n=3). (b) Representative chromatogram of drug release studied by high-performance liquid chromatography. 5-FU: 5-Fluorouracil, Cel: Celecoxib, CH: Chitosan, MSs: Microspheres, Ed: Eudragit S 100

Fig. 5: Therapeutic efficacy testing of Ed-CH-5-FU-Cel-MSs against human colon cancer cells, HT-29. Each experiment was carried out in triplicate (n=3). 5-FU: 5-Fluorouracil, Cel: Celecoxib, CH: Chitosan, MSs: Microspheres, Ed: Eudragit S 100