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RESEARCH ARTICLE

CLX-155: A Novel, Oral 5-FU Prodrug Displaying Antitumor Activity in Human Colon Cancer Xenograft Model in Nude Mice

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ABSTRACT

Introduction: Capecitabine is an oral prodrug of 5-FU, which interpatient pharmacokinetic (PK) variability related to liver function and severe adverse events (e.g., hand-foot syndrome, myelosuppression, and neurotoxicity) limits. CLX-155 is a novel oral 5'-DFCR prodrug involving 5'-DFCR as an intermediate for generating 5-FU, unlike capecitabine, which the liver does not metabolize. This study addresses the following research question: what is the activity of CLX-155 in a human colon cancer xenograft model in nude mice?

Methods: This study involved 50 Foxn1 athymic nude female mice implanted with the human colon cancer cell line HCT116 (5 million cells per site). Investigators randomized animals into five treatment groups (N = 10): vehicle control, CLX-155 at doses of 125, 250, and 500 mg/kg/day, or capecitabine 1000 mg/kg/day. Animals received oral treatment once daily for five days a week with two days off for a total of three consecutive weeks. Investigators evaluated treatment toxicity based on body weight loss. Calculations for tumor growth inhibition involved comparing changes in tumor volume on a given day to tumor volumes on Day 1.

Results: CLX-155 demonstrated statistically significant, dose-dependent tumor growth inhibition at all doses compared to vehicle control ($p<0.0001$). Tumor growth inhibition at Day 15 for CLX-155 treatment groups of 125, 250, and 500 mg/kg/day was 57.8%, 70.4%, and 90.6% respectively. Two animals in the CLX-155 500 mg/kg/day treatment group experienced complete tumor regression, and all animals in the CLX-155 treatment groups survived. Two animals in the CLX-155 250 and 500 mg/kg/day dosing groups experienced a decrease in body weight. In contrast, two mice in the capecitabine group exhibited clinical signs of hunchback and scaly skin, progressive weight loss, and eventual death.

Conclusion: CLX-155 demonstrated comparable tumor growth inhibition to capecitabine but at a lower dose, suggesting increased potency. In addition, CLX-155 exhibited improved tolerability and fewer adverse effects. These promising results support further investigation in Phase 1 clinical trials for managing colon cancer.

Keywords: Colon cancer, CLX-155, 5-FU prodrug, Xenograft, Preclinical activity, Antimetabolite

Introduction

5-Fluorouracil (5-FU) is a widely used antimetabolite anticancer agent. Food and Drug Administration approved this agent in 1962, 5-FU demonstrates efficacy in colorectal, pancreatic, esophageal, gastric, hepatocellular, cervical, breast, head, and neck cancers.[1,2] Due to its variable gastrointestinal (GI) absorption and rapid degradation, 5-FU administration is via the intravenous (IV) route.[3] After administration, 5-FU undergoes rapid transport into cells and is converted by phosphorylation into three primary active metabolites, including fluorouridine triphosphate (FUTP), fluorodeoxyuridine triphosphate (FdUTP), and fluorodeoxyuridine monophosphate (FdUMP).[4] FdUMP inhibits thymidylate synthase (TS) and thymidine formation, hindering DNA repair and replication. FdUTP acts as a DNA polymerase substrate, ultimately damaging DNA structure.[4] FdUTP inhibits transcription and maturation of rRNA due to incorporation instead of uracil, causing RNA damage.[4]

5-Fluorouracil's mode of administration and patient and hospital burden led to the development of the oral prodrug capecitabine. This agent utilizes a different metabolic pathway and is almost 100% bioavailable.[3] After being absorbed through the intestine, hepatic carboxylesterase converts capecitabine to 5'-deoxy-5-fluorocytidine (5'-DFCR). Cytidine deaminase, an enzyme with high liver, plasma, and tumor tissue concentrations, converts 5'-DFCR to 5'-deoxy-5-fluorouridine (5'-DFUR). Thymidine phosphorylase (TP), an enzyme in higher concentrations within solid tumors versus normal tissue, metabolizes 5'-DFUR to FU.[3] Because of TP's localization to the liver and tumor tissues, capecitabine results in less systemic toxicity than IV FU.[3] However, it possesses limitations, including interpatient variability of the PK of capecitabine and its metabolites related to enzymatic phenotypes and liver perfusion and function.[5]

Further, adverse reactions and cautions leading to dose adjustments affect capecitabine's use. Approximately 50% of patients experience severe hand-foot syndrome and severe GI toxicity.[6] Cautions for capecitabine include hepatic impairment, bone marrow suppression, and dihydropyrimidine dehydrogenase (DPD) deficiency.[7] Due to these limitations, capecitabine faces unmet needs concerning patients with liver and renal dysfunction, tolerability issues, and dose adjustments. Because of these unmet needs, capecitabine leaves a gap in care that is ready to be addressed.

The compound CLX-155 is a novel, oral 5'DFCR prodrug under evaluation as an antitumor agent. CLX-155 is a molecular conjugate of acetylated 5'-DFCR linked to caprylate, hydrolyzed by esterases in the intestinal wall to yield 5'-DFCR and caprylic acid, followed by 5'-DFUR and 5-FU. CLX-155's metabolism offers several interesting points that distinguish this compound from other antimetabolites. CLX-155 is not metabolized by the liver, providing the possibility of CLX-155 to avoid some of the shortcomings experienced with capecitabine and 5-FU. Also, the production of caprylic acid could contribute to antitumor activity, providing CLX-155 with two active moieties.[8,9]

Given these considerations and CLX-155's profile, this paper addresses the research question- what is the relative efficacy of CLX-155 in a human colon cancer xenograft model in nude mice? This work charts the following course to explore this question- methods, results, discussion of the relevance and implications, study limitations, and ideas for future research.

Methods

STUDY DESIGN

This parallel-design study (Figure 1) involved 50 Foxn1 athymic nude female mice sourced from Vivo Bio Tech.

The Institutional Animal Care and Use Committee (IAEC/JDC/2017-120) reviewed and approved procedures involving animal care and use prior to conduct. Animal care and use adhered to the principles outlined in the Guide for the Care and Use of Laboratory, 8th Edition, 2010 (National Research Council). The facility conducting the experimentation holds the Association for Assessment and Accreditation of Lab Animal Care International (AAALAC) accreditation.

Figure 1: Study Schema

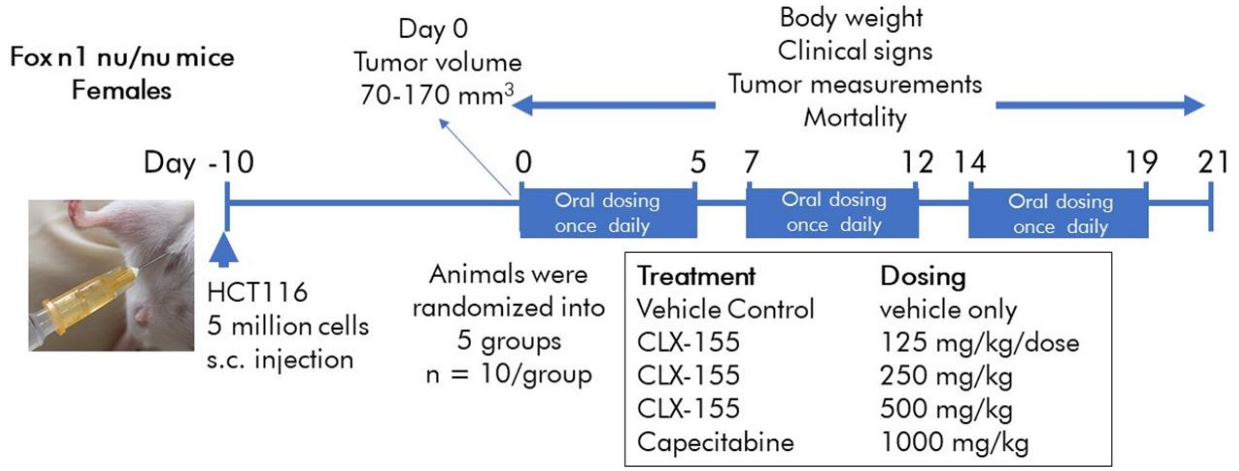

Figure 1. Overview of study schema from Day -10 to Day 21.

ANIMALS AND HANDLING

All animals resided in groups of five within individually ventilated cages in a dedicated rodent quarantine room within an immunocompromised facility for one week. Daily monitoring occurred throughout the one-week quarantine period to detect any clinical signs of disease. Following the completion of the quarantine period, healthy animals transitioned to an experimental room for seven days to acclimate to the experimental conditions.

Animals resided in a continuously monitored temperature and humidity-regulated aseptic and access-controlled environment (target ranges: temperature 22 ± 2°C; relative humidity 60 ± 4%; and 60 air changes per hour), with a 12-hour light/dark cycle, and under barrier (quarantine) conditions. Investigators routinely monitored the entire facility to detect any airborne infections. The animals received an autoclaved commercial diet (Nutrilab Rodent Feed, cylindrical-shaped pellets) and had free access to autoclaved water.

PREPARATION OF CANCER CELL INOCULUM

This experiment utilized the human colon cancer cell line HCT116, obtained from the American Type Culture Collection (ATCC), Manassas, Virginia, USA. This study used a Derived Xenograft (CDX) model adapted from[8], which demonstrated HCT116 colorectal tumor cell susceptibility to capecitabine treatment and has also been used in the evaluation of tumor growth inhibition of chemotherapy agents such as 5-FU, docetaxel, and flavopiridol.[10] The culture media used to grow the HCT16 cell line consisted of McCoy's 5a medium supplemented with 10% FBS and 1% penicillin-streptomycin. Investigators harvested cells by trypsinization at 70-80% confluence and then re-suspended cells in a serum-free medium prior to animal inoculation. Investigators implanted the HCT116 cell line (5 million cells/site) subcutaneously in the dorsal right flank. Injections contained viable HCT116 cells in serum-free medium at a concentration of 5 × 106/100 µL mixed with an equal volume of matrigel (1:1 ratio) for implanting at the subcutaneous site per mouse.[10] Each injection consisted of a total volume of 200 µL per site using a 1 mL BD syringe attached to a 23-gauge needle. Investigators measured the size of the HCT116 human colon tumor xenografts approximately ten days after cell injection and once the xenografts became palpable. Animals were randomized into five groups (N = 10) once the tumors reached an average size of ~130 ± 32 mm³, ensuring comparable average tumor volumes across all groups.

PREPARATION OF EXPERIMENTAL TREATMENTS

The administration of all compound formulations occurred within one hour of preparation. CLX-155 formulations consisted of 2600 mg of capryol 90, 200 mg of polysorbate 80, and 8 mL of water in sufficient quantities to make solutions of 15.625, 31.25, and 62.5 mg/mL for doses of 125, 250, and 500 mg/kg CLX-155 respectively. Investigators prepared the capecitabine 1000 mg/kg dose in 0.5% w/v hydroxypropyl methylcellulose (HPMC E15) in 40 mM citrate buffer, pH 6.0 in 0.2 µm filtered water vehicle for a capecitabine dose concentration of 100 mg/mL and a dose volume of 10 mL/kg.

TREATMENT GROUPS AND EXPERIMENTAL PROCEDURES

On Day 0, animals in Group 1 (Sham; N = 10) received an oral vehicle control at a dose volume of 8 mL/kg, animals in Groups 2 to 4 (N = 10 for each) received a dose of CLX-155 ranging from low, mid, to high doses (dose levels of 125

mg/kg/day, 250 mg/kg/day, and 500 mg/kg/day respectively), and animals in Group 5 received 1000 mg/kg/day of capecitabine. Researchers administered the doses through oral gavage at approximately the same time each day. They adjusted the dose volume (8 mL/kg for CLX-155 and 10 mL/kg for capecitabine) based on the most recently recorded body weight of each mouse. Doses were selected based on the results of a 7-day repeated dose range-finding toxicity study in Foxn1 nude mice. Treatment administration occurred once daily for all treatment groups, and animals in each group continued receiving these treatments once daily, five days a week for three weeks.

MEASUREMENTS AND ASSESSMENTS

The study team conducted daily mortality checks throughout the study. Investigators monitored animals daily for visible clinical signs (e.g., illness and behavioral changes) and tumors for necrosis, ulceration, wounds, and scars throughout the study. Recordings of body weights for all animals occurred on the first day of treatment and continued three times weekly. Evaluation of treatment toxicity relied on the presence of any body weight loss. Investigators recorded HCT116 xenograft growth on Days 1, 3, 5, 8, 10, 12, 15, 17, 19, and 22 and used a digital Vernier caliper to measure tumor length and width. Investigators measured tumor dimensions (length and breadth) for all animals on the first day of treatment (Day 1) and then three times per week. Tumor volumes involved calculating tumor length × (tumor width)^2 × 0.52. Calculations for tumor growth inhibition involved comparing the tumor volume on a given day compared to that on Day 1. Investigators terminated treatment and humanely sacrificed animals if they exhibited severe clinical signs of toxicity, greater than a 15% drop in body weight in a day, more than a 20% drop in body weight from pre-test level, or tumor volumes exceeding 2000 mm^3.

ANALYSIS AND STATISTICS

This study used Prism 5.0 for all statistical calculations. Assessment of the primary endpoint, tumor volume, involved a two-way ANOVA followed by Bonferroni's multiple comparison tests, and a *p*-value <0.05 compared to sham was considered significant. The percent of tumor growth inhibition involved the following formula: % TGI = [1 – (Treatment TV_{Final} – Treatment $TV_{Initial}$) / (Control TV_{Final} – Control $TV_{Initial}$)] x 100. Calculation of tumor growth rate involved the ratio between tumor volume on the day of measurements and tumor volume on the first day of drug treatment. This study expressed body weight (BW) as a percentage and calculated BW as follows: % BW change = (BW_{Final} –$BW_{Initial}$) / ($BW_{Initial}$) x 100. Complete response referred to a tumor with a volume <25 mm^3 for at least three consecutive measurements, while partial response indicated a tumor that decreased below 50% of its initial volume for at least three consecutive measurements.[10] Investigators expressed the results as means ± the standard deviation.

Results

DISPOSITION OF ANIMALS

This study enrolled fifty nude mice (Figure 2) and randomized them into their respective treatment cohorts (n=10/per cohort). In the vehicle control treatment group, four animals did not complete the study due to tumor volumes exceeding the ethical limit of 2000 mm^3. In the capecitabine 1000 mg/kg treatment group, two animals had expired, one on Day 14 and the second on Day 22.

No mortality or clinical signs of toxicity appeared in animals treated with CLX-155 at any dose level. However, two animals exhibited a decrease in body weight at the 250 and 500 mg/kg/day dose levels, with reductions of 24% and 34%, respectively. In contrast, two mice in the 1000 mg/kg/day dose group of capecitabine showed clinical signs of hunchback and scaly skin on Days 10 and 12. These mice also showed progressive weight loss, up to 18%, and eventual mortality.

Treatment with CLX-155 at all doses did not result in a statistically significant body weight loss. However, a dose-dependent response occurred in the percent mean body weight reductions. In the CLX-155 treatment group, the 125, 250, and 500 mg/kg/day groups had percent mean body weight reductions of -3.4%, -6.4%, and -9.9% respectively.

Figure 2: Animal Disposition
***n=4 mice discontinued due to tumor volumes reaching above the ethical limit of 2000mm³**
****n=2 mice found dead on Day 14 and Day 22**

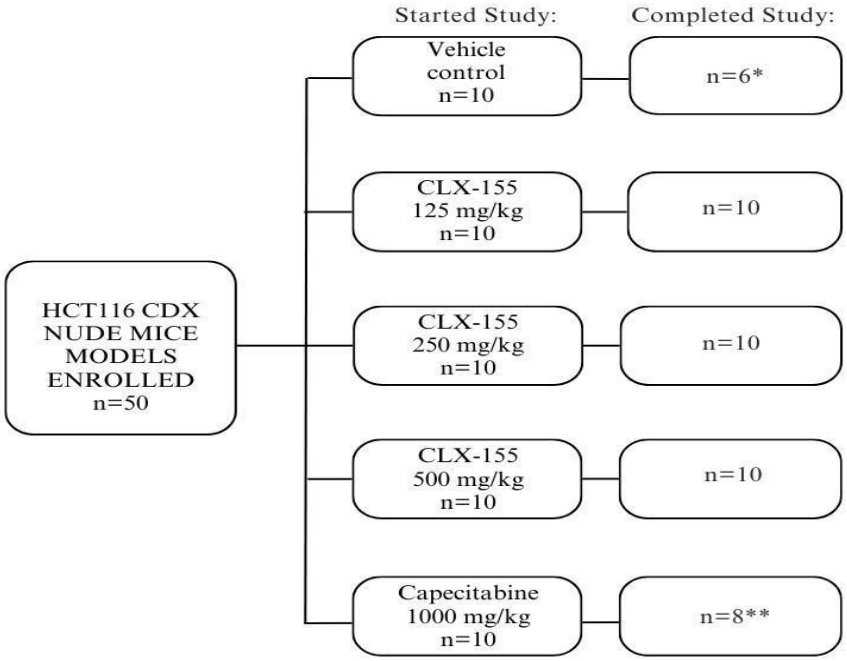

Figure 2. Number of animals in each treatment group (Vehicle control, CLX-155 125mg/kg, CLX-155 250mg/kg, CLX-155 500mg/kg, and Capecitabine 1000mg/kg) enrolled in the study and completed the study.

Tumor Volume Changes

The average initial tumor volume of HCT116 tumor xenografts was 130 +/- 32 mm3 on Day 1. The vehicle control group showed a 10-fold increase in tumor volume, averaging 1259 +/- 558 mm³ on Day 15. All treatment groups for CLX-155 and capecitabine demonstrated statistically significant tumor growth inhibition compared to the vehicle control (Figure 3). The average tumor rate in all treatment groups receiving CLX-155 or capecitabine reached statistical significance ($p<0.0001$) compared to the vehicle control by the end of the study. CLX-155 showed dose-dependent inhibition, with the 500 mg/kg dose showing evident efficacy (Figure 4). In the CLX-155 500 mg/kg/day dose group, two out of ten animals were complete responders. No CR or PR occurred in any of the animals in the capecitabine treatment group.

Figure 3: Average Tumor Volume Effects

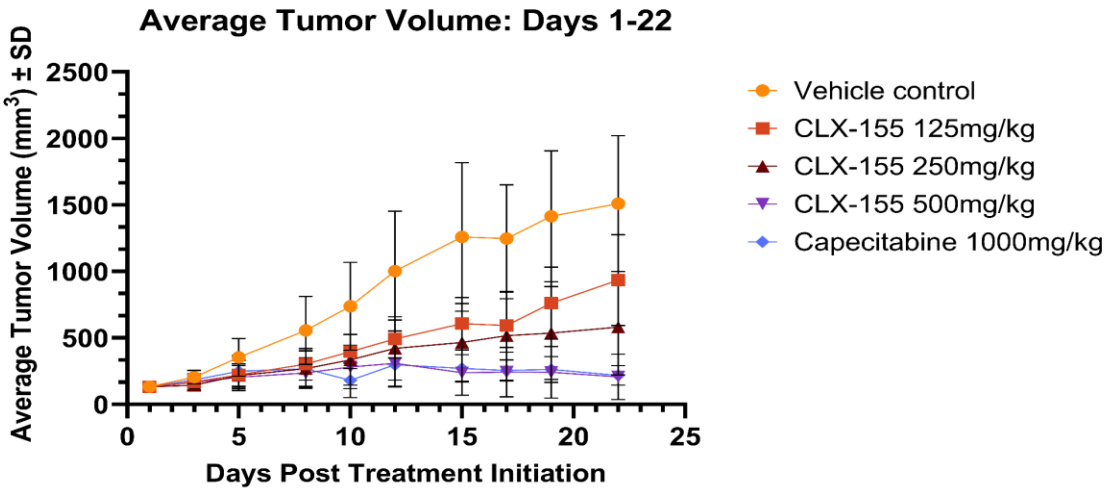

Figure 3: Average tumor volume ± SD (mm³) of animals measured on Days from 1 to 22 after treatment initiation. All treatment groups received treatment via oral route once daily for 5 days/week and 2 days off for a total of 3 weeks.

Figure 4. Average Tumor Growth Rate

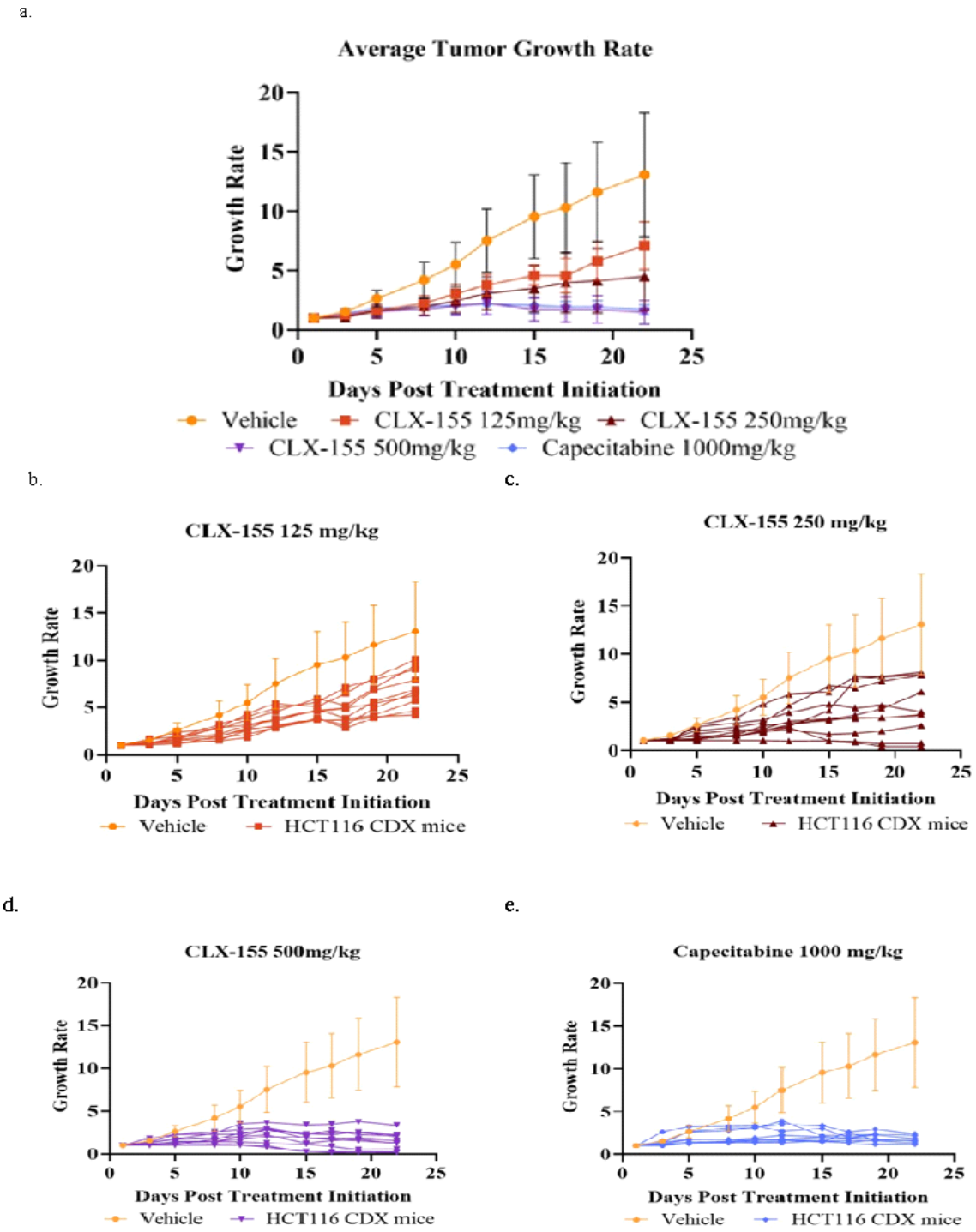

Figure 4. Average tumor rate of animals measured on Days from 1 to 22 after treatment initiation (a). All treatment groups received treatment via oral route once daily for 5 days/week and 2 days off for a total of 3 weeks: CLX-155, 125 mg/kg (b); 250 mg/kg (c); 500 mg/kg (d); and Capecitabine 1000 mg/kg (e), groups.

Discussion

This study compared the antitumor activity of CLX-155 with capecitabine in a human colon cancer xenograft model. The study's assessment of tumor growth inhibition revealed a consistent and dose-dependent response to CLX-155 across all dose levels. Of particular .significance was the comparable tumor growth inhibition at 500 mg/kg/day of CLX-155 to that of capecitabine at 1000 mg/kg/day. This observation not only underscores the potency of CLX-155 but also positions it as a viable alternative with efficacy on par with the standard treatment.

Studies commonly have used the cell line HCT116 in the colon cancer space. It has been extensively studied and established as a valuable model due to its tumorigenic potential, high motility, and invasiveness.[11] Notably, previous work has highlighted the sensitivity of HCT116 to 5-FU and has demonstrated dose-dependent apoptosis in HCT116 cells in response to increasing 5-FU concentrations.[12] The HCT116 cell line has also been used in xenograft nude mice models to evaluate the tumor growth inhibition of other antitumor agents such as 5-FU, docetaxel, and flavopiridol, and shown enhanced phase growth inhibition of HCT116 with these antitumor agents.[13]

Also, previous studies using xenograft models in mice have demonstrated capecitabine efficacy in colon, gastric, breast, cervix, ovarian, bladder, and prostate xenografts.[14,15] This agent showed antitumor activity in a variety of xenograft models with greater inhibition compared to 5-FU alone.[14,16,17] Specific to colon cancer, the ability of capecitabine to inhibit tumor growth using a variety of human colon cancer cell lines ranged from 17 to 101%, with capecitabine exhibiting the highest tumor growth inhibition in the HCT116 cell line at 101%.[17] Another study comparing the efficacy of capecitabine and 5-FU at their maximum tolerated doses in an HCT116 human colon cancer xenograft model demonstrated similar results, with capecitabine inhibiting tumor growth by 86% after seven weeks.[18] The data from this paper is comparable to the tumor growth inhibition rates observed with capecitabine in this study, which was 87.7% on Day 15.

A compelling aspect of CLX-155's efficacy was the occurrence of complete regression in two out of ten animals at 500 mg/kg/day. This outcome represents a noteworthy efficacy, suggesting a potential for achieving complete responses in a subset of treated subjects. In contrast, capecitabine did not yield complete or partial responses, emphasizing the potential superior efficacy of CLX-155 in inducing a more robust antitumor response. Additionally, CLX-155 demonstrated similar tumor growth inhibition to capecitabine at half the dose. This observation further supports the hypothesis that the unique mechanism of CLX-155, with its sequential release of active moieties, contributes to a more pronounced and comprehensive antitumor effect.

The rationale underlying these efficacy observations may be twofold. First, CLX-155 is a prodrug designed with a unique molecular structure—a conjugate of acetylated 5'-DFCR linked to caprylate. This molecule undergoes metabolic transformation *in vivo*, resulting in the sequential release of active compounds. Initial conversion yields the 5'-DFCR conjugate of caprylic acid (CLX-155 PM1), followed by subsequent steps generating 5'-DFCR, 5'-DFUR, and, ultimately, the active anticancer agent, 5-FU. The comprehensive comparison with capecitabine, a known prodrug of 5'-DFCR, provided a basis for evaluating CLX-155's efficacy by examining the parallel enzymatic pathways leading to 5-FU release. This unique pharmacological design introduces the potential for sustained and controlled release of active compounds, similar to continuous infusion administrations of 5-FU. 5-FU has a cell-cycle-specific mechanism of action, and efficacy relies on cancer cell contact time rather than dosage amount.[19,20] The prolonged exposure of tumor cells to successive active moieties could contribute to the observed dose-dependent tumor growth inhibition[21] and the remarkable complete regression outcomes seen with CLX-155. Further, the potential for sustained and controlled release mimics slow infusion administration seen with 5-FU, which may affect the efficacy of anticancer medications.[22] This concept may help explain the increased efficacy of CLX-155.

Second, CLX-155 showed a unique PK profile in mice in which the plasma AUC of 5-FU after CLX-155 (500 mg/kg) was 17% greater than that achieved with capecitabine (1000 mg/kg), suggesting a greater level of 5-FU generation with CLX-155 (data not shown). In clinical studies with capecitabine, the colorectal tumor concentration of 5-FU was 21.4 times greater than the plasma 5-FU levels.[23] Although this study did not measure tumor levels of 5-FU levels in the pharmacokinetic study, the marginally greater plasma levels of 5-FU with CLX-155 suggest potentially greater levels of 5-FU in the xenograft tumors, contributing to the comparable efficacy of CLX-155 to capecitabine (1000 mg/kg) at a lower dose of CLX-155 (500 mg/kg). The lower dose of CLX-155 compared to capecitabine also suggests a potential for less toxicity; however, this needs to be confirmed in further studies as this study's design was not to assess safety.

Unlike capecitabine, CLX-155 hydrolysis by intestinal esterase yields caprylic acid as another metabolite of CLX-155. Caprylic acid exerts anticancer activity on cultured human colorectal carcinoma (HCT-116), human skin epidermoid carcinoma (A-451), and human breast cancer cells (MDA-MB-231).[24] Caprylic acid produces antitumor effects by up-regulating apoptosis genes and down-regulating cell regulatory genes.[24] Fatty acid conjugation also enhances the efficacy of

gemcitabine on human breast cancer cells in vitro and *in vivo* in xenograft models.[25] Understanding the interplay between CLX-155 hydrolytic products, 5-FU and caprylic acid, could pave the way for novel strategies in cancer treatment.

Conclusion

CLX-155 produced anticancer activity in this model. The investigation demonstrated significant and dose-dependent tumor growth inhibition across all tested doses. Notably, at 500 mg/kg/day, CLX-155 exhibited a tumor growth inhibition comparable to that of capecitabine at 1000 mg/kg/day, suggesting a remarkable potency. Moreover, the observation of complete regression in two out of 10 animals at this dose highlights the potential for robust treatment responses.

Alongside the valuable insights gained from this study, it is essential to acknowledge its limitations, primarily associated with using the HCT116 human colon cancer xenograft model in Foxn1 athymic nude mice. While xenograft models help study antitumor efficacy, they inherently lack an intact immune system. The absence of immune responses in these mice may not fully represent the complex interactions between the immune system and tumor microenvironment, as seen in human subjects. The model used in this study focused on addressing the specific question as an initial activity signal. Thus, the model's lack of tumor metastasis is not fully representative of advanced disease. Given these points, it is necessary to evaluate further and confirm the efficacy of CLX-155 in other colorectal cancer models, including Genetically engineered mouse models (GEMMs), Patient-derived xenograft models (PDX), and Patient-derived organoid (PDOX) models.[26] Additionally, the novelty of CLX-155 introduces a unique aspect to the study, but it also prompts a cautious interpretation of the results. The specific metabolic conversions and subsequent release of active compounds *in vivo* need further elucidation, particularly in the context of potential variations across different tumor types or patient populations.

Despite these limitations, the study offers significant implications for developing CLX-155 as a potential anticancer therapeutic. The observed favorable safety profile and potent antitumor activity, particularly the occurrence of complete regression in a subset of animals, underscore the potential of CLX-155 as a compelling candidate for further clinical exploration. The comparison with capecitabine, a standard treatment, suggests that CLX-155 may be a promising alternative, potentially offering improved tolerability and efficacy. These findings contribute valuable insights into the potential of CLX-155 as an anticancer therapeutic. These findings set the stage for further exploration and development of CLX-155 as a potential treatment option for colorectal cancer and warrant continued investigations in diverse preclinical models and, eventually, in clinical trials.

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