UNIVERSITI TEKNOLOGI MARA

HYDROXYCHAVICOL IN COMBINATION WITH 5-FLUOROURACIL MODULATES PURINE METABOLISM IN COLORECTAL CANCER CELLS BY REDUCING HYPOXANTHINE, XANTHINE OXIDOREDUCTASE, AND REACTIVE OXYGEN SPECIES LEVELS.

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ABSTRACT

The development of resistance to 5-fluorouracil (5-FU) led to the need for combination therapies in colorectal cancer patients. In recent years, natural products have gained a lot of attention as adjuvants for chemotherapies due to their multi-targeting properties. Hydroxychavicol (HC), a bioactive compound found in *Piper betle* leaves, was reported to be a potent anti-apoptotic agent. The mechanism by which HC induces cell death is not fully understood, particularly in relation to purine metabolism. Hence, this study aims to elucidate the effect of 5-FU, HC, and HC in combination with 5-FU (HC+5-FU) in modulating the purine metabolism pathway of colorectal cancer (CRC) cell lines, HT-29 (Duke's B) and DLD-1 (Duke's C). The inhibitory concentration (IC_{50}) of HC, 5-FU, and HC+5-FU was determined by measuring the CRC cell viability via MTT assay. The most effective doses were then chosen in the subsequent experiments. The effect of HC+5-FU on the purine metabolism pathway was determined by measuring the levels of hypoxanthine (HPX), xanthine oxidoreductase (XOR) via Amplex xanthine/xanthine oxidoreductase assay, reactive oxygen species (ROS) via DCFDA assay in HT-29 and DLD-1 cells and elucidating the mitochondrial activity by analysing its mitochondrial membrane potential via JC-1 assay. Furthermore, to validate the findings on functional analysis, the expression of genes, equilibrative nucleoside transporter 1 and 2 (*ENT1* and *ENT2*), caspase 3 (*CASP3*), and B-cell lymphoma 2 (*BCL2*) were measured via qPCR. *ENT1* and *ENT2* transport hypoxanthine, whereas *CASP3* and *BCL2* regulate apoptotic cell death. The results showed that the treatments affect the cell viability of HT-29 and DLD-1 treated for 24 and 48h in a time-dose-dependent manner. The inhibition concentration of 50% (IC_{50}) of HC in HT-29 was 695.3 µM (24h) and 201.3 µM (48h), while for 5-FU, 244.8 µM (24h), and 123.1 µM (48h) respectively. In DLD-1, the IC_{50} of HC were 718.3 µM (24h) and 225.1 µM (48h), while for 5-FU, 98.3 µM (24h), 72.7 µM (48h). Following this, sub-effective doses of HC and 5-FU (55 µM) were combined to determine the IC_{50} of combination treatment. The IC_{50} of HC + 55 µM 5-FU treatment for HT-29 was 180.9 µM (24h) and 132.8 µM (48h). In DLD-1, the IC_{50} of HC + 55 µM 5-FU was 152.6 µM (24h) and 72.69 µM (48h). All concentration values were predicted by GraphPad Prism. HC+5-FU showed synergistic interaction in HT-29 treated for 24h, with a combination interaction (CI) of 0.49. However, treatment for 48h showed additive interaction in HT-29 (1.11). In both time points, treatment of HC+5-FU in DLD-1 showed additive interaction (0.77 and 1.08). The HPX, XOR, and ROS levels in HT-29 and DLD-1 treated with HC+5-FU (24 and 48h) were decreased compared to untreated cells. The combination treatment induced high mitochondrial depolarization. Gene expression analysis in HT-29 showed that *ENT1* was downregulated in 24h but upregulated in 48h HC+5-FU treatment compared to untreated and single doses (HC / 5-FU) treatment. Interestingly, these observations are contradictory in DLD-1. On the other hand, *ENT2* was downregulated in both HT-29 and DLD-1. Expression of *CASP3* was induced in both HT-29 and DLD-1 treated with HC+5-FU for 24 and 48h compared to untreated and single doses. *BCL2*, the anti-apoptotic gene, was downregulated in HT-29 and DLD-1 when treated with HC+5-FU. In conclusion, the induction of cell death in HT-29 and DLD-1 treated with HC+5-FU may be via the modulation of purine metabolism and the apoptotic pathway, in which the decrease of HPX and XOR may cause an imbalance of ROS and lead to the disruption of mitochondrial membrane potential. These observations were further supported by the downregulation of *ENT2*, which plays a role in HPX transport, the downregulation of the anti-apoptotic gene BCL2, and the upregulation of *CASP3*, a pro-apoptotic gene. Further investigation is needed on the potential of targeting the purine metabolism pathway as a novel therapeutic approach for colorectal cancer by elucidating its role in cancer formation and response to treatment.

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CHAPTER ONE
INTRODUCTION

1.1 Research Background

Colorectal cancer (CRC) is the third and second most common cancer, respectively, in men and women worldwide (Hashim et al., 2021). Mortality because of CRC is on the increasing trend, and it is leading at the second rank for cancer death in the world (Sung et al., 2021). The prevalence of CRC around the globe varies widely, and its prevalence has risen rapidly in many Asian countries over the past few decades (Allemani et al., 2015; Sung et al., 2021). Colorectal cancer accounts for about 11% of all cancers diagnosed yearly and cancer-related deaths worldwide (Bray et al., 2018). According to GLOBOCAN 2020, CRC is ranked second in the death section for all cancers in the world, with a total death of 935 173 (*GLOBOCAN*, 2020).

In Malaysia, CRC is the second most common cancer in both males and females (WHO, 2018). According to GLOBOCAN 2020, among the Southeast Asia countries, Malaysia ranks fifth in colorectum cancer (6,597 cases) and ranks 6th (29,530 cases) in mortality caused by CRC (*GLOBOCAN*, 2020). Malaysia, a developing country (Sung et al., 2015) contributed to the elevation of new cancer cases mainly due to the ageing population and high-income eating habits. Adverse risk factors such as obesity, lack of physical activity, western influence diets, and smoking were suggested to be the factors for increasing the risk of colorectal cancer (Chazelas et al., 2019; Dekker et al., 2019; Gibson et al., 2020; Gram et al., 2020; Li et al., 2021). Thus, the development of effective treatments for CRC is critical.

The potential treatments for colorectal cancer cells, which include chemotherapy, radiation, biological therapy, and surgery, will be chosen based on the cancer cell's stage. Azzani et al. (2019) stated that in CRC, only surgery is needed for stage I. However, as the stages increase (II-IV), chemotherapy becomes the foremost common treatment (Azzani et al., 2019). A common chemotherapy drug used for CRC is 5-fluorouracil (5-FU). The 5-FU main target is the thymidylate synthase enzyme (TYMS) which catalyses the formation of deoxythymidine-5'-monophosphate (dTMP) from 2'-deoxyuridine monophosphate using a cofactor called 5'10-methylene tetrahydrofolate via *de novo* pathway (Aspinall et al., 2015). 5-Fluorouracil is toxic to