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Original Article

Anti-proliferative and Anti-migratory Activities of Diphyllin on Human Colorectal Cancer Cells

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ABSTRACT

Background: Colorectal cancer (CRC) is the third leading cause of death, probably because of its invasive and metastatic potential. Agents that can mitigate the growth and prevent migration may benefit CRC. Diphyllin and its analogues belong to glycosylated compounds previously reported for their *in vitro* cytotoxicity against many viruses, candida, and human malignant cells. This study aims to evaluate the anti-proliferative and anti-migratory activities of diphyllin on human CRC cells. **Methods:** The MTT assay was performed to determine the 50% inhibitory concentrations (IC_{50}) in HT-29, SW-480, and HCT-15 CRC cells. The acridine orange and ethidium bromide (AO/EB) dual staining was used to ascertain the type of cell death. HT-29 cells were subjected to short-term 5-fluorouracil+oxaliplatin (5-FU+Ox) to eliminate the most sensitive cells. The remaining surviving cells were further treated with diphyllin alone or combined with 5-FU. The combination effect of diphyllin with 5-FU in 5-FU+Ox surviving HT-29 cells was determined by flow cytometry-based Annexin V apoptosis assay. The wound-healing assay was used to detect the anti-migratory activity of diphyllin on 5-FU+Ox surviving HT-29 CRC cells. **Results:** The IC_{50} values of diphyllin were found to be 2.9±0.38, 1.3±0.28, and 3.9±0.65 µg/mL against HT-29, SW-480, and HCT-15, respectively. Microscopic examination manifested apoptotic changes like chromatin condensation, nuclear fragmentation, and formation of apoptotic bodies in diphyllin treated CRC cells. Flow cytometry analysis revealed that diphyllin enhances the apoptosis in 5-FU+Ox surviving HT-29 cells. Wound-healing assay displayed the inhibitory activity of diphyllin in 5-FU+Ox surviving HT-29 cells. **Conclusion:** Diphyllin shows anti-proliferative activity by inducing apoptosis in HT-29, SW-480, HCT-15 cells and 5-FU+Ox surviving HT-29 cells and impairs their migration.

Key words: Anticancer, Apoptosis, Cytotoxicity, Flow cytometry, Wound-healing.

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INTRODUCTION

Cancer is a significant health burden affecting millions of people annually. Colorectal cancer (CRC) is the third most common malignancy, which is not only confined to developed countries anymore.[1] In general, cancer treatment includes surgery, chemotherapy, and radiotherapy. The selection of treatment strategy depends on the spread of tumors in primary and metastatic tissues, and usually, the combined approach is much preferred.[2]

Although cytotoxic chemotherapy is the primary choice in eliminating exponentially dividing malignant cells, it is associated with adverse effects.[3] More specific anticancer targeted therapy has been introduced to minimize toxicity to normal cells. Nevertheless, targeted therapy is generally combined with cytotoxic chemotherapy for better clinical outcomes. Another major issue with conventional chemotherapy is the development of drug resistance that may be superseded by targeting tumor cells at multiple levels. These resistant cells survive treatment and migrate to distant organs to produce metastasis.[4] A desirable anticancer drug intended to induce apoptosis and inhibit proliferation, migration, and angiogenesis can offer good clinical outcomes. Available cytotoxic and targeted therapy agents kill rapidly proliferating cells or target specific proteins involved in metastasis and angiogenesis. The paradigm has been shifted to develop an anticancer drug with multifaceted action and a distinct mechanism.

Diphyllin is a glycoside naturally expressed in many plant species and is chemically related to arylnaphthalene ligans. The pharmacological action of diphyllin is mainly attributed to its ability to interact with the vacuolar-ATPase (V-ATPase) pump that involves maintaining pH gradient across the cells.[5-6] In cancer, V-ATPase is associated with drug resistance, invasiveness, and metastasis.[7-8] Diphyllin cytotoxic effect on mammalian cells and viruses has been investigated.[9-11] Diphyllin and its analogues exhibit *in vitro* anticancer activity against liver, cervical and colorectal cancer cells.[12-15] Also, its derivatives were reported to show anti-migratory effects in gastric cancer cells.[16] The anticancer activity of diphyllin related compounds has been ascertained to inhibit V-ATPase in cancer cells.[17]

Cells tend to move from the primary site under certain physiological conditions and pathological states like cancer. Experimental methods to study mammalian cell migration are of great importance in cell biology, especially in immunology and cancer. *In vitro* wound-healing assay is the most reliable method to determine the migratory activity of cancer cells with or without the influence of anticancer agents. Moreover, it is a convenient and inexpensive method to evaluate the anti-migratory potential of compounds, especially for two-dimensional monolayer cultures.[18-19] Existing anticancer drugs primarily kill rapidly multiplying cells with limited or no action against specialized cells that drive metastasis. Continuous use of conventional anticancer drugs may lead to drug resistance. Compounds that interact with V-ATPase-associated proteins mitigate tumor cell activity and restore anticancer drug sensitivity.[20-22] Hence, it would be prudent to study the anti-proliferative and anti-migratory activity of diphyllin in colorectal cancer cells.

MATERIALS AND METHODS

Drugs and Chemicals

Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone (UK). Dimethyl sulfoxide (DMSO), trypsin-ethylene diamine tetra acetic acid (Trypsin-EDTA) and penicillin-streptomycin antibiotic solution were purchased from Hi-Media (USA). Diphyllin (CAS No. 22055-22-7), 5-fluorouracil (5-FU), oxaliplatin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), acridine orange (AO), and ethidium bromide (EB) were purchased from Sigma-Aldrich (USA). Binding buffer, propidium iodide (PI) and Annexin V-APC conjugate were purchased from BD bioscience (USA).

Cell Lines and Culture Conditions

The colorectal (HT-29, SW-480, and HCT-15) cells were procured from National Centre for Cell Sciences (NCCS), Pune, India. We followed biosafety level-2 practices for handling all cell culture experiments. Cells cultured in DMEM medium were incubated in a CO_2 incubator at 37°C. All experiments were conducted thrice in triplicates to confirm the findings.

The Cytotoxic Assay

The percentage of a viable cell population after treatment with diphyllin was evaluated by MTT assay.[23] Briefly, cells at 5×10^3 density per well were seeded in 96 well plates and placed for 12 h in an incubator (ESCO CLS-170T-8, Singapore). On the following day, various concentrations of diphyllin were added to cells and further incubated at 37°C for 48 h. A freshly prepared MTT solution (5 mg/mL) was added to each well without removing the media and incubated for 3-4 h. At the end of incubation, the formazan crystals were observed under a microscope and dissolved using DMSO (100 µL) solution. The absorbance of the purple color was recorded at 570 nm using a microplate reader (BIO-RAD 680 XR, USA). The inhibitory concentrations 50 (IC_{50}) of diphyllin were calculated for all CRC cells. We derived IC_{50} concentrations by performing three independent assays in triplicates using the following formula.

$$\text{Percentage of cell death} = \frac{(\text{OD of control cells} - \text{OD of treated cells})}{(\text{OD of control cells}) \times 100}$$

Acridine Orange and Ethidium Bromide (AO/EB) Staining

The AO/EB staining method was used to assess diphyllin induced morphological changes in CRC cells.[24] Using 6 well plate, approximately 5×10^5 cells/well were seeded and incubated with DMSO (vehicle control) and indicated doses of diphyllin for 12 h in a CO2 incubator at 37°C. Then, cells were washed with PBS, and a mixture (1:1) of AO/EB staining solution (100 µg/mL in PBS) was added. Cell images were captured using a fluorescent microscope (Thermo Fisher Scientific EVOS Imaging Systems, USA). A minimum of 100 cells from five random zones/well were counted manually and at least from three separate experiments.

Generation of 5-FU+Ox-surviving Colorectal Cancer Cells

We generated 5-FU+Ox surviving colorectal cancer cells based on a previously reported protocol.[25] Briefly, HT-29 cells at 70-80% confluence were treated with a combination of 50 µM of 5-FU and 1.25 µM of oxaliplatin (5-FU+Ox) for 48 h. This short exposure eliminates the most sensitive cells leaving behind the sturdy counterparts. Further experiments were conducted using 5-FU+Ox surviving HT-29 cancer cells.

Annexin V-PI Flow Cytometry Assay

We followed a previously described method and the manufacturer's instructions for this assay.[26] Briefly, 5-FU+Ox surviving HT-29 cells at a density of 1×10^6 cells/well were seeded in 6 well plates and incubated overnight. On the following day, DMSO (vehicle control), 5-FU, diphyllin alone, and in combination with 5-FU at indicated doses were added to the cells for 12 h. Cells were centrifuged for 5 min at 1500 RPM and resuspended in 1× binding buffer after a PBS wash. Annexin V-APC solution and PI (5 mg/mL) were added to 100 µL cell suspension and incubated for 15 min at room temperature in the dark. Cells were analyzed by flow cytometry within 30 min after washing and resuspending in 1× binding buffer. The gating strategy determined the percentage of live, apoptotic and dead cells. The apoptotic changes were differentiated based on their Annexin V and PI uptake, and the percentage of live, apoptotic and dead cells was calculated. Viable cells (both annexin-V and PI negative), early apoptotic cells (Annexin-V positive and PI negative) and dead cells (both annexin-V and PI-positive) were considered for analysis.

Wound-healing Assay

We carried out the wound-healing assay to evaluate the anti-migratory property of diphyllin in 5-FU+Ox survived HT-29 cells as reported previously.[18-19] HT-29 cells grown as a monolayer in a multi-well plate were washed with PBS, and a scratch was made using a sterile micro tip. Cells were rewashed and incubated in the presence of vehicle control and diphyllin. At regular intervals, cells were examined under an inverted microscope (Olympus CKX41, Japan), and images were captured at baseline, 12 and 24 h. The anti-migratory property of diphyllin was tested by observing the cell-free zone in control and treated cells.

Statistical Analysis

The IC_{50} values of diphyllin in CRC cells were obtained using OD values from the MTT assay and calculated from the inhibitor vs response curves plotted using the nonlinear regression analysis with GraphPad Prism version 7.0 software (GraphPad Software, Inc., La Jolla, CA, USA). One-way ANOVA followed by Tukey's post-hoc test was used to compare the groups, and a p value < 0.05 was considered significant.

RESULTS

Cytotoxicity Assay

Colorectal cancer cell lines, including HT-29, SW-480, and HCT-15, were treated with diphyllin under the indicated concentrations for 48 h, and then cell viability was evaluated using the MTT assay. Our results showed that diphyllin could inhibit the proliferation of HT-29, SW-480, and HCT-15 with an IC_{50} of 2.9±0.38, 1.3±0.28, and 3.9±0.65 µg/mL, respectively (Figure 1). It is observed that diphyllin inhibited the growth of CRC cells in a dose-dependent manner.

Acridine Orange and Ethidium Bromide Dual Staining

The fluorescent microscopy observation after AOEB staining showed cells emitting green, yellow-orange, and orange-red fluorescence depending on the stage of apoptosis. Furthermore, HT-29, SW-480, and HCT-15 cells treated with diphyllin displayed evidence of apoptosis with disorganized nuclei, highly condensed or fragmented nuclei with characteristic apoptotic body formation. On the other hand, the morphology of viable cells, especially in untreated cells, showed organized nuclei emitting green fluorescence (Figure 2). Thus, morphological changes indicated that diphyllin might promote apoptotic cell death in colorectal cancer cells compared to untreated cells. Late apoptotic cells appeared orange-red and expressed highly condensed or fragmented nuclei with characteristic apoptotic body formation.

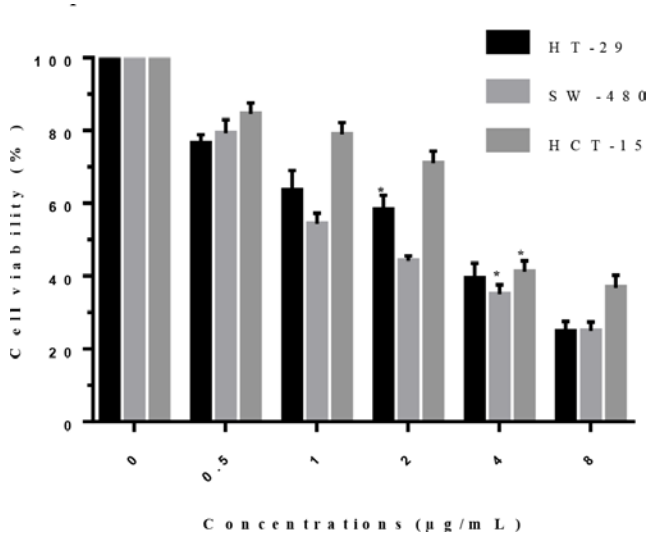

Figure 1: Effect of diphyllin on the proliferation of CRC cell lines, HT-29, SW-480, and HCT-15. The bars represent the percentage of viable SW-480, HT-29, and HCT-15 cancer cells. The data represent the mean ± SD from at least three independent experiments. *P < 0.05 significant difference compared to their preceding concentrations. The results were derived from three individual experiments performed in triplicates.

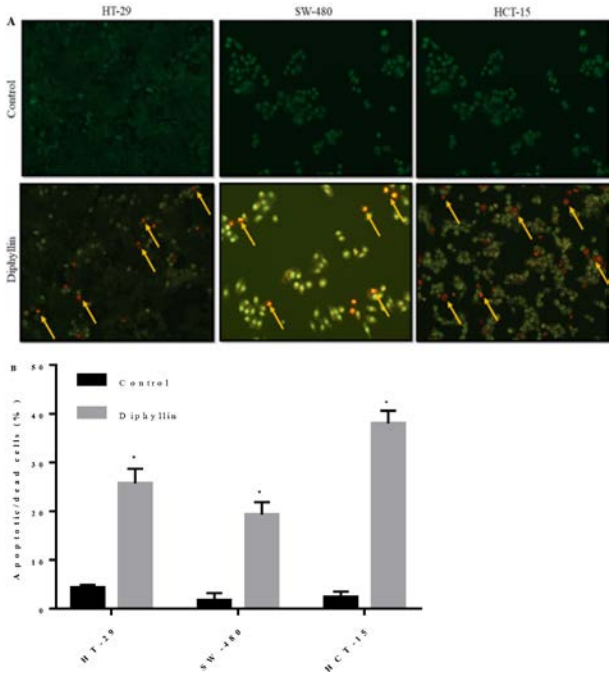

Figure 2: Assessment of diphyllin apoptotic morphological changes by AO-EB dual staining. A. Acridine orange enters both live and dead cells and emits green color, whereas apoptosis cells take ethidium bromide and emit yellow-red color. Arrows indicate apoptotic cells emitting orange-red color fluorescence. B. Compared to respective controls, diphyllin treatment significantly increased the percentage of apoptotic cells (p < 0.05) in all CRC cells. The results were derived from three individual experiments performed in triplicates.

Annexin-V Apoptosis Assay

The flow cytometry-based annexin V staining method assessed the apoptosis-inducing effect of diphyllin either alone or in combination with 5-FU against 5-FU+Ox surviving HT-29 cells. Negligible cell

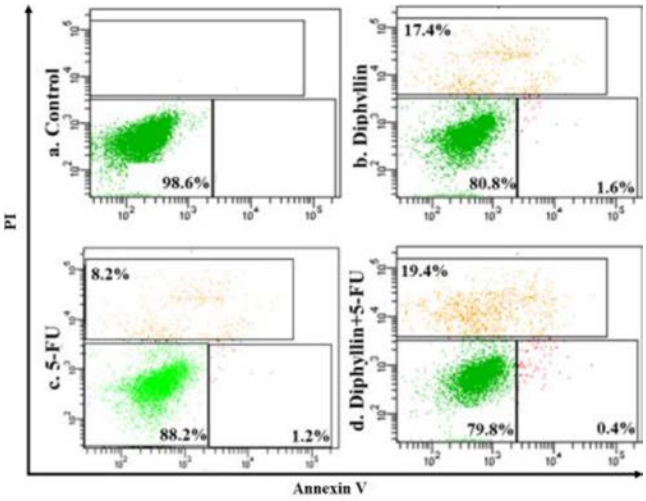

Figure 3: Diphyllin induced apoptosis detection in 5-FU+Ox survived HT-29 cells (a) Control (DMSO), (b) diphyllin, (c) 5-FU, (d) diphyllin and 5-FU combination. The lower left quadrants show viable cells, and the lower right quadrants are early apoptotic cells. Upper quadrants show dead cells, including late apoptotic cells. The results were derived from three individual experiments performed in triplicates.

death was detected in the control group. Compared to the control group (Figure 3a), diphyllin reduced the percentage of viable cells to 80.8 (Figure 3b) in FU+Ox surviving HT-29 cells. Furthermore, the percentage of viable cells (88.2%) in the 5-FU treated group (Figure 3c) was about 8% more than that of the 5-FU+diphyllin group (79.8%) (Figure 3d). The results indicate that diphyllin induced apoptosis in FU+Ox surviving HT-29 cells. The effect of diphyllin, either alone or in combination with 5-FU, showed an almost similar result.

Wound-healing Assay

We performed the wound-healing assay in 5-FU+Ox surviving HT-29 cells to determine the cell migration with or without the presence of diphyllin. The distance between the scratch edges was considered the cell-free zone to observe cell moment. Results indicated that diphyllin hindered the motility of CRC cells towards the wound area compared to control cells at 12 and 24 h. Further, diphyllin treated wells showed sparsely spaced cells than cells in control wells, indicating its ability to interfere with proliferation and migration (Figure 4).

DISCUSSION

The hallmark feature of cancer is exponential proliferation and metastasis, causing secondary tumors. The metastatic potential of cancer cells is attributed to their invasive and migration potential, which remains valid, especially for solid cancers.[27] Colorectal cancer spreads primarily to the liver, peritoneum, and other organs like the lungs, ovaries, and brain.[28] Conventional cytotoxic drugs kill rapidly proliferating cells but may not control their migration. The multifaceted anticancer agents with cytotoxic and anti-migratory properties may have certain advantages over merely cytotoxic drugs.

In the current investigation, diphyllin exhibited cytotoxicity in all three CRC cells at low IC_{50} concentrations. The compound significantly induced apoptosis in HT-29, HCT-15, and SW-480 CRC cells, resulting in morphological changes. Furthermore, diphyllin induced apoptosis in 5-FU+Ox surviving HT-29 cells. Diphyllin significantly prevented the movement of 5-FU+Ox surviving HT-29 cells into the cell-free zone indicating its anti-migratory action.

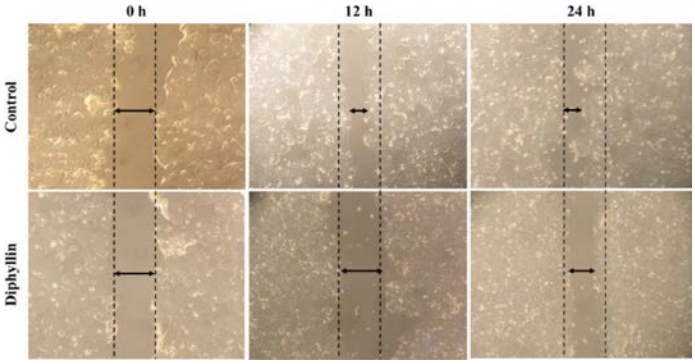

Figure 4: Representative images show the 5-FU+Ox surviving HT-29 cells in the wound healing assay. At 0 h, the scratch was created equally in monolayer cells and photographed at 12 and 24 h to compare the difference in the cell-free zone in control and diphyllin treated cells. Arrows indicate the width of the cell-free zone at different time points. The results were derived from three individual experiments performed in triplicates.

We chose three cell lines derived from different stages of colorectal adenocarcinoma. The IC_{50} concentrations of diphyllin were lowest against SW-480, followed by HT-29 and HCT-15 CRC cells. Similarly, IC_{50} concentrations of diphyllin and its analogs were lower against CRC cells when compared to oral and lung cancer cells. It has been demonstrated that gastrointestinal tract cancers were more sensitive to diphyllin like glycosides.[29-33] Though the exact mechanism is unknown, the presence of specific cellular targets in CRC cells might be a reason for the strong toxicity of diphyllin analogs.

Based on cytotoxic results, diphyllin was analyzed for its mode of cell death in CRC cells. The morphology of all three CRC cells which received diphyllin expressed typical apoptotic features like chromatin condensation and marginalization, nuclear fragmentation, and formation of apoptotic bodies. Cells undergo apoptosis naturally to maintain cell homeostasis, and disturbances in this pathway lead to many pathological conditions, including cancer. Apoptosis can also be induced by activating apoptotic machinery using anticancer agents.[34] Several existing anticancer drugs induce apoptosis by inhibiting topoisomerase enzyme, DNA synthesis, or causing DNA damage in malignant cells.[35] Whereas diphyllin is reported to inhibit V-ATPases pump in gastric adenocarcinoma to induce apoptosis. Interestingly, a diphyllin derivative induced G0/G1 arrest in hepatic cancer cells to induce apoptosis.[36] Another natural compound with the diphyllin in its core structure induces apoptosis by inhibiting autophagy in ovarian cancer cells.[37]

The backbone of colorectal cancer treatment is 5-fluorouracil based chemotherapy. However, the development of resistance to 5-FU combinations poses many challenges to clinicians.[38] The synergistic cytotoxic activity of some natural compounds with 5-FU has been reported.[39-41] The HT-29 cell line is reported to be a relatively resistant cancer phenotype that is selected to treat with the standard 5-FU+Ox regimen.[42] We initially exposed HT-29 cells to the standard 5-FU+Ox regimen. This treatment killed the sensitive subsets and spared moderately resistant HT-29 cells. When these 5-FU+Ox-survived HT-29 cells were subjected to diphyllin either alone or combined with 5-FU, they showed apoptotic phenomenon in annexin V assay.

Interestingly, the cell death caused by diphyllin is one-fold higher than that by 5-FU treatment. However, diphyllin and 5-FU could not significantly increase cell death, indicating no additional benefit of this combination. The failure of diphyllin to exert synergistic activity with 5-FU in inducing apoptosis is not known. However, the fact that these HT-29 cells used in the experiment were the ones that survived from short term 5-FU treatment might be the reason for the lack of response to 5-FU. In contrast, Moringa oleifera plant extract containing diphyllin glycoside exhibited a synergistic effect with 5-FU against CRC cells.[43] This phenomenon might be due to many other bioactive polyphenols in the extract. The antimetabolite 5-FU primarily acts by inhibiting the thymidylate synthase (TS) enzyme and falsely incorporating its metabolites into nucleic acids.[40] The tumor cells adopt various mechanisms to withstand the 5-FU treatment. On the other hand, diphyllin is reported to inhibit V-ATPase activity in cancer cells that usually deal with essential physiological functions of normal cells through pH regulation.[36]

In the present study, diphyllin alone showed better apoptosis-inducing activity than 5-FU in 5-FU+Ox-surviving HT-29 cells. Diphyllin and its derivatives were also reported to cause cell cycle arrest at various levels, ultimately responsible for apoptosis.[17,44-46] Furthermore, diphyllin impaired the migration of 5-FU+Ox surviving cells indicating its anti-migratory activity. Conversely, control cells significantly occupied the cell-free zone over the period. Our findings are in accordance with previous reports where diphyllin derivatives exerted similar effects.[13,16] The involvement of V-ATPase hyperactivity in tumor resistance, invasiveness and metastasis has been established.[5,7,47] In the present study, the 5-FU+Ox surviving cells with some armory become intuitively sensitive to diphyllin. This phenomenon might be due to the expression of specific cellular targets in CRC cells which can be further evaluated.

CONCLUSION

Diphyllin shows potent cytotoxicity on selected colorectal cancer cells in the present study. Diphyllin induces morphological changes in HT-29, SW-480, and HCT-15 CRC cells due to its apoptosis-inducing property. Additionally, the compound induces death in 5-FU+Ox surviving cells, which recovered from the 5-FU insult. Moreover, diphyllin exerts an anti-migratory effect on 5-FU+Ox surviving cells that is crucial to preventing cancer metastasis. Our findings, combined with previous evidence, may draw the researchers' attention to conduct studies on diphyllin like compounds against colorectal cancer, especially in resistant phenotypes.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

CRC: Colorectal cancer; **5-FU+Ox:** 5-Fluorouracil+oxaliplatin

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