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Inhibition of IRE1 RNase activity modulates the tumor cell progression and enhances response to chemotherapy in colorectal cancer

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Abstract

Background: Drug resistance is one of the clinical challenges that limits the effectiveness of chemotherapy. Recent reports suggest that the unfolded protein response (UPR) and endoplasmic reticulum (ER) stress-adaptation signalling pathway, along with increased activation of its inositol-requiring enzyme 1α (IRE1α) arm, may be contributors to the pathogenesis of colorectal cancer (CRC). Here, we aimed to target the IRE1α/XBP1 pathway in order to sensitise CRC cells to the effects of chemotherapy.

Methods: The CT26 colorectal cell line was treated with tunicamycin (TM), and then was exposed to different concentrations of 5- fluorouracil (5-FU), either alone and/or in combination with the IRE1α inhibitor, 4μ8C. An MTT assay, flow cytometry and RT-PCR were performed to determine cell growth, apoptosis and IRE1α activity, respectively. *In vivo* BALB/c syngeneic colorectal mice received chemotherapeutic drugs. Treatment responses, tumour sizes and cytotoxicity were assessed via a range of pathological tests.

Results: 4μ8C was found to inhibit the growth of CRC +, at a concentration of 10 μg/ml, without detectable cytotoxic effects and also significantly enhanced the cytotoxic potential of 5-FU, in CRC cells. *In vivo* experiments revealed that 4μ8C, at a concentration of 50μM/kg prevented tumour growth without any cytotoxic or metastatic effects. Interestingly, the combination of 4μ8C with 5-FU remarkably enhanced drug responses, up to 40- 60% and also lead to significantly greater (more?) inhibition of tumour growth, in comparison to monotherapy, in CRC mice.

Conclusions: Targeting the IRE1α/XBP1 axis of the UPR could enhance the effectiveness of chemotherapy in both *in vitro* and *in vivo* models of CRC.

Keywords: 4μ8C, Colorectal Cancer, IRE1α, XBP1s, UPR

Introduction

Colorectal cancer (CRC) is recognised as a significant health threat and is the second most fatal cancer globally [1]. Despite significant progress in prolonging overall survival via different treatments, the management of CRC remains a challenge [2,3]. One of the current therapeutic regimes for CRC is 5-fluorouracil (5-FU)-based therapy; however, cases of drug resistance have been reported, which are among the main reasons for chemotherapeutic failure and tumor recurrence [4]. Therefore, finding new methods to reduce or eliminate drug resistance have always been key concerns for clinicians treating these patients [5]. One of the main cellular responses during chemotherapy and the development

of drug resistance is ER stress (ERS) [6] . ERS is activated during changes in pathology or physiology of all living cells such as nutrient deprivation, lack of oxygen, reactive oxygen species (ROS) and the accumulation of unfolded or misfolded proteins. The unfolded protein response (UPR) is a compensatory mechanism to counteract the effects of ERS [7]. The UPR is activated and orchestrated managed by three ER-localized proteins including inositol-requiring transmembrane kinase/endonuclease 1α (IRE1α), pancreatic ER kinase (PERK) and activating transcription factor 6 (ATF6).

IRE1α is a transmembrane protein consisting of two N- and C-terminal domains; the N-terminal domain known as ER luminal domain which interacts with unfolded proteins and the C-terminal domain which is in the cytoplasmic region consisting of serine/threonine kinase and endoribonuclease (RNase) domains which initiate the UPR signalling. In response to the accumulation of unfolded proteins, dimerization and autophosphorylation of IRE1α is achieved by the kinase domain, with splicing and removal of a 26 nucleotide intron from XBP1 mRNA by the RNase domain of IRE1α [8]. The XBP1 spliced form (XBP1s) acts as a transcription factor and translocates to the nucleus to target the genes involved in ER-associated degradation (ERAD) of misfolded proteins, as well as protein entry to ER, folding capacity and biosynthesis of phospholipid [9]. The RNase domain of IRE1α is also required for degradation of the mRNAs encoding for ER proteins through the regulated IRE1-dependent decay (RIDD) pathway [10]. Overactivation of XBP1s, found in some diseases, such as cancers and metabolic conditions, and has also been implicated in tumor progression [11,12]. In this regard, inhibition of IRE1α expression, either by genetical or pharmacological means, may suppress the proliferation of cancerous cells, and thus may be considered as a suitable target for drug design [13,14]. Pharmacological inhibitors of IRE1 have been carried out with different strategies based on kinase or RNase domain. The compounds that target the RNase activity, including salicylaldehyde analogs (*e.g.* MK0186893) and umbelliferones (*e.g.* 4μ8c) [15]. 4μ8c, but not MK0186893, specifically inhibit the RNase activity of IRE1 without impact on its phosphorylation. Thus it is useful for studying the specific activity of IRE1 such as RIDD and XBP1 splicing [16]. Besides, using 4μ8c as type III inhibitors, has less toxicity, lower effect on immune responses, and decreases the cell proliferation in cancer cells [17]. The type I and II of IRE1 inhibitors are kinase-based compounds that inhibit or activate the IRE1 signalling through kinase inhibition without directly targeting the RNase domain. However, due to the high toxicity and off-target activity (such as interact with other kinases), these pharmacological inhibitors are less useful [17,18].

In this study, we first aimed to reduce the growth of tumor cells by inhibiting the RNase domain of IRE1α with 4μ8C, and secondly to increase the drug sensitivity by using 4μ8C and lower doses of 5-FU as chemotherapy drug. Our findings underline the importance of targeting the IRE1/XBP1 pathway in colorectal cancer, and also point towards the therapeutic importance of this pathway and its contribution to drug resistance.

Materials and Methods

Cell culture, cell viability and cell cytotoxicity

The CT26 colorectal cancer cell line was prepared from the cell bank of Pasteur Institute of Iran, Tehran. The cells were cultured in standard condition of RPMI1640 medium, (10%, v/v) of fetal bovine serum (FBS) and 1% of antibiotics (100 U/ml penicillin, and 100 µg/ml streptomycin). Then the cells were maintained at 37°C incubator, 95% humidity and 5% CO_2. To examine the CRC viability, a trypan blue exclusion test was applied followed by tunicamycin (TM) (1µg/ml), and also without TM treatment. TM causes ERS through inhibiting protein glycosylation. Briefly, the cells were seeded in an appropriate number, 24h before treatment, then the different concentrations of 5-Fu (Eber pharma) (5,10, 20, 50, 100 µg/ml), 4µ8c (Cat no HY-19707 MCE, USA) (1,2, 5, 10, 20 µg/ml), and combination of them were used, then the cells were trypsinized and stained with 0.4% trypan blue. The alive and dead cells were count under under an inverted light microscope (Zeiss, Germany).

The cytotoxicity of the cells was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. Briefly, the cells were treated with TM and different concentrations of 5-FU, either alone or in combination with 4µ8C, for a period of 24h. Then the cells were then incubated for 4h following by adding MTT solution (5 mg/ml) and dimethyl sulfoxide (DMSO) (100 µL). The optical density (OD) of the cells was read at 580 nm by ELISA reader (Thermo/Lab Systems Multiskan, USA). Then the cytotoxicity of the cells was calculated in compared to control cells, as reported elsewhere [19].

Cell death analysis

The dead cells such as apoptotic or necrotic cells were assessed by annexin/PI staining and measured by flow cytometric analysis [20]. Briefly, the treated and control cells were harvested and then stained with 1X of binding buffer, 1 M Annexin V Fluorescein Isothiocyanate (FITC), and 1 mg/mL PI solutions. Then, the cells were kept in a dark place at room temperature for 10 minutes. The analysis should be done immediately less than one hour with a FACS Calibur flow cytometer (FACS Calibur, BD Bioscience, USA).

RT-PCR and gel electrophoresis

Total RNA from all the treated and control cells were extracted using the RNX-Plus solution (CinaClon Co, Iran). The RNA concentration and optical density (OD) was measured by NanoDrop™ One C Spectrophotometer (Thermo Fisher Scientific Inc). The 1 µg of RNA was used for cDNA synthesis according to manufacturers' instruction (Bio fact, South Korea). RT-PCR was used in a total reaction of 20µl (cDNA template, forward and reverse primers, master mix and double distilled water) in the PCR set. The specific primers for both XBP1s and XBP1u were forward 5' GAACACGCTTGGGAATGGACAC3' and reverce5' AGAAAGGGAGGCTGGTAAGGAAC3'. The PCR program was run at 95ºC, 5 min, 38 cycles (95ºC, 25 Sec, 65ºC, 30 Sec and 72ºC 30S), then 72ºC for 30 Sec. The product of PCR was run on 2% gel electrophoresis. Then the quantitative data was analyzed by image J 152 software and reported as quantitative information.

Animals

The 8±12 week's old male BALB/C wild-type mice were obtained from the Pasteur Institute of Tehran, Iran. They were transferred to the animal care center and housed under standard conditions in a 12 h light and dark cycle and were given adequate mouse food and water. During their maintenance and also during the experiment, the animals were excluded from the study whenever any problems, such as unusual weight loss or gain, were observed. The project was approved in ethical committee at NIMAD institute, Iran, by 1398.265 approval ID.

Experimental CRC mice model and treatments

A total of 10^6 CT26 cells, in 100 µl RPMI media, were injected into the right flank of BALB/C wild-type (WT) mice. Following 1-2 weeks, when the tumor volume reached to 100-300 mm^3, the mice were randomly classified into four groups, each containing four mice. The mice were received an intraperitoneal injection of 100 µl DMSO as control and therapeutic regimes containing 5-FU (20mM/kg), 4µ8C (50µM/kg) and a combination of them two days in a week for three weeks. Body weights and tumor volumes were measured every other day and calculated with the following formula - $\frac{1}{2} * lengh * (width)^2$. Then the tumors and vital organs like kidneys and liver were surgically removed after 20 days of the last chemotherapy and kept in formalin for pathological analysis.

Pathologic complete response (pCR)

Pathologic complete response (pCR) was calculated by counting the number of cancerous cells in tissue samples after chemotherapy. Results of the scoring were reported as follows: RX means that the residual tumor cannot be distinguished; R0 means that no residual tumors was observed (100%); R1 indicated that single cells or rare small

groups of cancer cells were observed (near complete response, 60-100%), R2 indicated residual cancer with evident tumor regression partial response, 40-60%), R3 represented extensive residual cancer being present with no evidence of tumor regression (poor or no response, 0-40%).

Metastases and toxicity assay

The tumors and vital organs including livers, kidneys were made as paraffin-fixed embedded tissues. Then the sections of 5-10 µm from paraffin block were prepared with a microtome. Then the sections were stained with H&E according to routine laboratory protocol. The metastatic and mitotic cells were scored and counted in 1 mm^2 of a microscopic frame under light microscopy.

Statistical analyses

Results were obtained from three independent experiments, each performed in triplicate. The mean standard deviation (SD) and Student's t-test were performed using SPSS software, Ver. 16 or GraphPad Prism 8.3.0. In all experiments, $p<0.05$ was considered statistically significant.

Results

Combination therapy of 5-FU and 4µ8C reduces cell proliferation and induces cell death in CT26 CRC cells more than monotherapy of 5-FU

To examine the IRE1 activation in colorectal cancer cells, IRE1 RNase activity was inhibited by 4µ8C, and cell viability determined. 4µ8C is an IRE1 Inhibitor III capable of inhibiting the IRE1α RNase activity. Proliferation of the CT26 cells was reduced and cell death increased following 5-FU chemotherapy. As depicted in Fig.1A, in an unstressed situation, 5-FU could inhibit the cell proliferation significantly only at higher concentrations (100 µg/ml, p= 0.00014). However, following TM treatment (an ERS stress inducer), 5FU, 4µ8C and their use in combination reduced cell viability in a dose-dependent manner. For example, 5-FU at concentrations of 5, 10, 20, 50, and 100 µg/mL inhibited cell viability by 55.19% ± 3.16, 41.88%±1.36, 34.73% ± 2.09, 28.21%± 2.07, and 21.88% ± 0.32, respectively. Similarly, 4µ8C at concentrations of 1, 2, 5, 10 and 20 µg/mL decreased the cell viability by 77.50% ± 5.30, 70.94% ± 0.8, 47.25% ± 20.31% ± 0.89, and 16.85% ± 0.69, respectively. Interestingly, the combination of 5-FU and 4µ8C (5/5, 5/10, 10/5, 10/10 µg/mL) led to more growth inhibition in CRC cells, by 72.34% ± 1.17 (p=0.0019), 42.49% ± 5.8 (p=0.00022), 76.24% ± 10.80 (p=0.0382), and 15.26% ± 0.54 (p=0.0000187), respectively (Fig.1B). However, our results showed that 5-FU alone could induce ERS at higher concentrations, and the combination of 5-FU with 4µ8C did not produce significant changes. Thus, we used TM to create an ERS model to obtain better

visualization of the results. The data have shown increased sensitivity of the cells to chemotherapy when cotreatment with 4µ8C was used.

The MTT assay was carried out to determine the IC_{50} of drugs, the concentration that inhibits the cell growth by 50%. As shown in fig. 1C, D, E, the IC_{50} values for 5-FU and 4µ8C were 6.03, 3.7, respectively (Fig. 1C, 1D). The IC_{50} of the combination 5-FU and 4µ8C was 5 µg/ml for 5-FU and 5 µg/ml for 4µ8C, respectively (Fig. 1E).

Cell death analysis showed that the occurrence of apoptosis was not significant with the use of monotherapy. Although 5-FU at 5 µg/ml and 4µ8C at 10 µg/ml decreased proliferation of the CT26 cell line tested, neither drug induced cell death. However, high percentages of apoptotic cells (74.6%) resulted from the combination therapeutic regime of 5-FU with 4µ8C (Fig. 2).

Targeting IRE1 RNase domain decrease the splicing of XBP1 mRNA

4µ8C was used to target RNase domain of IRE1 to study the splicing activity of IRE1. Following TM treatment, XBP1s mRNA expression was found to be increased whereas the mRNA XBP1u level was decreased. The level of xbp1s/xbp1u was raised in the combination treatment of cells with 5-FU and 4µ8C in comparison to 5-FU alone, as observed in figure 3.

4µ8C could enhance the effectiveness of 5-FU, decrease the tumor growth, and increase the drug response in a mouse CRC model

We evaluated the effects of 5-FU alone, and in combination with 4µ8c, in the CRC mouse model in order to confirm our earlier findings. The doses of prescribed drugs were obtained from experimental results with no drug toxicity and metastasis. The combination therapy of 5-FU (20mg/kg) and 4µ8C (50µM/kg) reduce the tumor volume compared to the control and monotherapy groups significantly, $p<0.05$ from day 13 (Fig. 4). This decrease in tumor size of treated groups was positively correlated with the reduced mitotic cell counting. The mean mitotic rate was 14±1 in the 5-FU group compared to 9±1.2 in the 5-FU/4µ8C ($p < 0.001$). (Fig. 5A). The weights of all animals were checked during the experiment and there were no consequential discrepancies observed between the treatment and control groups. However, the variation of tumor volume to body weight (TV/BW) in different groups revealed that 5-FU/ 4µ8C reduced TV/BW more than 5-FU monotherapy, at day 21 ($p <0.001$). (Fig. 5B). The response to treatment effect was 40-60% in combination therapy of 5-FU/ 4µ8C group in comparison to monotherapy with 5-FU, which was less than 30% (Fig.6 and table 1), suggesting that the combination of 5-FU/4µ8C enhanced the chemotherapeutic response in

the *in vivo* model of CRC. The screening protocol of chemotherapy also confirmed that no signs of cytotoxicity or metastasis were detected in all treated groups (Fig. 7 and table 1).

Discussion

5-FU is one of the most commonly used drugs for treatment of CRC. However, its prescription often leads to development of drug resistance [21]. Recently, the association of chemoresistance with the molecular mechanism of ERS has been attracting in research attention [22]. Besides, it has also been shown that ERS and UPR activation can modulate the levels and activities of cell survival and autophagy-related regulators, thereby exerting crucial functions in controlling the drug resistance of cancer cells [22]. More importantly, 5-FU has also been linked to the stimulation of ERS in colon cancer [23]. The main goal of this study is focused on the enhancement of CRC susceptibility by targeting the IRE1α arm of UPR. Since the recognition of IRE1α as a suitable target for many cancers, pharmacological inhibition of its activity is seen to play an important role in therapeutic regimes [15]. Currently chemical manipulation of IRE1, such as targeting either the kinase domain or the RNase domain, is the main basis for drug development [15]. In this regard 4μ8C is an effective inhibitor of IRE1α RNase activity, with significant effects on the reduction of XBP1 splicing, the downstream effector of IRE1 and also on the reduced RIDD pathway [16]. Our results demonstrated that, in a controlled situation without using chemical inducers, 5-FU was able to diminish the cell proliferation in CRC cells only in high doses (100 μg/ml). Furthermore, 5-FU could also induce ERS and XBP1 slicing in CRC cell line as reported before (21). Therefore, the ability of 5-FU to induce drug resistance should also be considered. In contrast, by using TM, 5-FU was found to decrease CRC proliferation in a dose-dependent manner (5-100 μg/ml). To enhance the 5-FU sensitivity, specific IRE1α inhibition, 4μ8C was used under both unstressed and stressed conditions. Our results show that although 4μ8C inhibited IRE1α RNase activity in CT26 cells under non-stressed situations, the inhibitory concentration was only obtained at high doses of 20 μg/ml. However, under TM treatment, it can enhance the susceptibility of CRC to 5-FU chemotherapy at 5 and 10 μg/ml, suggesting that this inhibition is likely to occur independently of IRE1α RNase activity at higher concentrations or may be due to off-target effects. This situation is similar to the report in hepatoma cells which revealed that higher concentrations of 4μ8c appear to be independent of IRE1α RNase activity [24]. Besides, the previous studies demonstrated the role of IRE1 in the regulation of cell growth and ER membrane expansion, in both unstressed and ER stressed-cells [25]. Our results here demonstrate that inhibition of the IRE1α-XBP1 branch promotes apoptosis in the CT26 CRC cell line, and therefore could be

considered as potential target for chemotherapy. 4μ8C was found to successfully inhibit the IRE1 RNase activity and to enhance antitumor activity in CRC. The inhibition of RNase domain of IRE1 α is more stable than targeting kinase activity. There have also been some reports of pharmaceutical inhibitors that showed the importance of this domain in targeting IRE1 for cancer treatment - for example, using 4μ8C in hepatocellular carcinoma (HCC) or MKC8866 in triple-negative breast cancer [25,26]. To further support our data on 4μ8C activity, we established the CRC mice model. The previous experiment has demonstrated that although by increasing doses of 5-FU, the apoptosis was raised, severe diarrhea, histopathological damage and increased the mortality rate was occurred in mice model [27]. In this study, we have chosen the proper dosage according to the suggested intestinal administration and by performing the pilot study. Finally, 20 mg/kg of 5-FU was used alone and in combination with an inhibitor, 4μ8C which no toxicity was observed in any of the vital organs. Different doses of 4μ8C were also selected according to previous experience [28] , and the pilot study. Our results showed that 50 μM/kg of 4μ8C is more appropriate dose which did not show toxicity but reduced the tumor volume significantly. The doses lower that 50 μM/kg didn't show reduction in tumor size. Besides, the presence of 4μ8C did not promote cytoxicity in either of the treatment conditions but higher concentrations of the inhibitor (60 μM) were associated with apparent off-target or compensatory responses that were not observed at 10 μM in vitro assay [24]. The tumor volume of treated mice confirmed the considerable effects of combination therapy (5-FU and 4μ8C) on CRC mice, $p \leq 0.05$ from day 13 was observed. Indeed, the tumor volume of 5-FU/ 4μ8C therapy was significantly decreased in comparison to 5-FU monotherapy and controlled group. This treatment response also confirmed the efficacy of combination therapy, evaluated at 40-60% in comparison to monotherapy. In addition, no metastases in either kidney or liver were reported in all of the therapeutic groups, pointing to the safety profiles of the doses used in intraperitoneal administration. These findings are in line with similar studies of IRE1 inhibition, used in cancer mice models to enhance the chemotherapeutic effects [26]. Although our results revealed 4μ8C in combination with 5-FU did reduce tumor growth and increase the treatment response in CRC mice model, the pharmacokinetics of 4μ8C limits the application of this inhibitor on animal model [16].

Conclusions

Our results reveal that 4μ8C can successfully inhibit the IRE1/XBP1 branch of UPR, and that co-treatment of 4μ8C with 5-FU may enhance the sensitivity of CRC in an experimental mice model in comparison to monotherapy.

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Conflict of Interest

Authors declares that they have no conflict of interest.

Ethical approval

The project was approved by the ethical committee at NIMAD, Iran, by 1398.265 approval ID.

References

1. Rawla P, Sunkara T, Barsouk A. Epidemiology of colorectal cancer: Incidence, mortality, survival, and risk factors. Prz. Gastroenterol. 2019.

2. Grothey A, Van Cutsem E, Sobrero A, Siena S, Falcone A, Ychou M, et al. Regorafenib monotherapy for previously treated metastatic colorectal cancer (CORRECT): An international, multicentre, randomised, placebo-controlled, phase 3 trial. Lancet. 2013;

3. Falcone A, Ricci S, Brunetti I, Pfanner E, Allegrine G, Barbara C, et al. Phase III trial of infusional fluorouracil, leucovorin, oxaliplatin, and irinotecan (FOLFOXIRI) compared with infusional fluorouracil, leucovorin, and irinotecan (FOLFIRI) as first-line treatment for metastatic colorectal cancer: The gruppo oncologico nor. J Clin Oncol. 2007;

4. Pouya FD, Gazouli M, Rasmi Y, Lampropoulou DI, Nemati M. MicroRNAs and drug resistance in colorectal cancer with special focus on 5-fluorouracil. Mol. Biol. Rep. 2022.

5. De Rosa M, Pace U, Rega D, Costabile V, Duraturo F, Izzo P, et al. Genetics, diagnosis and management of colorectal cancer (Review). Oncol Rep. 2015;

6. Cao S, Tang J, Huang Y, Li G, Li Z, Cai W, et al. The Road of Solid Tumor Survival: From Drug-Induced Endoplasmic Reticulum Stress to Drug Resistance. Front Mol Biosci [Internet]. 2021;8. Available from: https://www.frontiersin.org/articles/10.3389/fmolb.2021.620514/full

7. Nikesitch N, Lee JM, Ling S, Roberts TL. Endoplasmic reticulum stress in the development of multiple myeloma and drug resistance. Clin. Transl. Immunol. 2018.

8. Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K. XBP1 mRNA Is Induced by ATF6 and Spliced by IRE1 in Response to ER Stress to Produce a Highly Active Transcription Factor. Cell [Internet]. 2001;107:881–91. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11779464

9. Hetz C. The unfolded protein response: controlling cell fate decisions under ER stress and beyond. Nat Rev Mol Cell Biol [Internet]. 2012;13:89–102. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22251901

10. Hetz C, Papa FR. The Unfolded Protein Response and Cell Fate Control. Mol Cell [Internet]. 2018;69:169–81. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1097276517304446

11. Wang M, Kaufman RJ. The impact of the endoplasmic reticulum protein-folding environment on cancer development. Nat. Rev. Cancer. 2014.

12. Oakes SA, Papa FR. The role of endoplasmic reticulum stress in human pathology. Annu Rev Pathol Mech Dis. 2015;

13. Xie Y, Liu C, Qin Y, Chen J, Fang J. Knockdown of IRE1α suppresses metastatic potential of colon cancer cells through inhibiting FN1-Src/FAK-GTPases signaling. Int J Biochem Cell Biol. 2019;

14. Jin C, Jin Z, Chen NZ, Lu M, Liu CB, Hu W Le, et al. Activation of IRE1α-XBP1 pathway induces cell proliferation and invasion in colorectal carcinoma. Biochem Biophys Res Commun. 2016;

15. Raymundo DP, Doultsinos D, Guillory X, Carlesso A, Eriksson LA, Chevet E. Pharmacological Targeting of IRE1 in Cancer. Trends in Cancer [Internet]. 2020;6:1018–30. Available from: https://linkinghub.elsevier.com/retrieve/pii/S2405803320302144

16. Cross BCS, Bond PJ, Sadowski PG, Jha BK, Zak J, Goodman JM, et al. The molecular basis for selective inhibition of unconventional mRNA splicing by an IRE1-binding small molecule. Proc Natl Acad Sci U S A [Internet]. 2012;109:E869-78. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22315414

17. Grandjean JMD, Wiseman RL. Small molecule strategies to harness the unfolded protein response: where do we go from here? J Biol Chem [Internet]. 2020;295:15692–711. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0021925817503981

18. Siwecka N, Rozpędek-Kamińska W, Wawrzynkiewicz A, Pytel D, Diehl JA, Majsterek I. The Structure, Activation and Signaling of IRE1 and Its Role in Determining Cell Fate. Biomedicines [Internet]. 2021;9:156. Available from: https://www.mdpi.com/2227-9059/9/2/156

19. Adibzadeh R, Golhin MS, Sari S, Mohammadpour H, Kheirbakhsh R, Muhammadnejad A, et al. Combination therapy with TiO2 nanoparticles and cisplatin enhances chemotherapy response in murine melanoma models. Clin Transl Oncol. 2020;

20. Mohammadalipour Z, Rahmati M, Khataee A, Moosavi MA. Differential effects of N-TiO 2 nanoparticle and its photo-activated form on autophagy and necroptosis in human melanoma A375 cells. J Cell Physiol [Internet]. 2020;jcp.29479. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1002/jcp.29479

21. Blondy S, David V, Verdier M, Mathonnet M, Perraud A, Christou N. 5-Fluorouracil resistance mechanisms in colorectal cancer: From classical pathways to promising processes. Cancer Sci. 2020.

22. Bahar E, Kim JY, Yoon H. Chemotherapy resistance explained through endoplasmic reticulum stress-dependent signaling. Cancers (Basel). 2019;

23. Kim JK, Kang KA, Piao MJ, Ryu YS, Han X, Fernando PMDJ, et al. Endoplasmic reticulum stress induces 5-fluorouracil resistance in human colon cancer cells. Environ Toxicol Pharmacol. 2016;

24. Stewart C, Estrada A, Kim P, Wang D, Wei Y, Gentile C, et al. Regulation of IRE1α by the small molecule inhibitor 4μ8c in hepatoma cells. Endoplasmic Reticulum Stress Dis [Internet]. 2017;4:1–10. Available from: https://www.degruyter.com/document/doi/10.1515/ersc-2017-0001/html

25. Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. Nat. Rev. Mol. Cell Biol. 2007.

26. Pavlović N, Calitz C, Thanapirom K, Mazza G, Rombouts K, Gerwins P, et al. Inhibiting IRE1α-endonuclease activity decreases tumor burden in a mouse model for hepatocellular carcinoma. Elife [Internet]. 2020;9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/33103995

27. Zhang S, Liu Y, Xiang D, Yang J, Liu D, Ren X, et al. Assessment of dose-response relationship of 5-fluorouracil to murine intestinal injury. Biomed Pharmacother [Internet]. 2018;106:910–6. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0753332218337260

28. Li X-X, Zhang H-S, Xu Y-M, Zhang R-J, Chen Y, Fan L, et al. Knockdown of IRE1α inhibits colonic tumorigenesis through decreasing β-catenin and IRE1α targeting suppresses colon cancer cells. Oncogene [Internet]. 2017;36:6738–46. Available from: https://www.nature.com/articles/onc2017284

Table caption and figure legends

Table 1. The effect of chemotherapy of 5-FU, 4μ8c and their combination on CRC syngeneic murine model tumors and vital organs.

Fig.1 Effect of different concentration of 5-FU and/or 4μ8C alone and in combination on the viability and cytotoxicity of CT26 CRC cells. The cells were treated by various concentrations of 5-FU (5, 10, 20, 50, 100 μg/ml), 4μ8C (1, 2, 5, 10, 20 μg/ml) and combination of them (5/5, 5/10, 10/5, 10/10 μg/mL) for 24 h, then viability and cytotoxicity were studied using trypan blue exclusion test and MTT assay, respectively. a) Un-stressed situation. b) Stressed situation by tunicamycin ™ induction. c) IC50 calculation for 5-FU. d) IC50 calculation for 4μ8C E) IC50 calculation for -FU and 4μ8C together. The results are from triplicated of three independent experiments and represented as means ± SD. Statistically different results are indicated with *($p< 0.05$), ** ($p < 0.01$) and ***($p<0.001$) compared to untreated cells. They were analyzed by GraphPad Prism 8.3.0.

Fig. 2 Effect of 5-FU and/or 4μ8C alone and in combination on cell death in CT26 CRC cells. Cells were treated with 5-FU (5 μ/ml), 4μ8C (10 μ/ml) and a combination of them for 24 h, then harvested and stained with PI/FITC for flowcytometric analyses by FACS Calibur flow cytometer (FACS Calibur, BD bioscience, USA).

Fig. 3 Tunicamycin ™ (1 μg/ml) is induced ER stress in CT26 CRC cell. Then the cells were treated by 5-FU (5μg/ml) and IRE1 inhibitor, 4μ8C (10μg/ml) alone and in combination together. RT PCR gel electrophoresis of XBP1u and XBP1s format. The band intensity of XBP1s and XBP1u was analyzed by image J software and reported as quantitative data.

Fig.4 The effect of 5-FU, and 4μ8C alone and in combination of them on tumor size and volume on BALB/C CRC model mice. Mice treated with 5-FU (20mg/kg), 4μ8C (50μM/kg) and combination of them. The tumor volume was measured every other day from 0-21 days of post 5-FU, 4μ8C and their combination administration. **a)** Tumor size and **b)** tumor volume of different groups. In 5- FU group, during the chemotherapy, the tumor volume decreases compared to the control ($p<0.05$ from day of 13); however, the increased rate was observed in compared to combination therapy. In 4μ8C group, although the tumor volume was diminished, an increasing trend was also observed in comparison to combination therapy from day 13. The significant reduction of tumor volume was observed in combination therapy group from day 7 in compared to control ($p<0.05$ from day of 13) and, also, monotherapy of 5-FU ($p<0.05$ from day of 19). * for $p<0.05$, ** for $p< 0.01$, *** for $p< 0.001$

Fig.5 The effect of 5-FU, and 4μ8C alone and in combination of them on mitotic and body weight of BALB/C CRC mice model a) Mitotic cell counting in 1 mm^2 of H&E staining slide in all treated and control group. **b)** The rate of tumor volume/body weight (TV/BW) of all treated and control animals. *p<0.05, ** p<0.01, *** p<0.001.

Fig.6 The effect of chemotherapy of 5-FU (20mg/kg), and 4μ8C (50μM/kg) alone and in combination on BALB/C CRC mice model.

Fig.7 The cytotoxicity effect of chemotherapy of 5-FU, and 4μ8C alone and in combination on BALB/C CRC model mice. Kidneys and livers in all groups of animals were removed after 21st day of the last chemotherapy by surgery and monitored by H&E staining. There were no signs of toxicity and metastasis associated with treatments in all groups of single therapy of 5-FU and 4μ8C and, also with a combination of them.